FRONTIERS IN THE PHARMACOLOGICAL MANIPULATION OF INTRACELLULAR cAMP LEVELS

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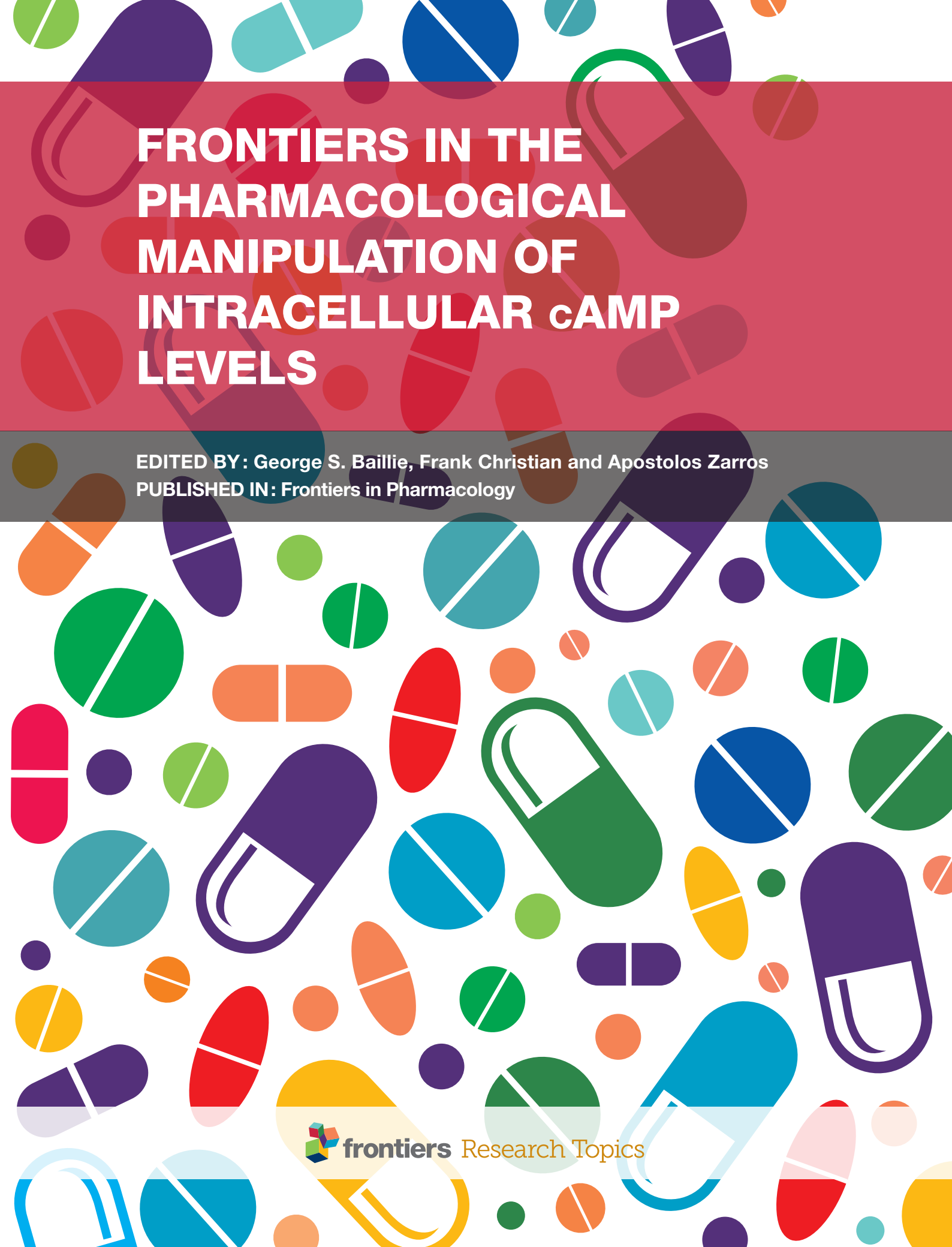

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FRONTIERS IN THE PHARMACOLOGICAL MANIPULATION OF INTRACELLULAR cAMP LEVELS

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Cyclic adenosine monophosphate (cAMP) is a second messenger of paramount biological importance, involved in the regulation of a significant number of cellular functions through the cAMP-dependent intracellular signal transduction pathways. The aim of this "Frontiers in Pharmacology" Research Topic was to attract contributions that highlight emerging ideas in the cAMP field that: (i) describe its role in cellular function and homeostasis, (ii) present the current approaches to its pharmacological manipulation, and (iii) clarify its central role in the development of more targeted therapeutic approaches toward a spectrum of diseases. The present collection of articles highlights, in a representative (but certainly not exhaustive) way, the research activity and emerging concepts in the field, while it also reveals the therapeutic potential that targeted pharmacological manipulation of intracellular cAMP levels could exert on a number of pathological conditions.

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EDITORIAL
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Editorial: Frontiers in the Pharmacological Manipulation of Intracellular cAMP Levels

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Keywords: cyclic adenosine monophosphate, cAMP, cellular function, signaling, homeostasis, disease, pharmacological manipulation, therapeutic applications

The Editorial on the Research Topic

Frontiers in the Pharmacological Manipulation of Intracellular cAMP Levels

Cyclic adenosine monophosphate (cAMP) is a second messenger of paramount biological importance, involved in the regulation of a significant number of cellular functions *via* the cAMP-dependent intracellular signal transduction pathways. Being the first second messenger described (Rall and Sutherland, 1958), cAMP has been extensively studied throughout the years and has revealed mainstream mechanisms of cellular signaling (Beavo and Brunton, 2002; Baillie et al., 2005; McCormick and Baillie, 2014). The aim of this Research Topic was to attract contributions that highlight emerging ideas in the cAMP field that: (i) describe its role in cellular function and homeostasis, (ii) present the current approaches to its pharmacological manipulation, and (iii) clarify its central role in the development of more targeted therapeutic approaches toward a spectrum of diseases.

To our belief, this aim has been successfully fulfilled. The present collection of articles highlights, in a representative (but certainly not exhaustive) way, the research activity and emerging concepts in the field, while it also reveals the therapeutic potential that targeted pharmacological manipulation of intracellular cAMP levels could exert on a number of pathological conditions. We organized the papers of this Research Topic/e-Book into three sections (A–C).

The first section (A: "*Frontiers in the role of cAMP in cellular function, homeostasis and disease*") opens with a very informative review article on the reciprocal regulation between cAMP signaling and the ubiquitin-proteasome system, as well as its relevance to certain human diseases (Rinaldi et al.). A more focused perspective on the importance of cAMP in cardiac physiology and pathophysiology is presented by the review article of Boularan and Gales and the mini review article of Froese and Nikolaev; both articles provide an up-to-date account of recent developments in the field and should be considered by readers as complementary to each other. Later in section A, readers will discover an elegant mini review on the role of cAMP signaling in neural plasticity, learning and memory (Lee), that summarizes findings generated by research on the fruit fly *Drosophila melanogaster* and briefly highlights implications of this signaling pathway to potential therapeutic applications. Section A concludes with an original article on the role of cAMP signaling in human trophoblast fusion (Gerbaud et al.), and with a perspective article on the spatiotemporal regulation of cAMP signaling in blood platelets (Raslan et al.).

The second section (B: "*Approaches to the study and pharmacological manipulation of intracellular cAMP levels*") includes an informative review article on state-of-the-art methodologies which can be utilized toward the study of cAMP-mediated signal transduction *via* G-protein coupled receptors (Wright et al.), as well as two excellent review articles on the pharmacological manipulation of intracellular cAMP levels through interference of the molecular interactions

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between the A-kinase anchoring proteins and other signaling protein intermediates (Calejo and Taskén; Nygren and Scott). This section also accommodates a contribution by Röck et al. with an original research article in which analysis of protein kinase A (PKA) dynamics following the integration of patient mutations into its catalytic (PKAc) and regulatory (RIa) subunits is presented. The section is completed with two analytical review articles that present recent developments in the utilization of genetically encoded tools for cAMP measurement in health and disease (Paramonov et al.; Patel and Gold).

The third section (C: *"Potential therapeutic applications"*) is introduced with a perspective article on the role of patients' sex toward a more targeted (cAMP pathway-regulated) therapeutic approach to brain tumors (Warrington et al.), followed by two original research articles on the role of cAMP signaling in the context of hematological malignancies and the potential therapeutic applications that could arise through its pharmacological manipulation (Dong et al.; Fernández-Araujo et al.). The current Research Topic / e-Book is concluded with a well-written review article on cAMP signaling in trypanosomatids that provides an overview of the complex functions of cAMP in these protozoan parasites, as well as a fine perspective on how potential targets for the trypanosomatid-specific cAMP pathway could provide efficient therapeutics for serious human diseases such as the sleeping sickness and other trypanosomatid-induced pathological entities (Tagoe et al.).

The recruited articles cover multiple aspects of the ongoing research in the cAMP field and allow an appreciation of the difficult task ahead in fully-understanding the complexity of this ubiquitous signaling cascade and taming it to develop efficient therapeutic applications. Hopefully, readers will consider this collection of papers as a small step toward these goals.

ACKNOWLEDGMENTS

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REVIEW
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A dynamic interface between ubiquitylation and cAMP signaling

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Phosphorylation waves drive the propagation of signals generated in response to hormones and growth factors in target cells. cAMP is an ancient second messenger implicated in key biological functions. In mammals, most of the effects elicited by cAMP are mediated by protein kinase A (PKA). Activation of the kinase by cAMP results in the phosphorylation of a variety of cellular substrates, leading to differentiation, proliferation, survival, metabolism. The identification of scaffold proteins, namely A-Kinase Anchor proteins (AKAPs), that localize PKA in specific cellular districts, provided critical cues for our understanding of the role played by cAMP in cell biology. Multivalent complexes are assembled by AKAPs and include signaling enzymes, mRNAs, adapter molecules, receptors and ion channels. A novel development derived from the molecular analysis of these complexes nucleated by AKAPs is represented by the presence of components of the ubiquitin-proteasome system (UPS). More to it, the AKAP complex can be regulated by the UPS, eliciting relevant effects on downstream cAMP signals. This represents a novel, yet previously unpredicted interface between compartmentalized signaling and the UPS. We anticipate that impairment of these regulatory mechanisms could promote cell dysfunction and disease. Here, we will focus on the reciprocal regulation between cAMP signaling and UPS, and its relevance to human degenerative and proliferative disorders.

Keywords: cyclic AMP, PKA signaling, proteasome, ubiquitination, AKAP

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cAMP Signaling

Since the discovery of cyclic adenosine 3′,5′-monophosphate (cAMP) in the late 1950s, significant advances have been made to better understand the link between the cAMP and the regulation of downstream signaling and cellular homeostasis. The principal elements of the cAMP cascade have been intensively studied, both at functional and structural side, delineating a complex and finely regulated network of signaling scaffolds and regulatory proteins (Walsh and Van Patten, 1994). cAMP levels are tightly regulated through the balance between two classes of enzymes: the adenylyl cyclases (ACs) and the cyclic nucleotide phosphodiesterases (PDEs). The main effector of cAMP is protein kinase A (PKA), whose role is fundamental in the propagation of the signal downstream to target substrates/effectors (Taylor et al., 2005). The duration and the amplitude of the propagating signal are controlled by a combination of different classes of ACs, protein kinases, PDE, phosphatases (PPs) and scaffold proteins (**Figure 1**).

G protein–coupled receptors (GPCRs) constitute a large family of membrane proteins that transduce signals from the extracellular microenvironment to inside cell (Rosenbaum et al., 2009). The binding of extracellular ligand to its cognate GPCR at the cell membrane activates AC, which in turn generates cAMP at discrete points along the plasma membrane. The mammalian ACs are encoded by nine independent genes differentially expressed in several cell types and tissues

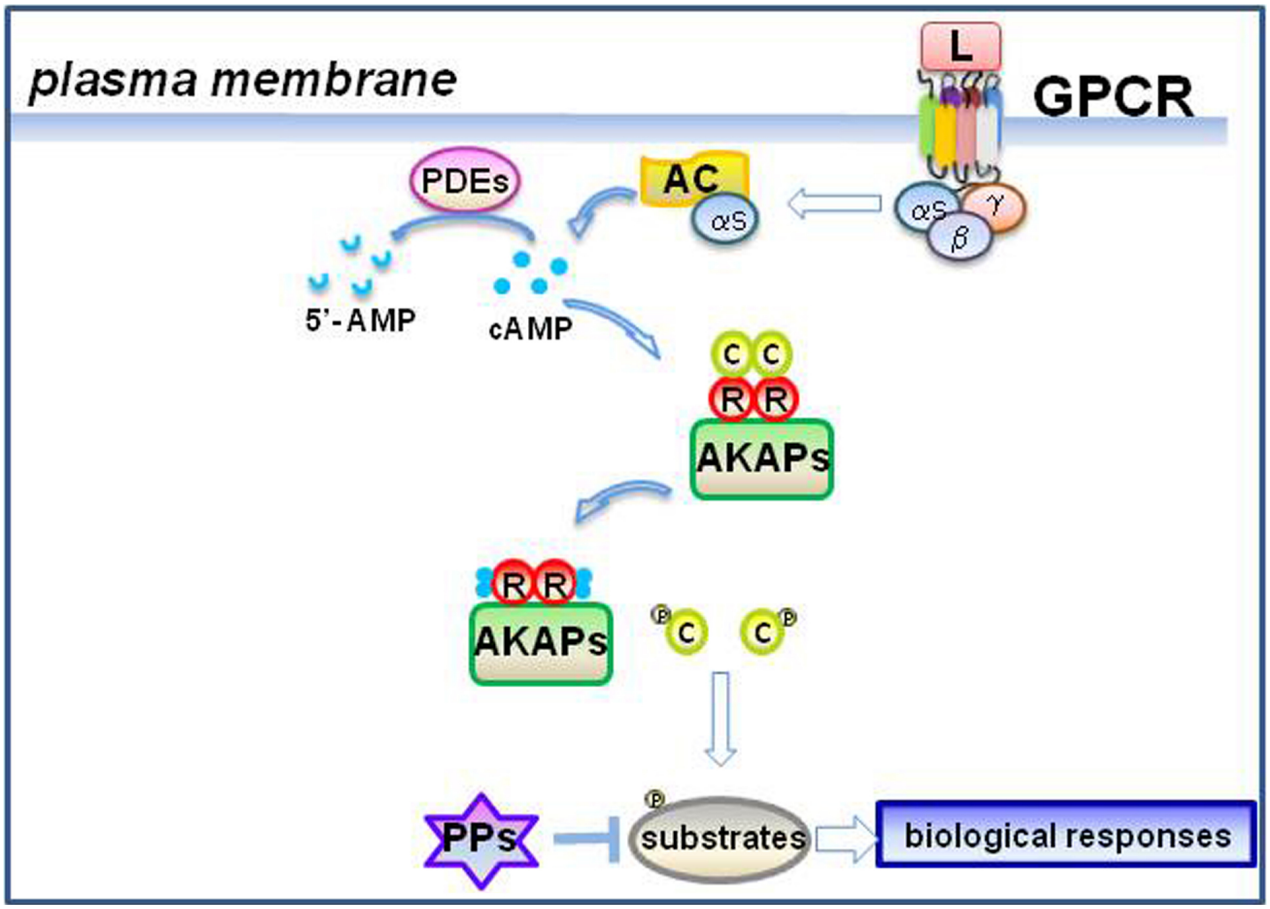

FIGURE 1 | GPCR stimulation and cAMP signaling. Ligand-induced activation of a GPCR dissociates heterotrimeric G proteins and activates the adenylyl cyclase (AC) through Gαs subunit (Rosenbaum et al., 2009). AC converts ATP into cAMP. cAMP binding to regulatory (R) subunits of AKAP-assembled PKA dissociates the holoenzyme and activates the catalytic (C) subunits (Taylor et al., 2005). Phosphorylation of cellular substrates by C evokes plenty of biological responses. Phosphodiesterases (PDEs) converts the cAMP in 5′-AMP and decrease cAMP signaling (Maurice et al., 2014). Dephosphorylation of substrates by protein phosphatases (PPs) contributes to attenuate the signal (Zhang et al., 2013).

(Iyengar, 1993). The cAMP-generating activity of ACs is stimulated by the interaction with the stimulatory α subunit of the G-proteins (Gαs). In the absence of ligand, Gαs forms a heterotrimeric complex with the β and the γ subunits. Once activated, the GPCR causes the dissociation of heterotrimeric G-proteins, with consequent activation of ACs by the Gαs subunit (Cooper and Tabbasum, 2014). Continuous or repeated pulses of hormone stimulation downregulate GPCR activation. This phenomenon, called receptor desensitization (Reiter and Lefkowitz, 2006), includes two phases: (1) acute desensitization, which involves the recruitment of β-arrestin to the activated GPCR, impairing the coupling between the receptor and G-proteins; (2) long-term desensitization which consists in the internalization and lysosomal degradation of the receptors (Sibley and Lefkowitz, 1985; Bouvier et al., 1989; Moore et al., 2007). Although stimulation by Gαs is the major mechanism of AC activation, different isoforms of ACs can receive signals from a variety of sources, as kinases (PKA, PKC and Calmodulin kinase) or Ca^{2+}, supporting and integrating distinct signal transduction pathways (Timofeyev et al., 2013; Zhang et al., 2013).

The cAMP-PDEs are enzymes that hydrolize the 3′,5′ phosphodiester bond in the second messenger cAMP, producing 5′-AMP (Maurice et al., 2014). By reducing the levels of cAMP, PDEs regulate the duration and amplitude of the cyclic nucleotide signaling. PDEs are encoded by 21 genes that generate 11 different families (PDE 1–11) that share structural similarities, but different substrate specificity, regulatory mechanisms and kinetics (Maurice et al., 2014). PDEs can hydrolyze cAMP (PDE4, PDE7, and PDE8), cGMP (PDE5, PDE6, and PDE9) or both cyclic nucleotides (PDE1, PDE2, PDE3, PDE10, and PDE11; Francis et al., 2011). The N-terminal regulatory region of PDEs controls the subcellular localization of the enzymes (Kenan et al., 2000). The differential distribution of PDEs within the cell generates intracellular microdomains of the second messenger that locally enhance the sensitivity and specificity of the signals carried out by cAMP (Lomas and Zaccolo, 2014). In this context, the use of cAMP biosensors, such as those utilizing fluorescence resonance energy transfer (FRET), contributed to dissect and visualize compartmentalized pools of cAMP that are generated in response to GPCR stimulation (Stefan et al.,

2007). The establishment of the so-called "signalosome" is based on the protein–protein interaction network among the unique combinations of cyclic nucleotides generators (AC), effectors (PKA, EPAC, and cAMP-gated ion channels), degrading PDEs and scaffolds proteins (A-Kinase Anchor Proteins, AKAPs).

Compartmentalized cAMP-PKA Signaling

In eukaryotes, most of the effects elicited by cAMP depend on the activation of PKA. This kinase consists of a tetramer composed of two regulatory (R) and two catalytic (C) subunits. The binding of cAMP to R subunit dissociates the PKA holoenzyme and releases the active C subunit, which in turn phosphorylates a wide array of cellular substrates, controlling different aspects of cell physiology (Taylor et al., 2008). The biochemical and functional features of PKA holoenzymes are largely determined by the structure, properties and relative abundance of the R subunits. The analysis of the kinetics of PKA activation/de-activation cycles contributed to understand the mechanisms of cAMP action on the effector kinase (Knighton et al., 1991). PKA stimulation by cAMP is followed by a refractory phase where a coordinated activation of Ser/Thr PPs, PKA inhibitors (PKIs) and changes in the ratio between R and C subunits eventually attenuate the signal (Armstrong et al., 1995; Canettieri et al., 2003).

The localization of PKA in the cell is mediated by scaffolding proteins, namely A-kinase anchoring proteins (AKAPs). AKAPs belong to a group of structurally different proteins that share the common feature to target the PKA holoenzyme in close proximity of its substrate (Michel and Scott, 2002). Each AKAP contains a PKA-binding motif that binds the R subunit of PKA and a targeting domain that directs the kinase to specific subcellular compartments. Biochemical and structural studies identified a conserved PKA-binding domain of AKAPs that forms an amphipathic helical wheel composed of 14–18-residues (Carr et al., 1992). The helical wheel binds with high affinity the N-terminal docking/dimerization (D/D) domain of the PKA-R dimer (Newlon et al., 1999, 2001). In particular, the hydrophobic residues of the helical wheel are located in the interior face, while charged residues align on the exterior surface. Although most of the AKAPs bind to RII subunit (Herberg et al., 2000; Carnegie and Scott, 2003), several RI-specific AKAPs have been characterized (Huang et al., 1997; Angelo and Rubin, 1998; Means et al., 2011; Burgers et al., 2012). The residues determining binding specificity of AKAPs to RI and RII have been partially defined (Alto et al., 2003). Disruption of the amphipathic helical wheel abrogates the binding to R subunits, both *in vitro* and *in vivo* (Welch et al., 2010). By modulating the dissemination of cAMP signals inside the cell, AKAPs control important biological responses, such as hormone secretion, metabolism, differentiation, cell growth and survival, synaptic transmission, learning and memory (Rubin, 1994; Alto et al., 2002; Tasken and Aandahl, 2004). AKAPs form a macromolecular complex, named transduceosome, that assembles components of cAMP generating systems (receptors and ACs), effectors (PKA and Epac) and attenuating enzymes (PDEs and PPs). This implies that complexes nucleated by AKAPs create intracellular domains where distinct signaling pathways converge and are locally attenuated or amplified, optimizing the biological response to extracellular stimuli (Feliciello et al., 2001, 2005; Dell'Acqua et al., 2006; Welch et al., 2010).

Feed-backward Regulation of cAMP-PKA by the UPS

The ubiquitin-proteasome system (UPS) is emerging as an important control mechanism of cell growth, survival and metabolism. Degradation of a protein via UPS involves modification of the substrate protein by the covalent attachment of multiple ubiquitin molecules. The ubiquitin-tagged protein is eventually degraded through the proteasome (Ciechanover, 2005). Defects of the UPS may represent the trigger of several important human disorders (Wang and Hill, 2015; Dantuma and Bott, 2014; Ortega and Lucas, 2014; Schmidt and Finley, 2014). The ubiquitylation is mediated by the attachment of ubiquitin to the ε-amine of lysine residues of target proteins. This process requires a series of ATP-dependent enzymatic steps catalyzed by E1 (ubiquitin activating), E2 (ubiquitin conjugating), and E3 (ubiquitin ligating) enzymes (Ciechanover, 2003). The result of this sequential cascade of events is the covalent attachment of ubiquitin molecules to lysine residues on the target protein. These modifications can involve either a single ubiquitin (mono-ubiquitylation) or a chain of ubiquitin (poly-ubiquitylation; Ramanathan and Ye, 2012). Poly-ubiquitylation of a substrate is mostly related to protein degradation through the proteasome (Ciechanover, 2005). By modulating the protein levels, the UPS influences many cellular processes. Polyubiquitylated proteins can also follow a non-degradative pathway (De Bie and Ciechanover, 2011). This mechanism may control the intracellular trafficking of the target protein or its activity (Bonifacino and Weissman, 1998). In this case, de-ubiquitinating enzymes (DUBs), by removing the ubiquitin moieties, can restore the localization/activity of the modified protein (De Bie and Ciechanover, 2011).

The cAMP-PKA signaling is regulated by- and can regulate the UPS at different steps, giving rise to a complex interactive and regulatory network that controls different aspects of cell fate (**Figure 2**).

At cell membrane, the ubiquitylation and consequent proteolysis of receptors by the UPS contributes to post-stimulus receptor desensitization (Bonifacino and Weissman, 1998). As example, following β adrenergic receptor 2 (β-2AR) stimulation, the adaptor protein ARRDC3 (arrestin domain containing 3) recruits the E3 ligase NEDD4 (neural precursor development downregulated protein 4) close to β-2AR. Concomitant inhibition of the deubiquitinase USP20 (Ubiquitin-specific-processing protease 20) by PKA favors ubiquitylation and degradation of the receptor by NEDD4 (Nabhan et al., 2010; Kommaddi et al., 2015). Agonist-induced ubiquitylation of both receptor and β-arrestins (β-receptor regulatory proteins) also contributes to regulate receptor endocytosis. Internalized GPCRs can undergo to degradation or be recycled back to the cell surface (Shenoy et al., 2001). β-adrenergic signal transduction is the major pathway involved in the maintenance of cardiac muscle contraction. Reduced response to β-adrenergic stimulation and pathological cardiac hypertrophy are hallmark of heart failure (Port and

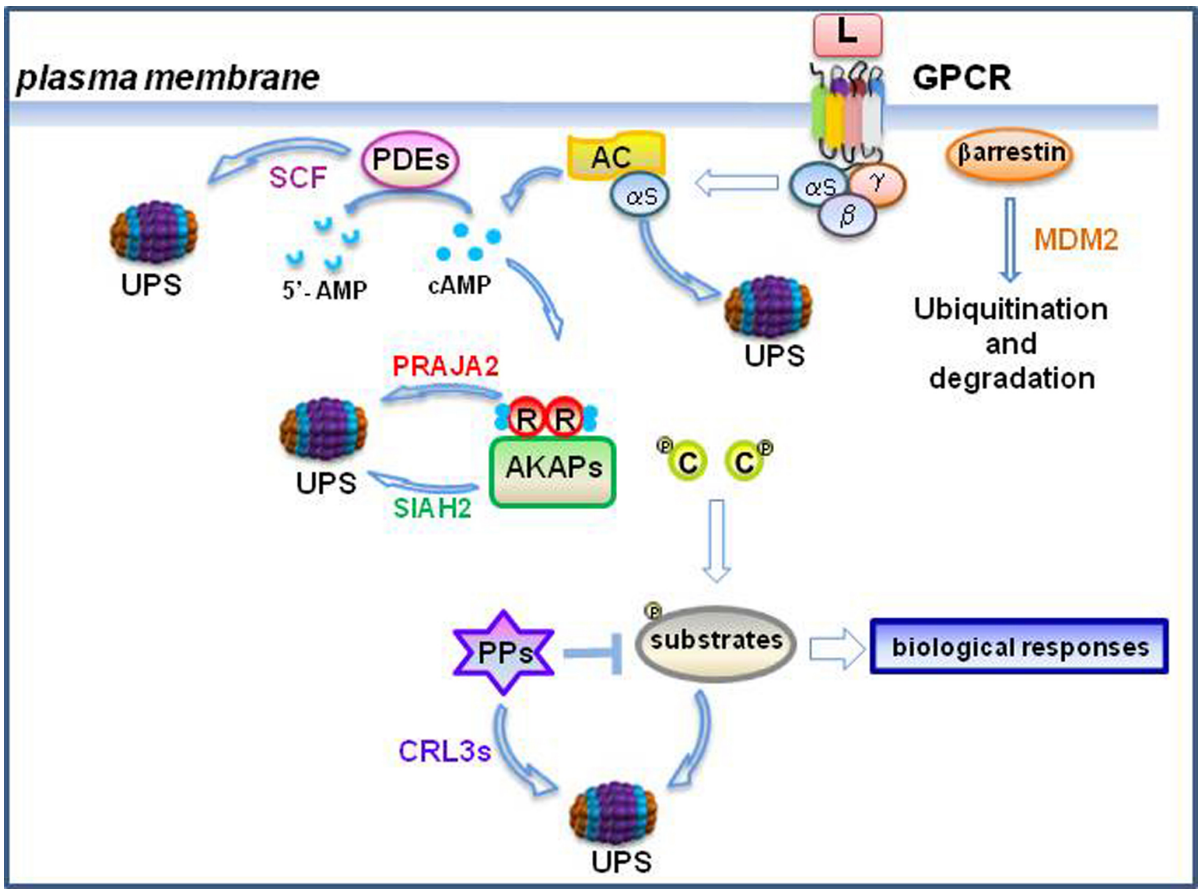

FIGURE 2 | Feed-back and feed-forward control of cAMP by the ubiquitin-proteasome system (UPS). In the burst phase, ubiquitylation of cAMP-phosphodiesterases (PDEs) through the E3 ligase SCF complex contributes to modulate cAMP levels (Zhu et al., 2010). R subunits undergo to proteolysis by the praja2-UPS pathway. Loss of R subunits sustains PKA signaling (Lignitto et al., 2011). Moreover, ubiquitylation of protein phosphatases (PPs) by cullin E3 ligases (CRL3s) further modulates phosphorylation-dependent downstream signaling (Xu et al., 2014). During the desensitization phase, agonist-induced ubiquitylation of both receptor and b-arrestins promotes receptor endocytosis and degradation, attenuating downstream signaling (Reiter and Lefkowitz, 2006). Gαs subunits are ubiquitylated and degraded by the UPS (Zha et al., 2015). During hypoxia, Siah2-mediated ubiquitylation and proteolysis of AKAP121 modulates mitochondrial activity (Carlucci et al., 2008a).

Bristow, 2001; Tilley, 2011) In this context, blunted response to agonist might be a consequence of decreased levels of Gαs subunit (Tang et al., 2008). Accordingly, recent evidence indicates that in hypertrophic hearts Gαs undergoes to extensive ubiquitylation with suppression of its downstream signaling. This eventually leads to cardiac contractility dysfunction (Jenie et al., 2013). Gβ subunits can also become a target of the UPS, contributing to feed-back regulation of GPCR signaling. Thus, Gβ2 binds to DDB1 (DNA damage-binding protein 1), a core component of CUL4B-based E3 ubiquitin ligase complex, and targets the GPCR kinase 2 (GRK2) to ubiquitylation by the DDB1-CUL4A-ROC1 ubiquitin ligase complex. Following GPCR activation, PKA phosphorylates DDB1 and induces its dissociation from Gβ2, increasing the levels of GRK2 and promoting receptor desensitization. Deletion of Cul4a gene resulted in cardiac hypertrophy and this phenotype can be partially rescued by concomitant deletion of GRK2 (Zha et al., 2015). These results unveiled a novel mechanism of feedback regulation of GPCR signaling based on a non-canonical function of Gβ2 protein, that acts as a component of the ubiquitin ligase complex that targets GRK2 for degradation.

It emerged that PDEs can be regulated by the UPS. Ubiquitin conjugation and proteasomal degradation of PDE4D by a cullin 1-containing E(3) ubiquitin ligase complex is induced through concomitant phosphorylation of PDE4D by casein kinase 1 (CK1) and glycogen synthase kinase 3β (GSK3β). A phospho-degron motif within the PDE4D was identified as responsible of ubiquitin-mediated proteolysis of the enzyme. Interestingly, protein PPs calcineurin (CaN) counteracts the effects of the SCF complex on PDE4D stability (Zhu et al., 2010), unveiling a complex regulatory mechanism of signal integration between PPs and kinases involved in the control of cAMP pathway.

The UPS can also regulate the PKA stability and signaling. praja2 is a widely expressed mammalian RING-H2 protein with intrinsic E3 ligase activity (Yu et al., 2002). praja2 acts as an AKAP that binds and targets PKA holoenzyme to the cell membrane, perinuclear region and cellular organelles. Co-localization of praja2-PKA complexes with PKA substrate/effector

molecules ensures efficient integration and propagation of the locally generated cAMP to distinct target sites. In course of agonist stimulation, praja2 couples ubiquitylation to proteolysis of the R subunits of PKA. By decreasing the ratio between R/C levels, praja2 sustains downstream signals carried out by PKA, positively impacting on specialized cell functions (Lignitto et al., 2011).

As major regulators of cAMP signaling, AKAPs can be regulated at post-translational level by the UPS. Thus, under normoxic conditions, mitochondrial AKAP121 assembles a multienzyme scaffold complex on the outer mitochondrial membrane that ensures efficient propagation of cAMP and src signals from sites of signal generation to mitochondria, enhancing oxidative phosphorylation, mitochondria remodeling, calcium homeostasis and cell survival (Cardone et al., 2004; Livigni et al., 2006; Dickey and Strack, 2011; Scorziello et al., 2013). Under hypoxic conditions, the RING E3 ligase seven in-absentia homolog 2 (Siah2) binds to- and ubiquitylates AKAP121. Ubiquitylated AKAP121 undergoes to proteasomal degradation. Disappearance of AKAP121 is accompanied by a significant drop of mitochondrial metabolic activity, leading to mitochondrial fission and cell death (Carlucci et al., 2008a,b; Merrill et al., 2011). In the ischemic mouse heart, infarct size and degree of cell death were blunted by genetic knock-out of Siah2. In hatching *Caenorhabditis elegans*, inhibiting Siah2 reduces life span, highlighting a role of the UPS-AKAP-PKA axis in the control of essential aspects of nematode aging (Kim et al., 2011).

Feed-forward Regulation of the UPS by cAMP

Besides auto-regulatory mechanisms, cAMP can also control the activity of E3 ligases. By modulating the ubiquitin pathway, PKA controls the biological activity of a wide number of cellular substrates, integrating signals generated by distinct hormones/growth factors. As example, p300 acts as scaffold and co-activator for transcription factors, facilitating chromatin remodeling and gene expression. p300 controls important biological functions, as cell proliferation, differentiation, apoptosis, and senescence (Rack et al., 2014). In lung cancer cells, the levels of p300 are tightly regulated post-translationally by the cAMP signaling. Thus, agonist-induced rise of cAMP levels promotes ubiquitin-dependent proteolysis of p300, downregulating nuclear gene transcription (Jeong et al., 2013). The Ca^{2+}/Calmodulin-dependent protein kinase III (CAMKIII), inhibits the elongation phase of translation by phosphorylating eukaryotic elongation factor-2 (eEF-2; Heise et al., 2014). Interestingly, CAMKIII protein levels are negatively regulated by isoproterenol stimulation of cAMP cascade. Degradation of the kinase requires the proteasome activity, linking the UPS to cAMP-dependent facilitation of protein translation (Wiseman et al., 2013).

A relevant role of cAMP-PKA axis in the epigenetic control of gene expression has been proposed. In eukaryotic cells, histone proteins are involved in the control of chromatin structure and remodeling. These are important mechanism(s) that cells adopt to regulate gene transcription (Tessarz and Kouzarides, 2014). Histones undergo to reversible post-translational modifications, such as acetylation. Acetylation/deacetylation cycles of histones are essential processes underlying gene expression and are catalyzed by families of histone acetyltransferases (HATs) and deacetylases (HDACs and sirtuins), respectively (Dekker and Haisma, 2009; Stasevich et al., 2014). Sirtuins have been implicated in a wide range of biological processes, such as transcription, DNA damage repair, and metabolism (Etchegaray et al., 2013). Sirtuin-6 (SIRT6) is a stress-induced gene that belongs to NAD^+-dependent Class III of histone deacetylases and controls the maintenance of telomere structure and length. By deacetylating histones, SIRT6 regulates genome stability and cell viability (Tennen et al., 2010). Loss of SIRT6 gene induces premature lethality and aging-related degeneration (Mostoslavsky et al., 2006). A link between sirtuins and cAMP signaling has been recently identified. Thus, cAMP stimulates ubiquitylation of SIRT6 protein and its consequent degradation through the proteasome. By reducing the levels of SIRT6, cAMP sustains radiation-induced apoptosis of lung cancer cells (Kim and Juhnn, 2015).

In neurons, cAMP-PKA signaling controls a variety of biological cues, as neurite outgrowth, morphogenesis and synaptic transmission and plasticity (Tasken and Aandahl, 2004). Most of the effects elicited by cAMP are mediated by a transcriptional control of gene expression. A post-translational mechanism of neurite extension which involves the UPS has been recently identified. Thus, neurotrophin-induced activation of PKA promotes praja2-dependent ubiquitylation and degradation of the neurite outgrowth inhibitor NOGO-A. By removing the inhibitory constrain of neurite extension imposed by NOGO-A, PKA-UPS drives a signaling circuit that promotes and sustains neuronal differentiation and synaptic activity (Sepe et al., 2014). In course of neuronal differentiation, phosphorylation of E3 ligases by PKA could also affect substrate recognition, switching the target selectivity between proteins with opposing functions. Thus, neurotrophin-stimulated phosphorylation of Smad ubiquitylation regulator factor 1 (Smurf1), a key component of TGF-β/BMP pathway, reduces degradation of polarity protein Par6 and enhances proteolysis of growth-inhibiting RhoA factor, eventually leading to axon outgrowth (Cheng et al., 2011).Similarly, in cisplatin-treated cancer cells, PKA phosphorylation of Smurf1 prevents degradation of the pro-apoptotic protein Nur77, triggering the mitochondrial apoptotic machinery (Lin et al., 2014).

Dephosphorylation of cellular substrates is mediated by distinct families of protein PPs, (Zhang et al., 2013). Among PPs, protein phosphatase 2 (PP2A) is a conserved Serine/Threonine phosphatase that regulates a wide number of signaling pathways. PP2A is composed of a dimeric core enzyme (structural A and catalytic C subunits), and a regulatory B subunit. In eukaryotes, C and A subunits of PP2A show high degree of sequence similarity, while the regulatory B subunits are highly heterogeneous. The assembly of the three subunits generates different PP2A holoenzymes, whose substrate specificity and intracellular localization are controlled by B subunits (Kiely and Kiely, 2015). PP2A dephosphorylates a variety of substrates, including cAMP-response element-binding protein (CREB). De-phosphorylation of CREB by PP2A attenuates cAMP-induced gene transcription (Wadzinski et al., 1993). PP2A is also involved in a variety

of cell functions, as proliferation, differentiation and cell death (Tsuchiya et al., 2014). During apoptosis, PP2A/C subunit is post-translationally regulated by the UPS. Thus, stimulation with tumor-necrosis factor-related apoptosis-inducing ligand (TRAIL) promotes the recruitment of PP2A/C, caspase-8 and Cullin3, a subunit of the cullin family of E3 ligases, into the death-inducing signaling complex (DISC). Within the complex, Cul3 targets PP2A/C for ubiquitylation and degradation by the proteasome. Downregulation of PP2A/C signaling and downstream gene transcription may account for the activation of the apoptotic machinery induced by TRAIL (Xu et al., 2014).

Dys-regulation of cAMP-UPS in Human Diseases

cAMP signaling is involved in a variety of different biological responses (Formosa and Vassallo, 2014). The complexity of the pathway and the high number of components involved ensure an efficient and a fine regulation of the signal transmission from the site of generation to downstream effectors. Genetic mutations or altered expression of any component of this sophisticated signaling cascade may lead to dys-regulation of the signaling, contributing to the onset and progression of human diseases.

In neurons, cAMP balance is crucial for physiological events underlying learning, memory and loco-motor activity (Gomez et al., 2002; Dell'Acqua et al., 2006). Several studies confirmed the pathogenic role of deranged cAMP signaling in neurodegenerative phenotypes (Satoh et al., 2009; Poppinga et al., 2014). As example, Huntington's disease (HD) is a genetic neurological disorder characterized by alteration of motor coordination that eventually leads to mental decline and behavioral symptoms. HD is caused by the expansion of a CAG repeat in the Huntington (HTT) gene, which induces accumulation of poly(Q)-expanded mutant HTT protein (mHTT; Labbadia and Morimoto, 2013). Accumulation of mHTT within neurons downregulates cAMP signaling, inhibits CREB-dependent gene transcription and profoundly affects neuronal activity and cell survival (Jeong et al., 2012). Recent evidence pointed to a role of mHTT in UPS-cAMP pathway. In particular, mHTT-mediated proteasome impairment inhibits the proteolytic turnover of R subunits within the striatum, increasing the R/C ratio and favoring reconstitution of inactive PKA holoenzyme. By limiting local activation of PKA, mHTT alters the stability of several proteins and impacts on neurons and loco-motor activity. Under these conditions, forced activation of PKA promotes phosphorylation of components of the proteasome (Rpt6) and rescues the impaired proteasome activity, favoring the removal of mHTT aggregates and improving loco-motor activity (Lin et al., 2013).

The UPS is an important control mechanism of cell growth, survival and metabolism. Removal of tumor suppressors or pro-apoptotic factors could, thus, play an important role in tumor growth. Changes in the levels, subcellular targeting or catalytic activity of the E3 ligases may exert major effect on cell growth and survival. Accordingly, dys-regulation of the UPS has been found in a wide array of human cancer (Landis et al., 1989; Weinstein et al., 1991; Palmer et al., 2000). Recent findings demonstrated that praja2, which regulates R subunit turnover, ubiquitylates and degrades MOB1, a core component of NDR/LATS kinase and positive regulator of the tumor-suppressor Hippo cascade (Hergovich, 2011; Lignitto et al., 2013). Removal of MOB1 by the praja2-UPS pathway attenuates the Hippo cascade and sustains glioblastoma growth *in vivo* (Lignitto et al., 2013). These findings uncover the existence of an intricate interplay between GPCR-cAMP signaling, UPS and tumor suppressor pathways in the control of cell proliferation and tumor growth.

Concluding Remarks

In the last decades, cumulative evidence uncovered a major role of PKA pathway in the control of important biological activities, ranging from differentiation, growth, metabolism, survival to more sophisticated brain activities. Derangement of the cAMP-PKA pathway has been pathogenically linked to the onset and progression of several neurodegenerative and proliferative disorders. So far, most of the cAMP-PKA effects have been attributed to phosphorylation/dephosphorylation events occurring at distal sites of cAMP generation. Emerging data suggest the existence of a cAMP-driven UPS circuitry that controls the turnover/stability of key elements of metabolic and proliferative pathways. At the same time, mounting evidence indicates that UPS by regulating the stability of components of the cAMP cascade controls directly the strength and duration of cAMP-PKA signals. Dys-regulation of this intricate interface between the cAMP and the UPS may underpin the pathogenesis of human diseases. Therefore, efforts are needed to discover new targets and mechanism(s) connecting the UPS to cAMP-PKA signaling, but also to construct a network that is able to predict and quantify the biological outcome (for example, degenerative versus proliferative phenotypes) of human genetic mutations affecting key elements of these transduction pathways. Understanding the complexity of such regulatory mechanisms and exploring further the biological significance of this kinase-ligase network will help to design novel tools and drugs that selectively restore a perturbed cAMP cascade in various human phenotypes.

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REVIEW
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Cardiac cAMP: production, hydrolysis, modulation and detection

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Cyclic adenosine 3′,5′-monophosphate (cAMP) modulates a broad range of biological processes including the regulation of cardiac myocyte contractile function where it constitutes the main second messenger for β-adrenergic receptors' signaling to fulfill positive chronotropic, inotropic and lusitropic effects. A growing number of studies pinpoint the role of spatial organization of the cAMP signaling as an essential mechanism to regulate cAMP outcomes in cardiac physiology. Here, we will briefly discuss the complexity of cAMP synthesis and degradation in the cardiac context, describe the way to detect it and review the main pharmacological arsenal to modulate its availability.

Keywords: GPCR, resonance energy transfer, phosphodiesterase, protein kinase A (PKA), Cyclic AMP

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Introduction

In cardiomyocytes, the influx of Ca^{2+} ions through voltage-dependent L-type Ca^{2+} channels (LTCC) plays an essential role in cardiac excitability and in coupling excitation to contraction of these cells. The depolarizing current through LTCC (ICa) contributes to the plateau phase of the cardiac action potential as well as to pacemaker activity in nodal cells (Shaw and Colecraft, 2013). This influx of Ca^{2+} triggers the release of intracellular stores of Ca^{2+} from the sarcoplasmic reticulum via the Ryanodine receptor (RyR), which results in activation of myofilaments contraction. Alterations in density or function of LTCC have been implicated in a variety of cardiovascular diseases, including atrial fibrillation (Van Wagoner et al., 1999) or heart failure (Mukherjee and Spinale, 1998). Cyclic adenosine 3′,5′-monophosphate (cAMP) is the main second messenger of the β-adrenergic receptor signaling inducing phosphorylation of the LTCC and the ryanodine receptor to increase the amount of intracellular Ca^{2+} necessary for heart contractility (responsible for positive chronotropic and inotropic effects during sympathetic stimulation) (Guellich et al., 2014). Moreover, catecholamine stimulated β-adrenergic receptor not only leads to cAMP effector dependent-troponin I phosphorylation to allow faster force development and shortening during systole and faster force relaxation and re-lengthening during diastole but also mediated cAMP effector dependent-phospholamban phosphorylation responsible for Ca^{2+} re-uptake in the sarcoplasmic reticulum and myofilament relaxation (lusitropic effects) (Bers, 2008). However sustained stimulation of this pathway may be detrimental thus leading to cardiac remodeling and development of heart failure (Brodde, 1993; Kiuchi et al., 1993). Thus, the proper physiological cardiac function relies on tight control of cellular cAMP concentration by fine-tuning the balance between cAMP synthesis and degradation. In mammalian cells, cAMP is produced by adenylyl cyclases (AC). Extracellular stimuli such as neurotransmitters, hormones, chemokines, lipid mediators, and drugs, can modulate AC activity to increase or decrease cAMP production by binding to a large number of transmembrane G protein-coupled receptors (GPCRs). The degradation of cAMP to AMP is catalyzed by phosphodiesterases (PDE) that are regulated by intracellular nucleotide concentrations, phosphorylation, binding

of Ca^{2+}/calmodulin and other regulatory proteins while cAMP efflux out of the cell is mediated by cyclic nucleotide efflux transporters. Over the years, several genetic models have been created to assess the role of the cAMP synthesis and hydrolysis proteins in cardiac physiology (**Table 1**). Once cAMP is produced it activates a set of diverse proteins, including

TABLE 1 | Cardiac phenotype for cAMP synthesis, hydrolysis and transporter proteins adapted from Guellich et al. (2014).

Class	Protein family	Substrate affinity	Protein	Cardiac function	Available model	References
AC			AC1	Modulates If pacemaker current		Mattick et al., 2007
			AC5	Regulates contractility β-adrenergic dependent	AC5 Knockout	Iwamoto et al., 2003; Okumura et al., 2003a,b; Tang et al., 2006
				Myocardardial contractility increases LV function increases heart rate, reduces inotropic, lusitropic and chronotropic response to β1 AR	AC5 Transgenic	Tepe et al., 1999; Esposito et al., 2008; Lai et al., 2013
			AC6	LV systolic and diastolic dysfunction LV contractility increases with βAR stimulation enhanced contractile function	AC6 Knockout AC6 Transgenic	Gao et al., 1999, 2008; Phan et al., 2007; Tang et al., 2008, 2010; Guellich et al., 2010
			AC8	Enhances basal intrinsic contractility	Cardiomyocyte specific AC8 Transgenic	Lipskaia et al., 2000
			sAC	Apoptosis of coronary endothelial cells heart rate increase	ADCY10 knockout	Kumar et al., 2009; M.G. Informatics[1]
PDE	PDE1	1–100 μM	PDE1A	Cardiomyocyte hypertrophy Cardiac fibroblast activation and cardiac fibrosis		Miller et al., 2011
			PDE1B		PDE1B Knockout	Yu et al., 1997; M.G. Informatics[2]
			PDE1C		PDE1C knockout mice	Vandeput et al., 2007; M.G. Informatics[3]
	PDE2	30 μM	PDE2A	PDE2 expression is increased in experimental heart failure L-type Ca2+ channel activity Contractility	PDE2A: embryonic death (EI7)	Hartzell and Fischmeister, 1986; Fischmeister et al., 2005
	PDE3	0.08 μM	PDE3A	Regulates β-adrenergic signaling, cardiac contractility, pacemaking, and output reduces cardiomyocyte apoptosis and prevents ischemia/reperfusion induced myocardial infarction cardiac contractility, LTCC activity	PDE3A knockout mice Cardiomyocyte PDE3A overexpressing mice	Tarpey et al., 2003; Ding et al., 2005a,b; Sun et al., 2007; Molenaar et al., 2013; Iwaya et al., 2014
	PDE4	1–4 μM	PDE4A	?	PDE4A knockout mice	Jin et al., 2005
			PDE4B	Arrhythmogenesis	PDE4B knockout mice	Leroy et al., 2011
			PDE4D	β-adrenergic signaling RyR2 hyperphosphorylation, arrhythmia	PDE4D knockout mice	Lehnart et al., 2005; Bruss et al., 2008
	PDE7	0.03–0.2 μM	PDE7A	?	PD17A Knockout	Yang et al., 2003
	PDE8	0.04–0.8 μM	PDE8A	Regulation of LTCC Ca^{2+} signaling, Ryr2 Ca^{2+} load	PDE8A knockout mice	Patrucco et al., 2010
Cyclic nucleotide efflux transporter			ABCC4	Enhances contractility and cardiac hypertrophy	MRP4 Knockout mice	Sassi et al., 2012
			ABCC5	?		

This table summarized the main cardiac functions and available model for the different class of proteins regulating cAMP availability: references for ACs (AC1, Mattick et al., 2007; AC5 KO, Iwamoto et al., 2003; Okumura et al., 2003a,b; Tang et al., 2006; AC5 Tg, Tepe et al., 1999; Esposito et al., 2008; Lai et al., 2013; AC6 KO, Tang et al., 2008, 2010; AC6 Tg, Gao et al., 1999, 2008; Phan et al., 2007; Guellich et al., 2010; AC8, Lipskaia et al., 2000; sAC, Kumar et al., 2009; M.G. Informatics), references for PDEs (PDE1A, Miller et al., 2011; PDE1B, Yu et al., 1997; M.G. Informatics; PDE1C, Vandeput et al., 2007; M.G. Informatics; PDE2A, Hartzell and Fischmeister, 1986; Fischmeister et al., 2005; PDE3A, (Tarpey et al., 2003; Ding et al., 2005a,b; Sun et al., 2007; Molenaar et al., 2013; Iwaya et al., 2014); PDE4A, Jin et al., 2005; PDE4B, Leroy et al., 2011; PDE4D, Lehnart et al., 2005; Bruss et al., 2008; PDE7, Yang et al., 2003; PDE8, Patrucco et al., 2010) and references for cyclic nucleotide transporter (ABCC4, Sassi et al., 2012).

[1]http://www.informatics.jax.org/marker/MGI:2660854. Informatix JAX.
[2]http://www.informatics.jax.org/marker/MGI:97523. JAX.
[3]http://www.informatics.jax.org/reference/marker/MGI:108413. JAX.

cAMPdependent-protein kinase (PKA) or cAMP-dependent exchange proteins (Epac), the two main cAMP effectors to mediate downstream signaling as well as cyclic nucleotide-gated ion channels (CNGC) and POPDC proteins (Beavo and Brunton, 2002). From the compartmentation hypothesis proposed by Brunton et al., in which cAMP microdomains are distinctly coupled to cellular functions (Brunton et al., 1981), a variety of technologies has been developed to study *in vivo* the different localizations and organization around macromolecular complexes to ensure a fine-tuned spatio-temporal compartmentation of cAMP production (for detailed reviews (Baillie, 2009; Edwards et al., 2012; Perera and Nikolaev, 2013). Recently, these tools led to the identification of a β2-adrenergic-dependent cAMP compartmentation defect in failing cardiomyocytes (Nikolaev et al., 2010). In this review, we will focus on cAMP in synthesis and hydrolysis in cardiology, the way to detect it and how to manipulate this cAMP pathway.

cAMP in the Cardiac Tissue

cAMP Synthesis

Adenylyl cyclases (AC) are ubiquitous enzymes that catalyze the conversion of Adenosine triphosphate (ATP) into cAMP and pyrophosphate. ACs structure consists in 12 transmembrane domains divided into 2 hydrophobics domains (6 transmembrane domains each) and 2 main intracellular loops called C1 and C2 that naturally dimerize to form the catalytic domain (**Figure 1A**). In mammals, 9 transmembrane and 1 soluble AC (sAC) encoded by different genes have been identified and have different regulatory mechanisms (Willoughby and Cooper, 2007). Mammalian ACs are strongly activated by Mn^{2+} or Mg^{2+} (Tesmer et al., 1999) and inhibited by millimolar concentrations of free Ca^{2+} probably acting as a Mg^{2+} competitor (Mou et al., 2009) but at submicromolar concentrations, Ca^{2+} can activate AC via calmodulin (CaM) throught its binding to a putative helical structure on the C1b region (Halls and Cooper, 2011). More precisely AC1, 3, and 8 are Ca^{2+}/CaM sensitive isoforms which localize in lipid rafts while AC2, 4, 7, and 9 are Ca^{2+}/CaM insensitive and are excluded from these membrane domains (Willoughby and Cooper, 2007). On the contrary, biochemical studies on membrane preparations revealed that AC5 and AC6 could be inhibited by Ca^{2+} (independently of CaM) in the submicromolar range (Guillou et al., 1999; Hu et al., 2002). Along with nitric oxide (NO), Hydrogen sulfide (H2S) is a biological gaseous transmitter able to modulate cAMP production. Using NO donors, the gasotransmitter NO is thought to attenuate forskolin-stimulated AC5 and AC6 isoforms activities without altering their basal activity on membrane from rat striatum (Hudson et al., 2001). Even if H2S is a poisonous gas used as a chemical reagent, H2S is endogenously formed in mammalian cells from cysteine by the action of cystathionine β-synthase with serine as a by-product at a concentration around 50–160 μmol/L (Goodwin et al., 1989). In the central nervous system, H2S enhances NMDA receptor-mediated response via cAMP production (Kimura, 2000) while in cardiac context it can also suppressed AC activity and, therefore, decreased forskolin-stimulated cAMP accumulation in different cell lines and tissue (Lim et al., 2008; Yong et al., 2008). In the cardiomyocytes, expression of AC1, AC5, AC6, AC8, and sAC has been detected and most function as modulators of inotropic and chronotropic β-adrenergic receptor (β-AR) signaling output (**Table 1**) but AC5 and AC6 represent the dominant isoform (Defer et al., 2000). Along with AC distribution within membrane microdomains (Efendiev and Dessauer, 2011), cAMP synthesis is spatially restricted by localization of activating receptors like β1-adrenergic receptors or β2-adrenergic receptors at caveolae or non-caveolae plasma membrane domains (Rybin et al., 2000; Ostrom et al., 2001).

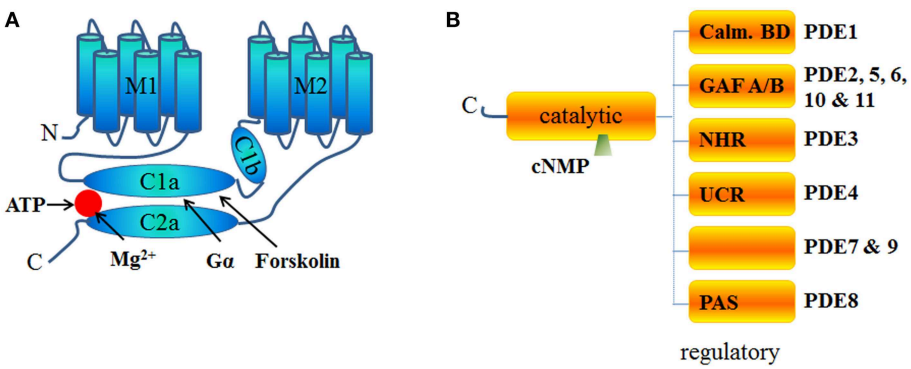


FIGURE 1 | Schematic structure of ACs and PDEs. (A) Structure of Adenylyl cyclase is comprised of 2 transmembrane domains (M1 and M2 6 helixes each) and 2 cytosolic domains (C1 and C2) subdivided into a and b domains. C1 and C2 contain the catalytic core, the Gα and the forskolin binding sites and other regulatory sites. C2b domain is almost inexistent in all AC isoforms. **(B)** PDEs are homodimers with the exception of PDE1 and PDE6 (usually heterotetramers). PDEs have an NH_2-terminal regulatory domain and share a conserved catalytic domain located in the COOH-terminal portion of the protein. The structure of the regulatory domain varies according the PDE isoform. GAF is an acronym for cGMP-specific PDE, Adenylyl cyclases and FhlA, NHR for N-terminal Hydrophobic Region, PAS for Per-ARNT-Sim and Calm. BD for calmodulin binding domain. No known domains are present in PDE7 or PDE9 regulatory C-terminal part. PDE4 proteins are classified as "long" or "short" isoforms, depending on the presence or absence of two highly conserved domains, Upstream Conserved Region 1 (UCR 1) and Upstream Conserved Region 2 (UCR 2) which interact to form a regulatory module that may influence catalytic activity by a PKA-dependent phosphorylation mechanism (Houslay, 2001; MacKenzie et al., 2002).

Moreover, specific A-kinase anchor proteins (AKAP) complexes (Kapiloff et al., 2014) have been identified as a potential molecular mechanism for the formation of specific cAMP microdomains (Kapiloff et al., 2014). The AKAPs constitute signaling hub proteins that scaffold on a same membrane domain the AC and the regulatory subunit of protein kinase A (PKA) cAMP effector, thus confining the enzyme activity to discrete locations within the cell. Cardiac myocytes exhibit at least four distinct AKAP complexes: AKAP79/150 (aka AKAP5) with AC5/6 (Nichols et al., 2010); mAKAPβ (aka AKAP6) with AC2/5 (Kapiloff et al., 2009), YOTIAO (aka AKAP9) with AC2/9 (Piggott et al., 2008), and AKAP18δ with PKA (Fraser et al., 1998) (**Figure 2**).

cAMP Elimination: Phosphodiesterases and Cyclic Nucleotide Efflux Transporters

Phosphodiesterases

Cyclic AMP is hydrolyzed exclusively by cyclic nucleotide PDEs classified in 11 families and encoded by at least 21 different genes with the existence of some splice variants (Omori and Kotera, 2007). PDEs are structured around a catalytic domain containing the cyclic nucleotide binding site conserved across all families and a regulatory N-terminus varying according to the different PDEs (**Figure 1B**). In the heart, 8 PDE families have been described: PDE1; PDE2, PDE3, PDE4, PDE5, PDE7, PDE8, and PDE9. Among them, PDE1, PDE2, and PDE3 are dual-specificity enzymes that can hydrolyze both cAMP and cGMP while PDE4, PDE7, PDE8 selectively hydrolyze cAMP and conversely PDE5, PDE9 selectively hydrolyze cGMP. Of the cAMP-hydrolyzing PDEs expressed in the heart, cGMP inhibits PDE3 and possibly PDE1, whereas PDE2 is activated by cGMP (detailed review in Zaccolo and Movsesian, 2007). Jurevicius and Fischmeister (1996a,b) provided the first direct evidence for PDE-mediated cAMP signaling compartmentation, showing that PDE inhibition allowed local β-adrenergic stimulation to enhance Ca^{2+} currents in frog ventricular myocytes (Jurevicius and Fischmeister, 1996a,b). Later on, imaging approaches confirmed that PDEs play a key role in shaping the intracellular cAMP gradient in rat neonatal cardiomyocytes (Zaccolo et al., 2000; Zaccolo and Pozzan, 2002). Like ACs, PDEs have also been shown to be compartmented by AKAPs complexes. Thus, specific cAMP hydrolysis-based PDE4 enzyme were shown to interact with mAKAP for PDE4D3 (Dodge et al., 2001); AKAP9 for PDE4D3 (Taskén et al., 2001), AKAP95 (aka AKAP8) for PDE4A (Asirvatham et al., 2004), AKAP149 for PDE4A (Asirvatham et al., 2004) (**Figure 2**).

Cyclic Nucleotide Efflux Transporters

In addition to PDEs and ACs, the intracellular concentration of cAMP is regulated by its efflux into the extracellular space through a specific transmembrane transport system named multidrug resistance proteins (MRP) (Cheepala et al., 2013) that belongs to the ATP-binding cassette (ABC) transporter

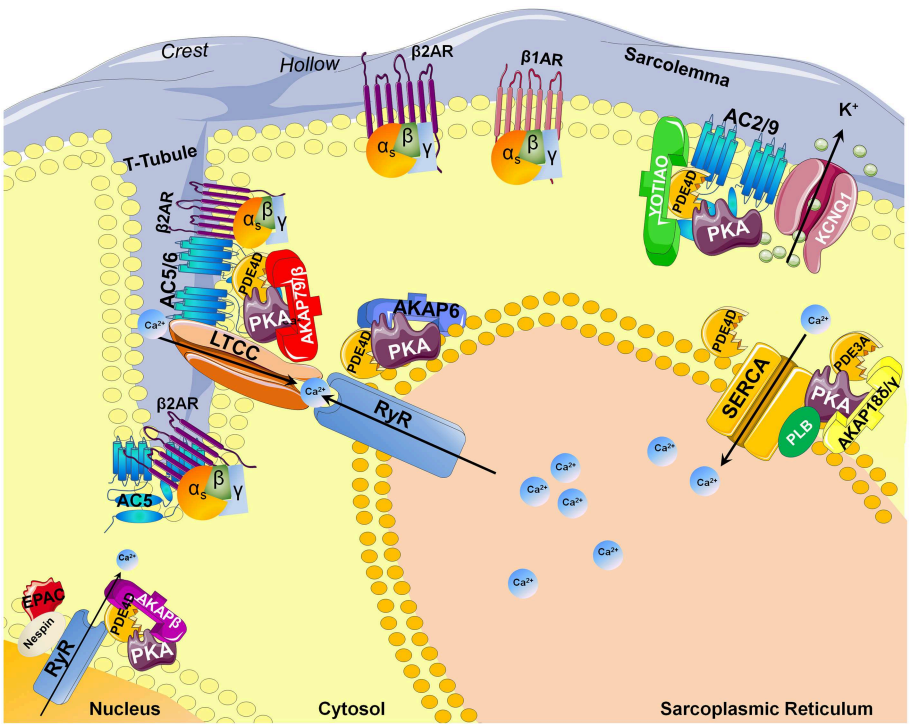


FIGURE 2 | AKAP-dependent AC and PDE compartmentalizations in the cardiomyocyte. Abbreviations stand for: AKAP, A-kinase anchor proteins; PKA, Protein Kinase A; β2AR/β1AR, beta adrenergic receptor; PLN, Phospholamban; EPAC, cAMP-dependent exchange proteins; AC, Adenylyl cyclase; RyR, Ryanodine Receptor; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; KCNQ1, potassium channel voltage gated KQT-like subfamily Q; PDE, Phosphodiesterase; T-tubule, Transverse tubule; LTCC, L-type calcium channel.

superfamily (subfamily C). Three of them (MRP4 aka ABCC4, MRP5 aka ABCC5, and MRP8 aka ABCC11) have the ability to actively extrude cAMP and cGMP from the cell (Kruh and Belinsky, 2003) and in cardiac myocytes, MRP4 has been shown to enhance cAMP formation, contractility, and cardiac hypertrophy (Sassi et al., 2012). The compartimentation of MRPs expression may also play an important role in the intra- and extracellular cAMP signaling processes. For instance, caveolin-rich membrane MRP4 localization (Sassi et al., 2008) could explain the local MRP4-modulated contraction of cardiac myocytes induced by activation of β-adrenoceptor (Sellers et al., 2012).

cAMP in Heart Failure

Heart failure (HF) occurs when the heart is unable to pump sufficiently to maintain blood flow to meet the body's needs. Around 2% of adults have HF and this percentage increases to 6–10% for people over the age of 65 (McMurray and Pfeffer, 2005). The HF syndrome arises as a consequence of an abnormality in cardiac structure, function, rhythm, or conduction. As stated in introduction, cAMP primarily, but not exclusively, controls beating frequency, force of contraction and relaxation, essentially through the β-adrenergic signaling pathway. This pathway is necessary for the beneficial effects of catecholamines on cardiac contractility. During heart failure set up, increased sympathetic activity drives the βAR overstimulation in cardiomyocytes, thus promoting higher intracellular cAMP signals for compensatory cardiac function in the heart (Baker, 2014). However, chronic βAR stimulation and uncontrolled cellular cAMP signals have been shown to affect heart function in a much more detrimental way responses such as cell apoptosis (Zhu et al., 2003) and the loss of pump function (Michel et al., 1990; Engelhardt et al., 1999; Lohse et al., 2003), ultimately leading to HF setup. During the ongoing of the disease, a down-regulation of β1AR expression (Nikolaev et al., 2010) is correlated with a modulation of Gαi proteins (Eschenhagen et al., 1992a,b) expression to attenuate cAMP synthesis. The ratio between β1AR and β2AR converts the latter to be the major βAR subtype in failing hearts. Interestingly, associated with this receptor expression imbalance, the β2AR dominant-induced cAMP signal is broadly distributed in the failing heart (Nikolaev et al., 2010) compare to a compartmentalized cAMP signal in physiological condition. The functional output of this broadly distributed cAMP signal in modulating contractile properties in failing hearts has to be studied. In failing cardiomyocytes, chronic β2AR stimulation also promotes CaMKII-dependent contractile responses which has a pronounced role in promoting the development of cardiac hypertrophy, myocyte apoptosis, cardiac dysfunction and arrhythmias by causing sarcoplasmic reticulum Ca^{2+} overload (Anderson et al., 2011). Inhibition of CaMKII is able to ameliorate cardiac remodeling and reduce cardiac arrhythmias after myocardial infarction (Zhang et al., 2005). Although the direct link between cAMP and CaMKII is still missing, the detrimental CaMKII activity in cardiomyocytes could be related to dysregulation of distribution of cAMP signals under chronic βAR stimulation. HF is a complex process where the various components in the cAMP signaling pathway constitute potential pharmacological targets.

Modulation of cAMP Concentration in the Cardiac Tissue

Modulation of cAMP Production

Targeting Adenylyl Cyclases

Pharmacological AC activators

The most prominent AC activator is forskolin (FSK). FSK, a diterpene extracted from the plant *Coleus forskohlii*, directly activates all AC isoforms except AC9. Despite a strong hydrophobic property, its action is not limited to the native membrane-bound form of the enzyme since it can readily stimulate some synthetic soluble ACs. FSK binds to the same cleft that contains the active site of AC (Tesmer et al., 1997) where it glues together its two cytoplasmic domains (**Figure 1A**) by a combination of hydrophobic and hydrogen-binding interactions (Zhang et al., 1997). Based on equilibrium dialysis experiments of the C1 and C2 domains of type AC5 and AC2, respectively, the C1/C2 complex binds only one Gsα, one ATP, and one FSK molecule (Dessauer et al., 1997). However, FSK has been shown to also inhibit a number of membrane transport proteins and channel proteins like Glucose transporter or voltage dependent K^+ channel (Laurenza et al., 1989). As Protein kinase C (PKC) activates AC2 by phosphorylating it on Thr-1057 (Böl et al., 1997), another alternative, but more restrictive one to activate AC, relies on the use of Phorbol 12-myristate 13-acetate (PMA) a phorbol diester and a potent tumor promoter known to activate PKC signaling. Thus, PKC-dependent phosphorylation of AC-C1 domain induces AC activation (Ebina et al., 1997). However, one must be cautious on the use of PMA as a specific AC-activator since PMA has also been reported to have actions on non-kinase proteins including chimaerins, RasGRP, and Unc-13/Munc-13 (Han and Meier, 2009; Kazanietz et al., 1995).

Pharmacological AC inhibitors

As schemed in **Figure 1A**, ACs are structured around 2 hydrophobic domains and 2 main intracellular loops containing the catalytical domain and the diterpene regulatory site. Based on this structure, ACs inhibitors can be divided into 4 groups (reviewed in Seifert et al., 2012): (i) the inhibitors competing with the ATP at the catalytic site like MANT-GTP (Gille and Seifert, 2003), (ii) the uncompetitive P-site inhibitors like 2′,5′-dideoxyadenosine-3′-tetraphosphate Vidarabine [aka 9-β-D-arabinofuranosyladenine (ara-A)] (Seifert, 2014) or NKY80 (a cell-permeable quinazolinone) to name a few, which work by stabilizing a pyrophosphate-bound transition state (Dessauer et al., 1999; Onda et al., 2001), (iii) the allosteric non-competitive inhibitors targeting the diterpene regulatory site like BODIPY-FS in presence of divalent cations (Erdorf et al., 2011), and (iv) the allosteric non-competitive inhibitors targeting alternated and unknown site like calmidazolium (Haunsø et al., 2003). Even though some specificity has been assigned to some of the molecules listed, to our knowledge, those inhibitors have not been accurately examined at all ACs isoforms, thus preventing

any formal conclusion only based on their use to assess the involvement of AC activity.

Targeting G-protein Coupled Receptor Signaling

We previously mentioned that cAMP constitutes the master second messenger of β-adrenergic receptor signaling which belong to the G protein-coupled receptors (GPCRs) family. According to conventional knowledge, 7 transmembrane GPCRs at the plasma membrane convert extracellular signals into intracellular ones through canonical heterotrimeric G proteins which transduce signals from GPCRs to secondary effectors thus leading to the second messengers production and the propagation of the signal through ensuing regulation of numerous downstream intracellular signaling targets (Gilman, 1987). G proteins localized on the cytoplasmic side of the plasma membrane and are composed of a guanine nucleotide binding α subunit (Gα) and a βγ dimer (Gβγ), both constitutively associated in the G protein inactive state. Upon GPCR activation, the Gαβγ protein associates with the receptor thus allowing GDP/GTP exchange on the Gα GTPase domain, leading to subsequent Gα-GTP and Gβγ dissociation both regulating downstream specific signaling targets (Denis et al., 2012). Intrinsic GTPase activity of the Gα then allows GTP hydrolysis and to turn off the G protein activity to its initial inactive Gαβγ associated state. G proteins have been classified into five subfamilies (Gi/o, Gs, Gq/11, and G12/13) according to the secondary effector of the Gα subunit (Denis et al., 2012). Thus, isoforms of the Gαi/o family classically inhibit ACs and cAMP production while, conversely, isoforms from the Gαs family activate ACs to favor cAMP production. It follows that modulation of the activity of cardiac expressed Gαi- or Gαs-coupled receptors either through the use of selective GPCR agonists and antagonists or G proteins activators or inhibitors will directly alter the G protein activity and cAMP availability.

GPCR agonists and antagonists

In the human genome, it is estimated that the GPCR superfamily consists in ∼600–1000 receptors (Lander et al., 2001; Vassilatis et al., 2003; Fredriksson and Schioth, 2005) where ≈ 200 have known cognate agonists and the larger part are still "orphan," i.e., without yet identified agonists (Vassilatis et al., 2003). Evaluation of GPCR expression *in vivo* has been largely hampered by lack of specific antibodies against this class of receptors. Thus, over the years, microarray technology allowed researchers to monitor the mRNA expression levels of thousands of GPCRs encoding genes. Based on the available genomic data (Hakak et al., 2003; Katugampola and Davenport, 2003; Tang and Insel, 2004; Regard et al., 2008; Moore-Morris et al., 2009), we tried to summarize the different GPCRs detected in the whole cardiac tissue (cardiomyocytes, endothelial cells, fibroblasts…) (**Table 2**), their classical G protein coupling and a selective agonist/antagonist for most of them. This list is non-exhaustive and selectivity or description of these compounds will not be detailed here. Thus, selective pharmacological targeting of Gαi- or Gαs-coupled cardiac receptors represents a way to modulate intracellular cAMP levels. It is noteworthy that the classical GPCR coupling has to be enlarged as a recent study shows dual agonist occupancy of the AT1-R and α2C-AR heterodimer, two GPCRs known to be coupled to Gαq and Gαi, respectively, created an original conformation different from the active individual protomers and triggered an atypical Gs/cAMP/PKA signaling (Bellot et al., 2015). Thus, co-stimulation or bivalent ligand development might be a new pharmacological area to regulate cAMP signaling (Berque-Bestel et al., 2008; Lezoualc'h et al., 2009).

Gα activators

Cholera toxin (CTX) is a specific Gαs potent activator secreted by the bacteria *Vibrio cholerae* which catalyzed the ADP-ribosylation of the Gαs proteins. The ADP-ribosylation blocks the Gαs catalytic activity and thus prevents the Gαs subunit to hydrolyze the GTP once activated, leading to the ensuing sustained Gs and AC activity (De Haan and Hirst, 2004). CTX administration in non-ischemic or ischemic heart contributes to the genesis of arrhythmia highlighting the essential role for Gαs in the regulation of cardiac physiology (Huang and Wong, 1989). More recently, *Pasteurella multocida* toxin (PMT), produced by toxigenic strains of the Gram-negative *Pasteurella multocida* bacteria, was identified as a potent and selective activator of Gαq, Gαi, and Gα13 by deamidating a glutamine residue in the switch II region of the Gα-GTPase domain (Orth et al., 2005, 2008). It was recently shown that, *in vivo,* PMT treatment in mice increased secretion and expression of connective tissue growth factor (CTGF) in cardiac fibroblasts to aggravate cardiac hypertrophy and fibrosis (Weise et al., 2015).

Gα inhibitors

Basically, all Gα subunits inhibitors share a common molecular mechanism by preventing the GDP/GTP exchange on the Gα-GTPase domain. A famous specific and highly effective Gαi inhibitor is Pertussis Toxin (PTX). PTX is a protein complex released by the bacterium *Bordetella pertussis* in an inactive form. PTX catalyzes the ADP-ribosylation of the Gαi subunit of the heterotrimeric G protein. The Gαi subunit remains locked in its GDP-bound inactive state, thus unable to interact with the receptor and to inhibit adenylyl cyclase activity (Hsia et al., 1984; Burns, 1988). PTX-pretreatment is classically used to delineate the involvement of Gαi-dependent signaling. It revealed for instance an increase in β-AR dependent inotropic response and cAMP accumulation in isolated ventricular cardiomyocytes (Melsom et al., 2014), confirming the dual coupling of β2-AR to both Gαi and Gαs (Xiao, 2001) in the cardiac tissue. On a purified Gα activity assay, suramin, an antimicrobial drug, was identified as a more selective inhibitor for Gαs (IC_{50} ≈ 250 nM) than for Gαo (IC_{50} ≈ 2 μM) or Gαi (IC_{50} ≈ 5 μM). Suramin exerts its effects by binding the effectors binding site on the Gα proteins (Freissmuth et al., 1996). It has to be noted that suramin is a large highly sulfonated and negatively charged molecule that limits its use to *in vitro* studies as it cannot cross the cell plasma membrane. Hohenegger and coworkers worked on suramin derivatives to increase specificity toward Gαs and identified two compounds (NF449 and NF503) that suppress the Gαs activation coupled to β-adrenergic receptors, whereas they affect the Gαi/Gαo- and Gαq-coupled receptors

TABLE 2 | GPCR expressed in heart: Gα coupling and pharmacological way to modulate their signaling.

Receptor	Subtype	Coupling	Example of agonist	Antagonist
α-Adrenergic	Ia, Ib, 1D,	Gq	Phenylephrine, Methoxamine	Corynanthiane, Prazosin
	2a, 2c	Gi	UK14304, B-HT92O	Yohimbine, RX821002
β-adrenergic	β1, β2, β3	Gs, Gi	Isoproterenol	Alprenolol, Pindolol, Propranolol
Adenosine	Adoral,	Gi	CHA, CPA	CPX, CPT, N-0840
	2a,2b	Gs	CGS21680, DPMA, HENECA	KW6002, Alloxazine, SCH-58261
Adrenomodulin	CGRP	Gi, Gs		
Angiotensin	AT1a	Gq, Gi	AngII, AngIII L162313	Losartan, Candesartan
	AT2	Gi	AngII, AngIII CGP42112A	PD123319, L-159686
Apelin	APJ	Gi	Apelin13	
	AVPR1a	Gq	vasopressin	Relcovaptan
Bradykinin	Bdkrbl, 2	Gq	Lys-BK	B9430
Calcium sensing	Ca-SR	Gs		
Cannabinoid	CB1	Gi	THC, CP-5594O, Nabilone	SR141716A, AM25I
Chemokine receptor	CX3CR1,	Gi	Fractalkine	
	CXCR2	Cri	IL-8, GCP2	
	CXCR4,7	Gi	SDF1α	AMD3100
	CXCR6	Gi	CXCL16	
	CCBP2,	Gi		
	CCR1, 5	Gi	MIP1α	
	CCR2	Gi	MCP-1	
	CCR10	Gi	CTACK	
	XCR1	Gi	xCL1	
Complement component receptor	C3aRl, C5R1	Gi		
Corticotropin releasing hormone	CRHR2	Gs	CRF, UCN1	Astressin
Cysteinyl leukotriene	Cystl1	Gq	LTD4	Cinalukast
Dopamine	Drd2	Gi	U-91356A, TNPA	L-741626
	Drd3	Gi	PD128907, BP897	Nafadotride, GR103691
Endothelin	ET-A, ET-B	Gq	ET1	PD142893
Frizzled	Fzdl, 2, 3, 5, 6, 7, 8			
Galanin	GalR2	Gq	Galanin, GALP	Galantide
GLP1		Gs		
Glucagon		Gq, Gs		
Gonadotropin receptor	LGR6, 7	Gi, Gq		
GPCR5	Raig2, GPCR5c	?		
Growth hormone secretagogue-receptor	GHS-R1a	GS, Gq		
Histamine	H1	Gq		Pyrilamine
	H2	Gs	Amthamine	Cimetidine
	H3	Gi	Immethridine	Ciproxifan
Latrophilin	Lphn1, 2	011-15	alpha-Latrotoxin	
Mas	Mas1, GPR168	?		
Melanin concentrating hormone	SLC-1	?	MCH	SNAP794I
Melanocortin	MC3R	Gs	γ2-MSH	SHU9119
Melatonin	MT1	Gi, Gq	Melatonin, S20098	Luzindole
	MT2	Gi	Melatonin, S20098	Luzindole
Muscarinic	M2, M3	Gi, Gq	Bethanecol, Xanomeline, Metoclopramine	Gallamine, Atropine, Scopolamine
Neuromedin U	NMUl, NMU2	Gi, Gq	NMU	
Neuropeptide Y receptor	NPY1, 2	Gi	NPY	BIBP3226, BIIE0246
Nucleotide	P2Y1	Gq	2MeSADP	BzATP, Suramin
	P2Y2	Gq, Gi	UTPγs	Suramin
	P2Y4	Gq, Gi	UTP	ATP

(Continued)

TABLE 2 | Continued

Receptor	Subtype	Coupling	Example of agonist	Antagonist
	P2Y5	Gi, G12/13		
	P2Y6	Gq	UDP	Suramin
	P2Y11	Gq, Gs	BzATP	Suramin
	P2Y13	Gi	2MeSADP	Ap4A
	P2Y14	Gi	UDP-glucose	
Opioid	MOP	Gi	DAMGO	Cyprodime
	DOP	Gi	DPDPE	Naltrindole
	KOP	Gi	Enadoline	GNTI
Opsin	Opn4	Gi, Gq		
Oxytocin	OXTR	Gq	Oxytocin, Carbetocin	
P518RF amide	SP9155	?	P518	
Vasoactive Intestinal Peptide receptor	VIPR2	Gs, Gq	VIP	acetyl-His-PheLysArg-VIP-GRF
PAR	PAR1	Gi, Gq, G12/13	Thrombin, Trypsin	BMS200261
	PAR2	Gi, Gq	Trypsin	
	PAR4	Gq	Thrombin, Trypsin	t-cinnamoylYPGKF
Platelet-activating factor receptor	PTAFR	Gq	PAF	Israpafant
Prolactin releasing peptide	GR3			
Prostacyclin	Ptgir	Gs		
Prostanoid	EP1	Gq	Iloprost	SH-19220
	EP4	Gs	ONO-AE1-734	AH23848
Relaxin-H2	LGR7, Rxfp4	Gi		
Serotonin	5HTR1a	Gi	R(+)-8-OH-DPAT	Spiperone
	5HTR1b	Gi	Sumatriptan, CGS12066	GR55562, SB216641
	5HTR2b	Gq	BW723C86	YM348
	5HT4	Gs	BIMU8	GR113808
Smoothened	Smoh	Gi		
Somatostain	SSTR3	Gi	L-796778	NVP-ACQ090
	SSTR4	Gi	NNC26-9100	s
Sphingosine	Edg1	Gi	S1P, FTY720-P	VPC23019
	Edg5	Gq,Gi G12/13	S1P	JTE-013
	Edg3	Gq, Gi, G12/13	S1P, FTY720-P	VPC23019
LPA	Edg2	Gi, G12 13	1-oleyl-LPA	VPC32183
Substance P	NK-1	Gq	substance P	GR-82334
Thromboxan	Tbxa2r	Gq	Thromboxan	Seratrodast
Urotensin	GPR14	Gq	UII	$[Cha^6]U\text{-}II_{(4\text{-}11)}$
Other GPCR expressed in Heart				
CD97	GPR77(C5L2)			
ELTD1	GPR82			
EMR1	GPR107			
TM7SF3	GPR108			
GPR1	GPR116			
GPR10	GPR120			
GPR17	GPR124			
GPR2l	GPR125			
GPR22	GPR133			
GPR27	GPR135			
GPR30	GPR137			
GPR31	GPR137b			

(Continued)

TABLE 2 | Continued

Other GPCR expressed in Heart	
GPR34	GPR146
GPR4	GPR153
GPR44	GPR161
GPR48	GPR175
GPR54	GPR182
GPR56	GPR183 (Ebi2)

This table summarized the list of mRNA encoding for GPCRs detected in the whole cardiac tissue (cardiomyocytes, endothelial cells, fibroblasts…) extracted from (Hakak et al., 2003; Katugampola and Davenport, 2003; Tang and Insel, 2004; Regard et al., 2008; Moore-Morris et al., 2009), the main known Gα protein coupling, an example of an agonist and an antagonist (this list is non-exhaustive and selectivity for each molecule is not discussed).

(A1-adenosine and angiotensin II receptor, respectively) to a much lesser extent (Hohenegger et al., 1998). Lately, BIM-46174 and BIM-46187 were classified and used as pan Gα inhibitors targeting Gαs, Gαq/11, Gαi/o, and Gα12/13 family (Prévost et al., 2006). However, these cell permeable compounds have not be tested toward all the individual members of Gα subunit family and more recently Kostenis and colleagues found that BIM-46187 was more selective to inhibit Gαq depending on the cellular context (Schmitz et al., 2014).

Gβγ complex inhibitors

Smrcka and coworkers described small molecule Gβγ inhibitors that selectively block Gβγ-binding interactions to their effectors, including M119 and its highly related analog, gallein (Lehmann et al., 2008). These compounds blocked interaction of Gβγ and GRK2 *in vitro* and reduced β-AR–mediated membrane recruitment of GRK2 in isolated adult mouse cardiomyocytes (Casey et al., 2010). The authors showed M119 enhanced both adenylyl cyclase activity and cardiomyocyte contractility in response to β-AR agonist (Casey et al., 2010). More recently, in a screen for the identification of OXE receptor antagonists, Gue1654 was discovered as a biased inhibitor that selectively prevent Gβγ signaling without affecting the Gα pathway (Blättermann et al., 2012). The molecular mechanism underlying Gue1654 action is still under investigation.

Targeting Tyrosine Kinase Receptor Signaling

It has to be noted that AC have been involved in the mechanisms of action of insulin and other peptides of the insulin superfamily like Insulin-like Growth factor I, relaxin and mollusc insulin-like peptide which are ligands for tyrosine kinase receptors (TKR) (Pertseva et al., 2003). Earlier, it was shown that in the heart EGF, another TKR, triggered some AC mediated effect (Nair and Patel, 1993). At a molecular level, TKR dependent activation of AC can rely on the activation of PI3K, PKCζ, or the Gβγ complex (Wilson et al., 1996; Standaert et al., 1997; Molina-Munoz et al., 2006). Thus, modulating activities of TKRs and their signaling regulators constitute an alternative approach to modulate cAMP but such compounds will not be described in this review.

Modulation of cAMP Degradation

Phosphodiesterases Inhibitors

The cardiostimulatory action of PDE make their inhibition as a promising therapeutic approach for the treatment of heart failure by sustaining cAMP production and action. Methylated xanthines, like theophylline, caffeine, or Iso-butyl-methyl-xanthyl (IBMX), are long known to act as competitive nonselective PDE inhibitors (Hess et al., 1975) but they also exhibit nonselective PDE action like adenosine receptor antagonist activities (Ukena et al., 1986). Over the years, several more specific and selective PDEs inhibitors have been developed. Representative selective inhibitors that can be used in cardiac tissue are listed below. Originally, 8-MM-IBMX was thought to be PDE1 selective (Rybalkin et al., 2002), but an extensive *in vitro* study characterized more potent and more selective compounds able to inhibit PDE1 activity like SCH51866 (Dunkern and Hatzelmann, 2007). The first specific inhibitor developed for PDE2 was EHNA [erythro-9-(2-hydroxy-3-nonyl)adenine] with an IC50 value of ∼1 μM (Podzuweit et al., 1993) but a screen of compounds developed by Bayer showed that BAY60-7550 (an EHNA analog) was 100-fold more potent and 50-fold more selective for PDE2A over other PDEs compared to EHNA (Boess et al., 2004). Cilostamide-dependent PDE inhibition was discovered in 1970's (Hidaka et al., 1979) but cilostamide and its derivative selectivity for PDE3 family was described by Sudo et al. (2000). The prototypical PDE4 inhibitor is rolipram; originally named ZK62711, that was discovered in 1976 (Schwabe et al., 1976) but its use was limited by its associated side effects, particularly those affecting the gastrointestinal tract (Barnette and Underwood, 2000). Thus, in 2010, potency and selectivity of roflumilast and its active metabolite have been studied for all PDE (Hatzelmann et al., 2010). Roflumilast does not affect PDE enzymes apart from PDE4 family, and has a subnanomolar inhibitor activity toward all PDE4 splicing variants tested (Rabe, 2011). As the PDE4 family is encoded by 4 genes (PDE4A, B, C, or D) and 27 splice variants, identification of selective PDE4 subtypes inhibitors has been boosted and especially for PDE4B that can be selectively inhibited for example by triazine derivative (Hagen et al., 2014). ASB16165 was characterized as a specific and highly potent inhibitor for PDE7A with an IC50 value of 15 nM for human PDE7A (Kadoshima-Yamaoka et al., 2009). PDE8s are inhibited by dipyridamole, despite this drug is also known as a relatively nonselective cGMP specific PDE5 inhibitor (Soderling et al., 1998) while two studies have described a newly available PDE8 inhibitor developed by Pfizer, PF-04957325 (Vang et al., 2010; Shimizu-Albergine et al., 2012).

Cyclic Nucleotide Efflux Transporters: Description and Inhibitors

As mentioned earlier, ABCC [ATP-binding cassette (ABC) transporter superfamily (subfamily C)] regulates cAMP efflux into the extracellular space to decrease cAMP availability. Three of them (ABCC4, ABCC5, and ABCC11) are expressed in cardiac tissue but ABCC4 is the most studied and has been shown to enhance cAMP formation, contractility, and cardiac hypertrophy (Sassi et al., 2012). Non selective inhibitors including MK-571, dipyrimamole or indomethacin (Reid et al., 2003) have been described to dually inhibit ABCC transporters and PDEs (Xie et al., 2011). Thus, the interpretation of experiments using those compounds has to take in account their side activities. In 2014, a high throughput screening identified Ceefourin 1 and 2 as highly selective ABCC4 inhibitors (Cheung et al., 2014). The authors described a micromolar inhibition of ABCC4 over other members of ABCC transporter families but no data are available concerning their effect on PDE activity (Cheung et al., 2014).

Optogenetics Methods to Modulate cAMP Availability

The genome of *Beggiatoa*, a sulfide-oxidizing bacterium, revealed the presence of a DNA sequence encoding for a cytosolic adenylyl cyclase directly linked to a BLUF (blue light receptor using FAD) type light sensor domain. This photoactivatatable adenylyl cyclase (bPAC) shows a low cyclase activity in the dark but that increases about 300-fold upon light activation (Stierl et al., 2011). Efetova et al. pioneered the use of bPAC to distinguish between the functions of alternative cAMP effectors in the *in vivo* regulation of a *Drosophila melanogaster* physiological process (Efetova et al., 2013) while Von Zastrow's group recently used the bPAC fused to different targeting sequences to assess the role of cAMP compartmentation in GPCR signaling (Tsvetanova and von Zastrow, 2014). Recently, a red light-activated PDE was engineered by recombining the photosensor module of *Deinococcus radiodurans* bacterial phytochrome with the effector module of *Homo sapiens* PDE2A (Gasser et al., 2014). Compare to the bPAC system, the red-shifted activation of this new tool will allow the creation of interesting animal model to study the spatio-temporal cAMP signaling pathway. This concept was declined for multiple targets referenced and collected by the CHROMus project (Shui et al., 2014) and applied to GPCR where the cytosolic part of the Rhodopsin receptor was replaced by the β2-AR receptor part to create a photoactivable Gαs coupled GPCR (Airan et al., 2009).

Modulation of cAMP Effectors

cAMP has four direct intracellular targets: protein kinase A (PKA), the exchange protein activated by cAMP (EPAC), the cyclic nucleotide gated ion channels (CNGC) and the popeye domain containing protein (POPDC). cAMP output signaling can be modulated by targeting its effectors.

PKA Inhibitors and Activators

Inactive PKA relies on an heterotretamer consisting of two regulatory (R) and two catalytic (C) subunits (**Figure 3A**). Two principal isoforms of the R-subunit (type I and II) each further subclassified into α and β subtypes (Hofmann et al., 1975) and three isoforms of the C-subunit have been described in mammals (Cα, Cβ, and Cγ) (Uhler et al., 1986; Beebe et al., 1990). RIIα is the major isoform expressed in the heart but can also be found in the brain (Skalhegg and Tasken, 2000). RIα is also expressed in the cardiac tissue and the central nervous system while RIβ and RIIβ are respectively found in the spinal cord or brain and liver or fat tissue (reviewed in Skalhegg and Tasken, 2000). In regard to its molecular mechanism of activation, two cAMP molecules bind to each R-subunit and induce a conformational rearrangement of PKA which initiates the functional dissociation of the regulatory from the catalytic subunits (Murray, 2008).

The classically used PKA inhibitors, H89 (isoquinolone derivative) and KT5720 (synthesized from fungus *Nocardiopsis* sp.) act as competitive antagonists of the cAMP nucleotide for the binding site on the PKA regulatory subunit (Kase et al., 1987; Engh et al., 1996). Studying the specificity for commonly used inhibitors for a range of protein kinases, Davies and coworkers found unspecific effect for H89 and KT5720 as they were found to inhibit other kinases at lower concentrations than those used to prevent PKA activation (Davies et al., 2000). For instance, H89 is able to inhibit ROCK, S6K, PKBα, or MSK1 while KT5720 inhibits PDK1 and PHK (Davies et al., 2000). Alternate PKA inhibitors were developed including Rp-cAMPs and its derivatives. Those inhibitors act as competitive antagonists of the cyclic nucleotide binding domain on the regulatory PKA subunit. A study in *Dictyostelium* has characterized both selectivity and degradation of such compounds (Schaap et al., 1993) and demonstrated that those molecules can indeed inhibit proteins containing other cAMP binding domain. Finally, the protein kinase inhibitor peptide (PKI) remains likely the most specific way to interfere with PKA as it binds to the free catalytic subunit and prevents phosphorylation of PKA targets (Dalton and Dewey, 2006). However, high concentration of this peptide can also inhibit PKG signaling (Glass et al., 1992). By opposition to PKA inhibitors, 8pcpt-cAMP and its derivative (Sp-5,6-DCl-cBiMPS) are cell permeable cAMP analogs that can bind the PKA-cAMP binding site and promote the activation of PKA downstream effectors (Sandberg et al., 1991).

Epac Inhibitors and Activators

Epac (exchange protein activated by cAMP) constitutes with PKA the main direct cAMP effector and has been identified by two independent group in 1998 (de Rooij et al., 1998; Kawasaki et al., 1998). In mammals, two isoforms of Epac (Epac1 and Epac2), products of independent genes have been identified which contain a cAMP binding domain (that is homologous to that of PKA R subunits) and other conserved domains (**Figure 3B**). Its activation relies on a conformational rearrangement of the protein promoted by cAMP binding. Epac1 is mostly abundant in the heart, kidney, blood vessels, adipose tissue, central nervous system, ovary, and uterus, whereas Epac2 splice variants (Epac2A and Epac2B) are mostly expressed in the central nervous system (Epac2A), adrenal gland (Epac2B), and pancreas (Epac2A) (de Rooij et al., 1998; Kawasaki et al., 1998; Niimura et al., 2009). Once activated, Epac proteins activate the Ras superfamily small G proteins Rap1 and Rap2 (for review Cheng et al.,

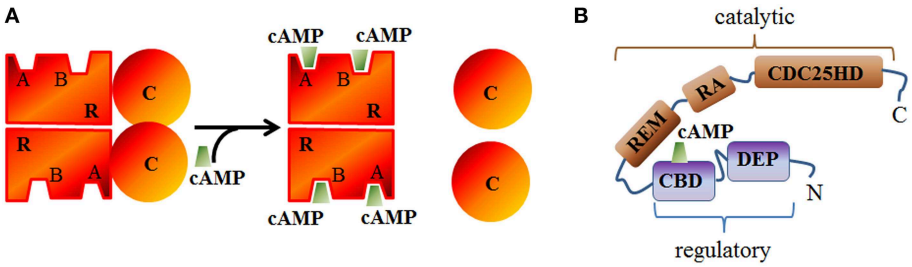


FIGURE 3 | Schematic structure of PKA and EPAC. (A) The catalytic (c) subunit of cAMP-dependent Protein Kinase (PKA) is a serine/threonine protein kinase associated, in the absence of cAMP, with the regulatory (R) subunit to form the inactive PKA holoenzyme. cAMP can bind to A or B sites in the regulatory subunits and induces the dissociation of the catalytic subunits. **(B)** Epac structure showing the conserved cAMP binding domain (CBD), Disheveled/Egl-10/pleckstrin (DEP) domain, RAS exchange motif (REM) domain, RAS association (RA) domain, and CDC25-homology domain (CDC25HD).

2008) by functioning as guanine nucleotide exchange factors. In cardiomyocytes, Epac proteins are involved in the formation of gap junctions to coordinate cardiac contractions through gating ions and small molecules (Somekawa et al., 2005) and enhances intracellular Ca^{2+} release during cardiac excitation-contraction coupling (Pereira et al., 2007). A High throughput screening assay to identify Epac inhibitors without affecting PKA activity led Cheng and coworkers to the identification of ESI-05 as an isoform specific inhibitor of Epac2 but not Epac1 (Tsalkova et al., 2012) and ESI-09 an pan inhibitor of Epac1 and 2 (Almahariq et al., 2013). CE3F4 compound (Courilleau et al., 2012) was identified as a specific Epac1 inhibitor without influence on PKA activity and its isoform selectivity for EPAC1 toward EPAC2 was demonstrated later (Courilleau et al., 2013). CE3F4 could be of interest in the therapeutic of cardiac pathophysiology as Epac1 is involved in β-adrenergic receptor-induced cardiomyocyte hypertrophy (Métrich et al., 2008). The compound usually named 007 [8-(4-Chloro-phenylthio)-2'-O-methyl-cAMP] is a cAMP analog activating Epac but not PKA (Enserink et al., 2002) but it has to be noted that 007 can behave as an inhibitor of PDEs which may indirectly increases cyclic nucleotide concentration (Poppe et al., 2008).

CNGC Inhibitors

The family of cyclic nucleotide gated channels (CNGC) comprises two groups: cyclic nucleotide gated (CNG) channels and the hyperpolarization-activated cyclic nucleotide-gated (HCN) channels. Both types are members of the six transmembrane channel superfamily and contain a cyclic nucleotide binding domain in their cytosolic C-Terminus that serves as an activation domain. Upon cyclic nucleotide binding, CNG channels gates the flow of monovalent cations such as Na^+ and K^+ to cross the plasma membrane and have a greater sensitivity for cGMP than for cAMP (for review Podda and Grassi, 2014). HCN cations channels open upon hyperpolarization and cAMP enhance their activity by shifting the activation curve to more positive voltage (Scicchitano et al., 2012). Four members exist in mammals (HCN1–HCN4) and are known to regulate the If current to control heart rate and rhythm by acting as a pacemaker current in the sinoatrial node (Wahl-Schott et al., 2014). If current is regulated by various neurotransmitters and metabolic stimuli (Pape, 1996) and are promising pharmacological targets in the treatment of cardiac arrhythmias. Thus, the most extensively studied HCN channels blocker is ZD7288 (BoSmith et al., 1993) but If current can also be blocked by ivabradine (Bucchi et al., 2002, 2006), zatebradine, and cilobradine (Van Bogaert and Pittoors, 2003). Ivabradine derivatives led to the discovery of HCN selective blockers with EC18 identified as a selective blocker for HCN4 and MEL57A induced mHCN1 inhibition (Melchiorre et al., 2010; Del Lungo et al., 2012).

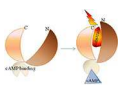
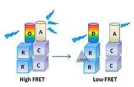
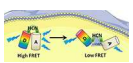
POPDC Inhibitor

The Popeye domain-containing gene family consists of 3 genes (podc1, podc2, and popdc3) encoding a 3 transmembrane proteins that bind cAMP through their conserved cytoplasmic Popeye domain with an affinity (IC_{50}) of 120 nM, which is comparable to the affinities reported for PKA (100 nM) (Froese et al., 2012). These proteins are essential for stress mediated modulation of cardiac pacemaking (Froese et al., 2012; Schindler et al., 2012). To our knowledge, no pharmacological inhibitors have been reported to investigate specific POPDC protein function so that the only way to modulate their activities so far is the use of genetic tools (small interfering RNA technology or gene knockout).

Methods for cAMP Detection in the Cardiac Tissue

The number of technologies that enables the functional screening of cAMP production has expanded over the years. Consequently, the choice of the technology will define the scope of the conclusions that can be drawn. Those methods can be divided into two groups: the direct methods allowing an "absolute" cAMP concentration quantification and the indirect methods which give a relative representation of cAMP availability. Thus, as summarized in **Table 3**, direct methods are generally more sensitive than indirect one since lacking any mediator but cannot accurately sense low cAMP levels produced in subcellular compartments. The advantages and limits of the common systems are listed below and basic principle for each technique is shown on **Table 3**. As previously pinpointed, cAMP availability is

TABLE 3 | Comparison of cAMP detection system.

Class	Method	Principle	Localization	Sensitivity	Signal in presence of cAMP
Biochemical	Radiometric		Homogenate	10 nM	↘
	Fluorescence polarization		Homogenate	10 nM	↗
	Enzyme or fluorescence detection		Homogenate	1 nM	↘
	HTRF		Homogenate	1 nM	↘
	AlphaScreen		Homogenate	1 nM	↘
	Enzyme complementation		Homogenate	1 nM	↗
	Electroluminescence		Homogenate	1 nM	↘
Integrative	cAMP binding on circularly permutted luciferase		Whole cell	100 μM	↗
	CNGC based		Whole cell	nd	↗
Reporter gene	CRHB response element		Whole cell	nd	↗
RET Based	PKA based		Cytosol		↘
	Epac based		Plasma membrane Endosome Cytosol		↘ ↘ ↘
	CNGC based		Membrane Membrane Membrane	0.1 μM 1 μM 50 μM	↘ ↘ ↘
Click chemistry	Copper free Azide-alkaline cycloaddition		Derivative cAMP syntesis	nd	↗

fine-tuned by a tight balance between its synthesis and immediate hydrolysis/efflux/use so that at one time point, cAMP is not enough amenable to quantification assays. Thus, accumulation of cAMP is often mandatory in most of cAMP detection assays with the common use of the pan PDEs inhibitor IBMX (when cAMP production needs to be measured) or FSK pretreatment

(when cAMP production inhibitory function wants to be outlined).

Direct Methods: Biochemical Approaches

cAMP has been long quantified through a radioactive functional assay based on affinity chromatography purification using ^{3}H-ATP-preloaded cells lysates. After lysis and cAMP production can be estimated by measuring the ratio between ^{3}H-cAMP over ^{3}H-ATP + ^{3}H-cAMP separated on affinity column (Piñeyro et al., 2005). Despite its high sensitivity, this technique often requires the presence of PDEs inhibitor and is not suitable for high throughput screening (HTS) strategy. Most of HTS biochemical methods relies on the general principle that cAMP accumulation is being detected by competition for a specific cAMP antibody between free unlabelled cAMP present in the sample to evaluate and a labeled form (radioactive, fluorescent, or enzymatic) of cAMP (Williams, 2004). Radiometric assays allow detection of cAMP using competition with ^{125}I labeled cAMP for anti-cAMP antibody immobilized on a solid scintillant plate. In those assays, the radiometric signal decreases proportionally to the amount of cAMP present in the sample (Horton and Baxendale, 1995). Fluorescence polarization cAMP assays monitor the light emitted from a fluorescent-tagged cAMP following excitation by a polarized light source. When the labeled cAMP is bound to an antibody more polarized light will be produced upon excitation (Prystay et al., 2001; Huang et al., 2002). To increase the signal to noise and to avoid cell autofluorescence detection, Cisbio developed an HTRF (Homogeneous time resolved fluorescence)-based cAMP assay. This assay is still based on a competitive immunoassay using cryptate-labeled anti-cAMP antibody and d2-labeled cAMP (Degorce et al., 2009). Alpha-screen technology uses acceptor beads conjugated to an antibody that recognizes cAMP and streptavidin-coated donor beads. When brought into close proximity by the presence of biotinylated cAMP, an oxygen radical dependent light is emitted. The cAMP extracted from a cell lysate will compete with the biotinylated cAMP and reduce the emitted light. This kind of assay is also available with an enzymatic based detection method where cAMP found in test sample competes with a fixed amount of Horse Radish Peroxydase-linked cAMP for binding to an anti-cAMP immobilized antibody (Bouchard et al., 2006). The electroluminescence technique (Mesa Scale Discovery) is another competitive immunoassay based on the displacement of ruthenium-labeled cAMP for an anti-cAMP antibody. The electrochemical reaction is initiated upon substrate and electrical charge addition and produce light which is inversely proportional to the cAMP present in the sample (Filip et al., 2004). The immuno-based competition can also be revealed using an enzyme complementation method (DiscoverX). In this assay, a fragment of β-galactosidase (β-gal) is conjugated with cAMP and act as an enzyme donor (ED) (Golla and Seethala, 2002). This ED-cAMP conjugate and cellular cAMP compete for binding to an anti-cAMP antibody. In presence of the enzyme acceptor (EA), the active enzyme will be reconstituted and will be able to subsequently hydrolyze a substrate to produce a chemiluminescent signal that is directly proportional to the amount of cAMP in the cells. Despite being sensitive and specific, all those techniques require cells or tissue disruption making the real-time and sub-cellular analysis of cAMP quantification impossible. Moreover if those assays are highly efficient to measure cAMP production, their use to bring to the fore cAMP inhibition is challenging and require FSK pretreatment.

Indirect Methods

Integrative Methods

Promega developed an assay based on the GloSensor Technology, a genetically modified form of firefly luciferase into which a cAMP-binding protein domain has been inserted (Fan et al., 2008). The firefly luciferase is circularly permuted and upon cAMP binding, a conformational change induces luciferase enzyme reconstitution which produces light in presence of its substrate. This technique is sensitive enough to assess role of endogenous receptors but requires transfection of the biosensor, thus limiting the quantification of the cAMP to the transfection efficiency (expression heterogeneity between cells) which can lead to high results variability. Maintaining advantages of integrative methods (sensitivity and kinetic) but avoiding transfection variability, Rivero-Müller's group developed a derived detection method CANDLES (Cyclic AMP iNdirect Detection by Light Emission from Sensor cells). Briefly, a stable cell line expressing a GloSensor plasmid is co-cultured with cells expressing the receptor to be tested and through cell-cell interaction via gap junctions, cAMP produced by the cell of interest can be transferred to the sensor cells to activate GloSensor plasmid (Trehan et al., 2014). Finally, given that the GloSensor is a cytosolic probe, it could be not appropriate to detect low concentrations of compartmentalized cAMP at the plasma membrane.

Reporter Gene Methods

The reporter gene method is a homogeneous, simple and inexpensive but indirect method to detect cAMP-downstream signaling. This assay is based on the specific activation of the transcription factor CREB (cAMP response element binding protein) upon cAMP production which induces a reporter gene under the control of a CRE element (cAMP Response Element) promoter. Various reporter genes have been used over the years: fluorescent proteins, luciferase, β-galactosidase or β-lactamase. Far downstream of the cAMP activation cascade, this method is sensitive but unable to give kinetics or localization information.

Resonance Energy Transfer Methods

The methods described above are unable to define the cellular localizations of cAMP production. Thus, visualization was achieved using resonance energy transfer (RET) techniques described by the Theodor Förster in 1940's (Forster, 1946). RET is a mechanism relying on an energy transfer between a donor chromophore that may transfer energy to another acceptor chromophore through non-radiative dipole–dipole coupling upon distances proximity conditions (Hebert et al., 2006; Kiyokawa et al., 2006). The name FRET "Förster resonance energy transfer" which includes the commonly used term FRET "Fluorescence resonance energy transfer" and

BRET "Bioluminescence resonance energy transfer" is a non-radiative transfer of energy occurring between two fluorescent chromophores for FRET or between an enzyme generating luminescent signal upon addition of its substrate and a fluorescent acceptor partner in the case of BRET technology. The efficiency of this energy transfer is inversely proportional to the sixth power of the distance between donor and acceptor, making RET extremely sensitive to very small changes in distance thus allowing an accurate sensing of change in protein conformations for instance (Hebert et al., 2006). BRET is a first line assay for HTS screening as it avoids the consequences of fluorescence excitation and has a better Stokes' shift over FRET but is not recommended for imaging technique to identify localized cAMP compartmentalization. Thus RET-based methods have been developed to detect cAMP production and extensively reviewed (Williams, 2004; Willoughby and Cooper, 2008; Sprenger and Nikolaev, 2013; Calebiro and Maiellaro, 2014). All the methods rely on the use of the downstream cAMP effectors PKA, Epac or CNGC either that all directly bind cAMP molecules related to the expression of a specific cAMP-binding motif. Briefly, either full-length cAMP-effector probes or single cAMP domain sensor extracted from the different effectors are fused to an energy donor and acceptor allowing the generation of a basal RET signal in the absence of cAMP production. Upon cAMP binding, a conformational rearrangement in the RET-based sensor will lead to a modification of the RET signal. Since the pioneering studies using those RET probes to study cAMP availability in cardiac tissue (Zaccolo et al., 2000; Zaccolo and Pozzan, 2002), many efforts have been made these last years to improve signal to noise ratio, RET efficiency (optimizing donor-acceptor couple, linker optimization), cAMP binding affinity (mutagenesis on single domain or full length protein probes), RET detection methods (e.g., Sensitized emission vs Fluorescence lifetime imaging microscopy for FRET-based probes, Renilla luciferase variants for BRET-based probes) (Willoughby and Cooper, 2008; Sprenger and Nikolaev, 2013). Moreover with the prominently recognized mechanism for cAMP compartmentation, several group restricted the expression of those RET based probes to subcellular localization using for example plasma membrane targeting sequence or endosome localization (Klarenbeek and Jalink, 2014; Sprenger et al., 2015).

Copper Free Azide-alkaline Cycloaddition: a "Click Chemistry"

In chemical synthesis, click chemistry is a process that generates by joining small units together. The azide alkyne Huisgen cycloaddition using a Copper (Cu) catalyst is one of the most popular reactions within the Click chemistry concept between an azide and a terminal or internal alkyne to give a 1,2,3-triazole (Rostovtsev et al., 2002; Tornøe et al., 2002). To avoid Cu toxicity, Baskin *et al.* developed a Cu-free click reaction with comparable kinetics to Cu dependent cycloaddition, but adapted for dynamic *in vivo* imaging (Baskin et al., 2007). In a recent study, this copper free method was applied to detect cAMP derivative (8-azido cAMP) *in vivo* after the addition of difluorinated cyclooctyne (DIFO) as a reagent (Ito et al., 2013). If this approach will enable to visualize and quantify derivative cAMP endogenous modulators at the single cell level without exogenous transfection protocol, it has to be noted that the molecule used is a cAMP derivative so the signal observed will be the result of

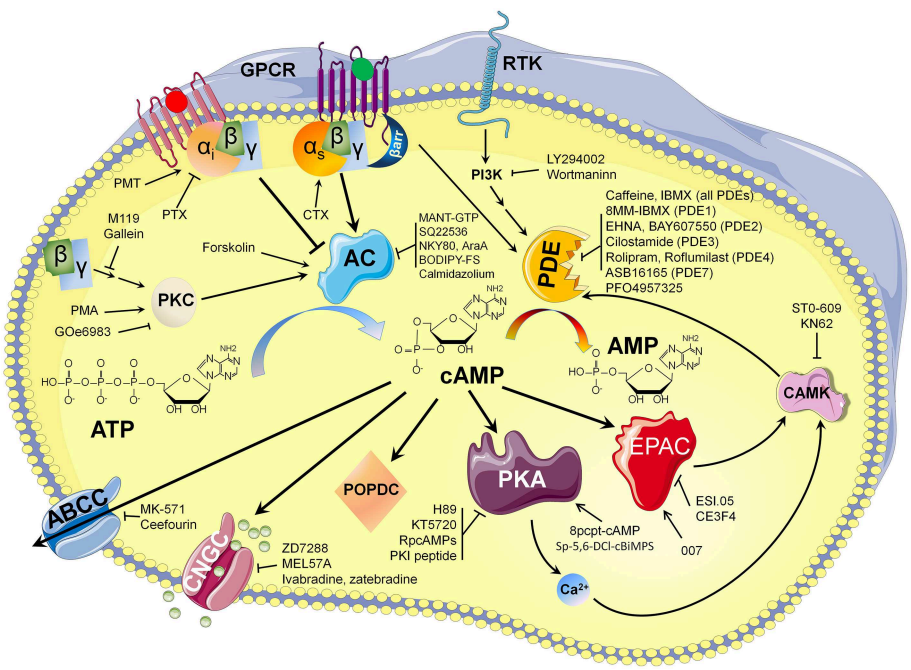


FIGURE 4 | cAMP synthesis and hydrolysis: pharmacological way to modulate its availability.

ACs/PDEs activities in competition with cAMP endogenously produced.

Conclusion

The development of optical methods that allow monitoring of cAMP dependent signaling in living cells and the growing list of molecules (summarized in **Figure 4**) available to modulate cAMP availability played a fundamental role in revealing an unexpected level of cAMP organization in cardiac tissue. It is likely that new optical methods development, with higher temporal and spatial resolution, will improve our knowledge of cAMP dependent signaling microdomains located on the cell surface or other intracellular membranes for individual cells within heart architecture.

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MINI REVIEW
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Imaging alterations of cardiomyocyte cAMP microdomains in disease

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3′,5′-cyclic adenosine monophosphate (cAMP) is an important second messenger which regulates heart function by acting in distinct subcellular microdomains. Recent years have provided deeper mechanistic insights into compartmentalized cAMP signaling and its link to cardiac disease. In this mini review, we summarize newest developments in this field achieved by cutting-edge biochemical and biophysical techniques. We further compile the data from different studies into a bigger picture of so far uncovered alterations in cardiomyocyte cAMP microdomains which occur in compensated cardiac hypertrophy and chronic heart failure. Finally, future research directions and translational perspectives are briefly discussed.

Keywords: cAMP, microdomain, cardiomyocyte, hypertrophy, heart failure, FRET, biosensor

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3′,5′-cyclic adenosine monophosphate (cAMP) is an important second messenger and critical regulator of cardiac function. Stimulation of cardiac contractility by catecholamines and their receptors, in particular β-adrenoceptors (β-ARs) which are central to the well-established physiological fight-or-flight response, leads to generation of cAMP that acts in distinct subcellular microdomains (Fischmeister et al., 2006; Zaccolo, 2009; Perera and Nikolaev, 2013). Such microdomains are formed around specific scaffolding proteins (i.e., A-kinase anchoring proteins or AKAPs) which create multiprotein signalosomes. They contain local pools of kinases targeted to their substrates, certain subsets of phosphodiesterases (PDEs) which are enzymes responsible for local cAMP degradation, protein phosphatases and other molecules. All of them act together to confer specificity of multiple substrate phosphorylation and therefore plethora of physiological responses engaged by the same second messenger cAMP (Buxton and Brunton, 1983; Mauban et al., 2009; Zaccolo, 2009; Diviani et al., 2011). In this mini review, we highlight most recent developments and latest research on cardiomyocyte cAMP microdomains in healthy and diseased cardiomyocytes.

cAMP Compartmentation in Healthy Cardiomyocytes

In healthy cells, cAMP microdomains are supposed to provide specificity of A-kinase (PKA) substrate phosphorylation at different functionally relevant locations. In terms of contractility, several cAMP microdomains exist around calcium handling proteins such as L-type calcium channels (LTCCs), ryanodine receptors (RyRs), and phospholamban (PLN) which regulates the activity of the cardiac sarcoplasmic/endoplasmic reticulum (SR) calcium ATPase 2a (SERCA2a; Bers, 2002; Lompre et al., 2010). Each of these microdomains contains at least one specific AKAP and one PDE isoform (see **Figure 1A**). Each PDE family, e.g., PDE4, is comprised of several subfamilies such as 4A, 4B, 4D which have slightly different catalytic domain structures. Every subfamily usually has several isoforms (e.g., 4D3, 4D5) each having a unique N-terminal sequence responsible for differential subcellular localization (Conti and Beavo, 2007). Functional LTCCs are localized in

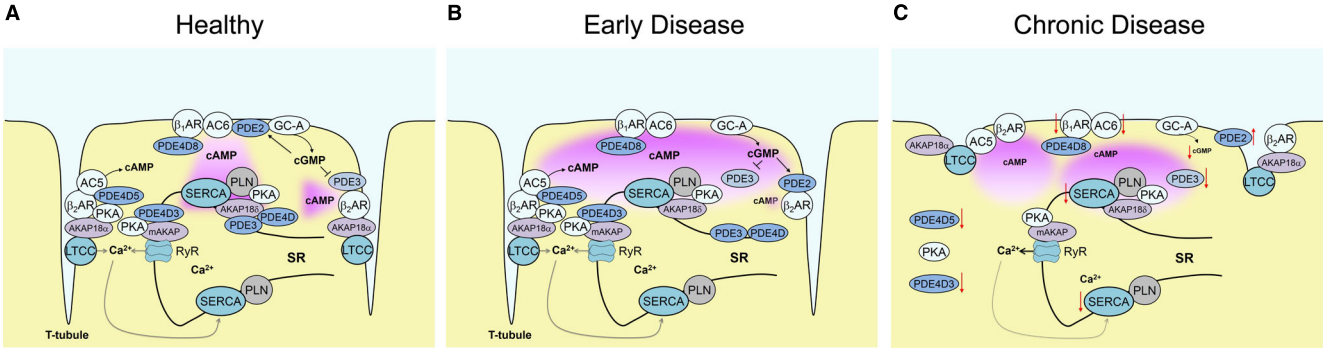

FIGURE 1 | Alterations of local cardiomyocyte cAMP signaling in early and late disease. (A) cAMP microdomains in healthy cells formed around major calcium cycling proteins—L-type calcium channels (LTCCs), ryanodine receptor (RyR) and SERCA. Each microdomain contains local pools of PKA anchored to AKAPs and specific subsets of PDEs. β_2-AR and AC5 are part of a signalosome around LTCC in the T-tubules controlled by PDE4D5 and PDE3 activities, while AC6 is located together with β_1-AR at the outer membrane to produce far-reaching cAMP signals which are "channeled" to subcellular microdomains (e.g., to SERCA) by joint action of several PDEs. **(B)** Alterations of local cAMP signaling in early cardiac disease such as compensated hypertrophy. While, the total whole-cell activities of PDEs are not yet altered, there is a relocation of PDE2 between β_1- and β_2-AR, decrease of local PDE3 activity at β_2-AR, and PDE3/PDE4 at SERCA. This leads to a change in cGMP/cAMP cross-talk, favoring cGMP-mediated augmentation of the contractile β_1-AR-cAMP pool, and to disruption of the cAMP "channeling" from β_1-AR to SERCA2a. **(C)** In chronic cardiac disease such as heart failure, multiple structural and functional alterations take place. They include partial loss of the membrane-associated T-tubules, changes in whole-cells PDE activities, delocalization of PKA, and downregulation and/or desensitization of β_1-AR, ACs, SERCA and GC-A which all impact on receptor-microdomain coupling and functional cAMP responses.

cardiomyocyte transverse (T)-tubules, plasma membrane invaginations rich in caveolin (Gu et al., 2002; Insel et al., 2005). Together with AKAP15/AKAP18α (Fu et al., 2011), AKAP79 (Nichols et al., 2010) and PDE4B (Leroy et al., 2011) they form a signalosome which is crucial for β-AR/cAMP-dependent regulation of LTCC current and contractility. RyRs at the junctional SR have been claimed to be a part of mAKAP-orchestrated signalosome which also contains PDE4D3 (Lehnart et al., 2005). PLN forms a complex with AKAP18δ (Lygren et al., 2007) and one of the PDE4D and PDE3A isoforms (Beca et al., 2011, 2013) to regulate diastolic calcium uptake. Each of these complexes should also include a local pool of type II PKA molecules. There are two types of regulatory PKA subunits RI and RII, together with catalytic subunits they form either PKA type I or type II complexes. While PKA type II has been shown to phosphorylate the above mentioned calcium handling proteins, the exact nature of PKA type I substrates remains unclear (Stangherlin et al., 2011). Apart from channel recordings and substrate phosphorylation analysis, which provide only indirect measure of the downstream PKA-mediated signaling, it has been challenging to directly visualize cAMP levels in these specific microdomains until novel biophysical techniques became available.

Advent of fluorescence resonance energy transfer (FRET) based biosensors enabled a real-time monitoring of cAMP in intact cells (Sprenger and Nikolaev, 2013). Very early experiments in neonatal cardiomyocytes could directly visualize discrete microdomains where cAMP increases and activates PKA upon β-AR stimulation (Zaccolo and Pozzan, 2002). There are even different pools of cAMP associated with type I and type II PKA responsible for phosphorylation of different substrates and oppositely regulated by cGMP due to its action on either cGMP-activated PDE2 or cGMP-inhibited PDE3 (Stangherlin et al., 2011). Development of further biosensors, adenoviruses and transgenic mice which serve as a way to express such sensors in cells and tissues, enabled measurements in adult myocytes which revealed tight regulation of cAMP by various PDE families (Warrier et al., 2005; Leroy et al., 2008) and spatial differences between β_1- and β_2-AR-cAMP signals, the former having more diffuse far-reaching and the latter highly confined nature (Nikolaev et al., 2006).

Even deeper insights into cAMP compartmentation in relation to membrane structure of living cardiomyocytes were provided by scanning ion conductance microscopy (SICM) combined with FRET. SICM is a non-optical imaging technique which utilizes an electrolyte-filled glass nanopipette as a scanning probe fixed on a three-axis piezo-actuator stage (Korchev et al., 1997). The current flow through pipette is decreased whenever it approaches cell membrane, and by keeping this current change and thereby the distance between pipette tip and cell membrane constant, one can scan the morphological profile of the membrane with nanometer resolution. In general, SICM as a multimodal imaging technique can be applied to study not only cell/tissue structure, but also to record ion channel currents in precise membrane locations, to analyze cell volume and contractility (Miragoli et al., 2011). Using combination of SICM with FRET, which allows local nanopipette-based receptor stimulation and concomitant cAMP imaging, it was uncovered that β_1-AR is localized across the whole membrane, while, in contrast, β_2-AR is located exclusively in the T-tubules of healthy cells (Nikolaev et al., 2010). Moreover, β_2AR are strictly compartmentalized in caveolin3-rich microdomains (Wright et al., 2014) to produce confined cAMP signals limited by local PKA and PDE4 activities. This receptor can also switch from stimulatory to inhibitory G-proteins to limit cAMP production upon prolonged exposure to high agonist concentrations (Liu et al., 2009).

Not only AKAPs but also cAMP synthetizing enzymes adenylyl cyclases (ACs), with the most predominantly expressed cardiac AC5 and AC6, can center cAMP signalosomes in myocytes and other cells (Cooper and Tabbasum, 2014). A recent elegant electrophysiological study by Timofeyev et al. (2013) using AC5 and AC6 knockout myocytes revealed that AC5 is mainly localized in T-tubules where it interacts with caveolin and β_2-ARs. Together with a local PDE, caveolin is thereby involved in compartmentation of β_2-AR-cAMP signals in this microdomain. In contrast, AC6 associated the β_1-AR is localized outside the T-tubules and is responsible for β_1-AR-mediated augmentation of LTCC current. Interestingly, yet another functional population of β_1-ARs is targeted to T-tubules and the AC5-PDE signalosome (Timofeyev et al., 2013).

To gain more specific insights into cAMP dynamics directly in the microdomains around calcium handling proteins, our group has recently generated targeted FRET biosensors and expressed them in myocardium of transgenic mice to directly monitor local cAMP in freshly isolated adult cardiomyocytes. First, the cAMP sensor Epac1-camps was targeted to caveolin-rich membrane microdomains (to generate pmEpac1-camps) where it should localize in close proximity to LTCC and β_2-AR (Perera et al., 2015). This new sensor uncovered differential PDE-dependent regulation of β_2- and β_1-AR stimulated cAMP pools at the membrane, the former one predominantly confined by PDE3 and the latter one by balanced actions of PDE4, 3, and 2 (Perera et al., 2015). Second, a fusion of Epac1-camps with PLN was used to target the cAMP sensor to the SERCA2a microdomain (Sprenger et al., 2015). Here, high basal PDE3 and PDE4 effects were detected which confine this microdomain and prevent PLN phosphorylation by high cytosolic cAMP levels. More interestingly, upon β-adrenergic stimulation, local, and cytosolic PDE3 and PDE4 act in concert to "channel" cAMP from the membrane to SERCA2a and enable functional response in this microdomain (Sprenger et al., 2015; **Figure 1A**), a phenomenon which has previously been observed in HEK293 cells when measuring membrane, cytosolic and nuclear cAMP pools (Terrin et al., 2006). However, particularly exiting findings could be made when subjecting both FRET sensor transgenic mouse lines to an experimental model of cardiac disease.

Remodeling of cAMP Microdomains in Early Cardiac Disease

While alterations in cAMP pathway have been extensively studied in chronic disease (see below), not much is known about changes in local cAMP signaling in early compensated cardiac hypertrophy. To address this question, pmEpac1-camps and Epac1-camps-PLN mice were subjected to transverse aortic constriction which induces only a mild compensated phenotype in the FVB/N1 mouse background. Interestingly, in this case, no changes in total whole-cell PDE activities and no β_1-AR or guanylyl cyclase A (GC-A, membrane receptor which produces cGMP upon natriuretic peptide stimulation) desensitization can be detected. Instead, there was a subcellular relocation of PDE2 between β_1- and β_2-AR, and local decrease of the major PDE3-mediated control at the β_2-AR (**Figure 1B**). This leads to a change of cGMP/cAMP cross-talk in a way that cGMP, which is produced by GC-A stimulated with increased levels of natriuretic peptides in hypertrophy, leads to augmentation of the far-reaching β_1-AR-cAMP pools coupled to increase in force and frequency of contraction (Perera et al., 2015). This might represent a compensatory mechanism aimed to initially maintain cardiac output under the conditions of increased pressure overload during disease, before the transition to a decompensation at some later time-point. However, the exact local mechanisms which accompany this transition remain to be defined. It is also not clear in which particular membrane microdomains GC-A is localized and whether this localization is changed in disease.

The study using cAMP biosensor targeted to SERCA2a demonstrated that cardiac hypertrophy leads to local decrease of PDE3 and PDE4 effects which confine this microdomain from the bulk cytosol. Furthermore, it causes changes in PDE composition at various subcellular locations in a way that leads to impairment of the above described PDE3/4-dependent "channeling" of cAMP from β_1-AR to SERCA2a (**Figure 1B**, Sprenger et al., 2015). In the future, it would be exciting to dissect which individual PDE3 and PDE4 isoforms are involved in any individual microdomain, how they are regulated by calcium signaling and positive or negative feedback loops, and how all these processes are affected by cardiac disease. This can be done using PDE knockout mouse models, as previously demonstrated for healthy neonatal mouse myocytes using pmEpac-camps and its parential cytosolic sensor (Leroy et al., 2011; Mika et al., 2015). Future developments should also provide new biosensors for other microdomains, such as the one associated with RyR, various other signalosomes and organelles.

cAMP Microdomain Alteration in Chronic Cardiac Disease

In human and rodent failing myocytes, a series of well-established signaling alterations occurs, including desensitization/downregulation of β_1-AR, GC-A, ACs, SERCA2a, and impairment of PKA-dependent phosphorylation of major contractile substrates (Lohse et al., 2003). Structurally, SICM studies in failing human and rat cardiomyocytes revealed a loss of cell-surface T-tubules as well as disruption of Z-groove structure (Lyon et al., 2009). The whole-cell activities of major PDE families were reported to be down- (for PDE3/4; Ding et al., 2005; Abi-Gerges et al., 2009) or upregulated (PDE2; Mehel et al., 2013). Loss of membrane T-tubules leads to redistribution of β_2-AR to detubulated areas where it gets uncoupled from its microdomain and generates far-reaching cAMP signals (Nikolaev et al., 2010; see **Figure 1C**). Altered cAMP compartmentation worsens PKA substrate phosphorylation and calcium cycling. Several open question still remain. Is there any PDE relocation also in chronic disease? Are there any differences between various clinical types of heart failure and what is the time course of deleterious events (detubulation, receptor relocation, microdomain remodeling) during progression of disease? Still unclear are the exact molecular mechanisms behind the loss of T-tubules and their link to calcium cycling, improvement of which correlates with restoration of the membrane structure (Lyon et al., 2012).

Better understanding of cAMP dynamics in various functionally relevant microdomains and especially of their changes in disease should ultimately provide more precise ways of therapeutic correction. To improve cAMP or cGMP flow in the microdomains, one can imagine approaches aimed at depletion of specific PDEs or PKA from desired signalosomes. More specific PDE inhibition and treatments aimed at improvement of membrane structure, receptor localization and protein composition of the microdomains can also be considered. These developments should enable more targeted and specific cardiac therapeutics.

Author Contributions

AF and VN discussed the concept, wrote and edited the manuscript.

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frontiers in Pharmacology

MINI REVIEW
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Global and local missions of cAMP signaling in neural plasticity, learning, and memory

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The fruit fly *Drosophila melanogaster* has been a popular model to study cAMP signaling and resultant behaviors due to its powerful genetic approaches. All molecular components (AC, PDE, PKA, CREB, etc) essential for cAMP signaling have been identified in the fly. Among them, adenylyl cyclase (AC) gene *rutabaga* and phosphodiesterase (PDE) gene *dunce* have been intensively studied to understand the role of cAMP signaling. Interestingly, these two mutant genes were originally identified on the basis of associative learning deficits. This commentary summarizes findings on the role of cAMP in *Drosophila* neuronal excitability, synaptic plasticity and memory. It mainly focuses on two distinct mechanisms (global versus local) regulating excitatory and inhibitory synaptic plasticity related to cAMP homeostasis. This dual regulatory role of cAMP is to increase the strength of excitatory neural circuits on one hand, but to act locally on postsynaptic GABA receptors to decrease inhibitory synaptic plasticity on the other. Thus the action of cAMP could result in a global increase in the neural circuit excitability and memory. Implications of this cAMP signaling related to drug discovery for neural diseases are also described.

Keywords: ***Drosophila melanogaster*, synaptic plasticity, associative learning and memory, rutabaga, dunce, cAMP homeostasis**

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Since its discovery in 1958 (Sutherland, 1992; Pittenger et al., 2012), the cyclic AMP signaling pathway has been shown to regulate a plethora of cellular functions including energy metabolism, gene expression, development, apoptosis and exocytosis. This second messenger molecule is synthesized from ATP by a family of enzymes called adenylyl cyclases (ACs), activated by G-protein coupled receptors (GPCRs). Considering the importance of this intracellular signaling, it is not surprising that cAMP levels in the cell are negatively regulated by another group of enzymes—phosphodiesterases (PDEs). An increase in cAMP levels activates protein kinase A (PKA) which then phosphorylates target proteins including other kinases, transcriptional factors and ion channels. Therefore, its effects can be achieved by short- or long-term fashion. The latter is a protein synthesis-dependent process and thus mediated by a well-known transcription factor cAMP response element-binding protein (CREB).

Many isoforms of ACs and PDEs have been identified in the nervous system, indicating critical roles of cAMP in neural function (Pittenger et al., 2012). Indeed, cAMP signaling in the brain is known to mediate numerous neural processes from development, cellular excitability, synaptic plasticity, learning and memory, pain and motor function to neurodegeneration and drugs of abuse (Pierre et al., 2009; Bollen and Prickaerts, 2012; Kandel, 2012; Pittenger et al., 2012). In this commentary, I will specifically focus on the role of cAMP signaling in neural excitability, synaptic plasticity, learning and memory. Other important functions of cAMP signaling in the nervous system

can be found in several outstanding reviews elsewhere (refer to Pittenger et al., 2012).

The cAMP signaling pathway mediates synaptic plasticity in both vertebrates and invertebrates. In a sea slug *Aplysia*, it was demonstrated that cAMP signaling mediates short- and long-term facilitation (LTF) at sensorimotor synapses (Brunelli et al., 1976; Schacher et al., 1988; Kaang et al., 1993; Bartsch et al., 1995; Kandel, 2012). The LTF was dependent on the action of CREB and new protein synthesis, and subsequently shown to mediate memory formation in *Aplysia*. In the rodent hippocampus, cAMP is involved in long-term potentiation (LTP) at excitatory glutamatergic synapses (Frey et al., 1993; Weisskopf et al., 1994; Silva et al., 1998). All these findings support the idea that cAMP-dependent synaptic plasticity is responsible for behavioral learning and memory at the whole organism level in both invertebrates and vertebrates. The fruit fly *Drosophila melanogaster* has been widely used to study molecular and cellular mechanisms of learning and memory due to its sophisticated genetic approaches. In addition, *Drosophila* nervous system contains all molecular components (e.g., AC, PDE, PKA, CREB, etc) essential for the cAMP signaling pathway. *Drosophila* AC gene *rutabaga* and PDE gene *dunce* have been intensively studied to understand the role of cAMP signaling in the nervous system. These two mutant genes were originally identified on the basis of associative learning deficits (Dudai et al., 1976; Chen et al., 1986; Levin et al., 1992; Busto et al., 2010). Further, the cAMP signaling pathway in the fly regulates synaptic plasticity at both peripheral neuromuscular junction (NMJ) as well as central synapses, where alterations in facilitation and post-tetanic potentiation were observed in mutant flies (*dunce* and *rutabaga*) with defects in cAMP signaling (Zhong and Wu, 1991; Cheung et al., 1999; Lee and O'Dowd, 2000; Ganguly and Lee, 2013). All these findings strongly support that *Drosophila* is an excellent model system to study the role of cAMP signaling in synaptic plasticity and the resultant behavior—learning and memory.

In this mini review, I summarize the findings on the role of cAMP in *Drosophila* neuronal excitability, synaptic plasticity, and learning and memory. The main emphasis is to understand distinct mechanisms (global versus local) regulating excitatory and inhibitory synaptic transmission related to cAMP homeostasis. At the end, I comment on implications of this research on disease therapy.

Global Missions of cAMP Signaling

Neuronal Excitability

One common effect of cAMP on neural function is to modulate cellular excitability. In an *Aplysia* sensory neuron, cAMP increased spike duration and excitability (Goldsmith and Abrams, 1992). Excitability of the rodent hippocampal neurons was also increased by cAMP signaling which subsequently enhances LTP (Gruart et al., 2012). Further, striatal neuronal excitability has been shown to be regulated by cAMP signaling (Threlfell and West, 2013).

In *Drosophila* NMJ, the excitability of a motor neuron is regulated by cAMP signaling (Zhong and Wu, 1991, 2004). This change increases the excitability of presynaptic terminals, thus influencing release of neurotransmitter (NT) glutamate. This cAMP effect on excitability was also observed in *Drosophila* central nervous system (CNS). In contrast to mammalian CNS, acetylcholine is the primary excitatory NT in *Drosophila* CNS (Restifo and White, 1990; Lee and O'Dowd, 1999). These cholinergic neurons play a critical role in almost all higher brain function in *Drosophila* as glutamatergic neurons do in mammalian CNS. Due to difficulties in recording electrical signals from a single neuron in the fly brain, *Drosophila* primary neuronal culture has become a good alternative to study ionic and synaptic currents from central neurons (O'Dowd, 1995; Lee and O'Dowd, 1999; Ganguly and Lee, 2013). When fly cholinergic neurons were focally exposed to a popular AC activator forskolin (FSK), action potential (AP) frequency was drastically increased. GABAergic neurons also showed an increase in AP in response to the focal application of FSK (Ganguly and Lee, 2013). This change is a direct effect of cAMP as excitatory cholinergic inputs to GABAergic neurons were blocked by an acetylcholine receptor (AChR) blocker curare. In *Drosophila*, live cholinergic and GABAergic neurons can be easily identified using a live fluorescent marker (e.g., RFP or GFP; Wiemerslage et al., 2013). Increased excitability by cAMP is primarily achieved through PKA, which mediates a phosphorylation-induced reduction in potassium channel conductance (Wright and Zhong, 1995; Delgado et al., 1998). Taken together, all these findings show that cAMP-PKA signaling directly enhanced the excitability of all types of neurons—not only inhibitory GABAergic (Ganguly and Lee, 2013) but also excitatory cholinergic and glutamatergic neurons (Zhong and Wu, 1991, 2004; Lee and O'Dowd, 2000). Further, these findings indicate that cAMP signaling increases pre-synaptic NT release through enhanced excitability.

Synaptic Plasticity in CNS

Synaptic transmission is a primary way to communicate between neurons in the brain. The synapse is not static, rather dynamically changes its strength which is known as synaptic plasticity, an important subcellular mechanism underlying learning and memory (Bhalla and Iyengar, 1999; Kandel, 2001). A key molecule involved in this plasticity is the second messenger cAMP.

The cAMP-PKA pathway is known to regulate synaptic plasticity (e.g., LTP) in the mossy fibers and CA3 pyramidal cells in hippocampal slices (Nicoll and Malenka, 1995; Silva et al., 1998). The cAMP signaling pathway has been shown to increase the pre-synaptic vesicle release probability (Chen and Regehr, 1997), through enhanced vesicle docking before exocytosis (Sudhof, 2004). In *Aplysia*, PKA increases an influx of Ca^{2+} into the pre-synaptic neuron, facilitates vesicle fusion, and glutamate release resulting in short term facilitation by inhibiting the S-type K^+ channels (Kandel, 2001). Studies in *Drosophila* NMJ have shown that cAMP alters pre-synaptic release probability and facilitation (Zhong and Wu, 1991), which can be achieved by the reduction of K^+ currents (Zhong et al., 1992) or by activation of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels (Cheung et al., 2005). We have demonstrated that cAMP signaling can regulate functional plasticity, independent of differentiation, at excitatory cholinergic synapses between cultured *Drosophila* neurons (Lee and O'Dowd,

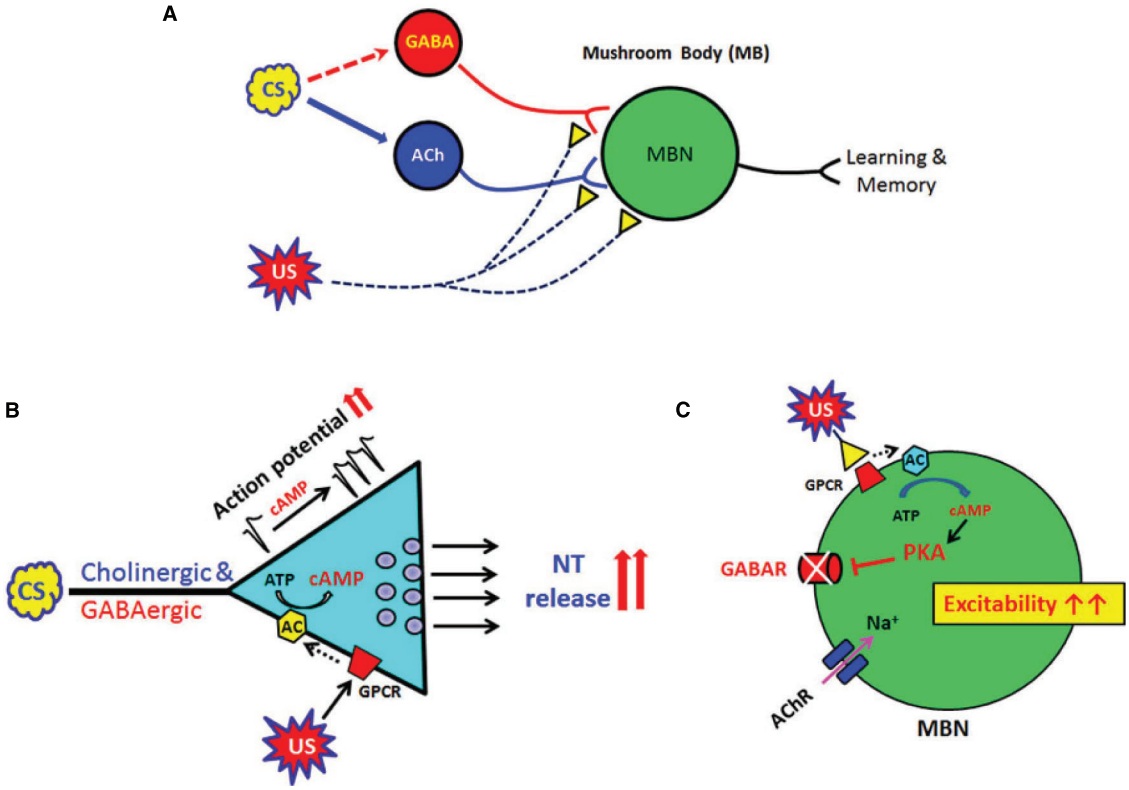

FIGURE 1 | Global and local actions of cAMP signaling in neural plasticity, learning and memory in *Drosophila*. (A) A diagram showing *Drosophila* neural circuits which mediate associative learning and memory formation. Sensory information (CS) is mainly transduced through excitatory cholinergic synaptic inputs to the fly learning and memory center, mushroom body (MB). Inhibitory GABAergic inputs to MB are known to shape this sensory information transduction although its sensory processing circuits remain to be explored (dotted arrow). US (reward or punishment) is mediated through modulatory synaptic inputs such as dopaminergic or serotonergic. Association of the CS and US in MB is the basis of *Drosophila* learning. **(B)** Cyclic AMP has global (or general) roles to increase presynaptic excitability and neurotransmitter release. These changes are found in all central neurons including excitatory cholinergic and inhibitory GABAergic neurons. **(C)** In contrast, cAMP suppresses ionotropic GABA receptors in the postsynaptic neuron and thus enhances overall excitability in MB neural circuits. Acetylcholine receptors (AChRs) in *Drosophila* postsynaptic neurons were not affected by cAMP signaling (Lee and O'Dowd, 2000). Conditional stimulus (CS); MB neurons (MBN); unconditional stimulus (US).

2000). Presynaptic GABA release was also greatly increased by an AC activator FSK (Ganguly and Lee, 2013). Further, it has been shown that facilitation is impaired in cAMP signaling mutants (Zhao and Wu, 1997; Lee and O'Dowd, 2000; Ganguly and Lee, 2013). All the findings show that the cAMP-PKA pathway plays an important role in regulating plasticity at excitatory as well as inhibitory synapses in *Drosophila* CNS. Therefore, global actions of cAMP signaling on synaptic plasticity appear to be enhanced presynaptic release of NT via increasing presynaptic excitability in all types of neurons including excitatory cholinergic and inhibitory GABAergic neurons (**Figure 1**).

Local Mission of cAMP Signaling

Cyclic AMP-PKA signaling generally (and globally) increases neuronal excitability and presynaptic release of NTs in *Drosophila* nervous system. This increase was observed not only at excitatory but also inhibitory synapses. This can be contradictory because an increase in inhibitory synaptic transmission by cAMP is expected to reduce overall strength of excitatory synaptic transmission in certain neural circuits. Therefore, we further examined the role of cAMP signaling in inhibitory GABAergic synaptic transmission in *Drosophila* (Ganguly and Lee, 2013). When postsynaptic cAMP signaling was specifically blocked by a membrane-permeable PKA inhibitor, the frequency of GABAergic synaptic currents was increased by focal application of FSK like excitatory cholinergic synaptic currents (see above). However, without the blocker in a postsynaptic neuron, GABAergic synaptic transmission was suppressed by FSK even if presynaptic GABA release was increased (Ganguly and Lee, 2013). This confirmed that PKA suppresses GABAergic synaptic transmission by regulating postsynaptic GABA receptor sensitivity through phosphorylation. Among three *Drosophila* ionotropic GABA receptors (RDL, LCCH3, and GRD; Harvey et al., 1994; Hosie et al., 1997), the GABA *resistant to dieldrin* (RDL) receptor subunit is widely expressed in several regions of the *Drosophila* brain (Harrison et al., 1996) and its expression in the fly learning and memory center mushroom body (MB) is inversely correlated to olfactory learning (Liu et al., 2007). Based on the observation that RDL containing GABA receptors mediate the majority of GABAergic synaptic currents in *Drosophila* (Lee et al., 2003; Ganguly and Lee, 2013), the action of cAMP on GABAergic synaptic

currents is likely through the GABA RDL subunit. PKA-mediated phosphorylation of RDL subunits and subsequent GABA receptor internalization may occur specifically (and locally) in the postsynaptic region (Mou et al., 2011; Vithlani et al., 2011).

Drosophila brain has shown the presence of a large number of inhibitory GABAergic interneurons, some of which innervate into the MB (Yasuyama et al., 2002; Busto et al., 2010). These interneurons have been shown to be important for various forms of information processing and behaviors including learning and memory (Olsen and Wilson, 2008; Isaacson and Scanziani, 2011; Wilson, 2013). Strengthening in the efficacy of excitatory transmission causes enhanced neural circuit plasticity. Therefore, the suppression of inhibitory transmission by a common second messenger like cAMP is expected to further increase the neural circuit excitability. In *Drosophila*, cAMP-PKA signaling increases excitability at the cholinergic synapses (Yuan and Lee, 2007) but decreases the conductance of postsynaptic GABA receptors (Ganguly and Lee, 2013). These findings demonstrate a novel dual regulatory role of cAMP by showing that it increases overall presynaptic function globally, but acts locally on postsynaptic GABA receptors to decrease GABAergic plasticity. Thus the action of cAMP results in further increases in neural excitability (**Figure 1**).

Cyclic AMP Homeostasis, Neural Circuits, Learning, and Memory

A number of *Drosophila* mutants showing defects in the cAMP-signaling cascade were originally identified on the basis of associative learning deficits (Dudai et al., 1976; Livingstone et al., 1984; Chen et al., 1986; Levin et al., 1992). Among them, a *Drosophila* Ca^{2+}/CaM-dependent AC *rutabaga* is known to function as a coincidence detector during learning and memory consolidation (Tomchik and Davis, 2009; Gervasi et al., 2010). A typical associative learning task is comprised of two different stimuli—conditional (CS; sensory input such as smell or visual) and unconditional stimuli (US; reward or punishment). In *Drosophila*, excitatory cholinergic and GABAergic inputs are considered to deliver CS signals while a variety of synaptic modulators (e.g., dopamine, serotonin, etc) are involved in the transduction of US signals (**Figure 1**). The US modulators typically stimulate GPCR to activate AC while synaptic inputs from CS neural circuits increase cellular excitability and also Ca^{2+}/CaM. Therefore, AC serves as a coincidence detector of CS and US. A *Drosophila* PDE4 homolog *dunce* is also known to cause defects in short-term memory (Dudai et al., 1976; Chen et al., 1986). Both *rutabaga* and *dunce* enzymes in *Drosophila* show changes in the strength of *Drosophila* excitatory synapses (Zhong and Wu, 1991; Lee and O'Dowd, 2000) as well as inhibitory GABAergic synapses (Ganguly and Lee, 2013). Therefore, cAMP-dependent synaptic plasticity must be an essential feature for neural circuits in mediating learning and memory.

Interestingly, consequences of *rutabaga* and *dunce* mutants are opposite in terms of intracellular cAMP levels. The former decreases basal cAMP levels and thus does not able to temporally increase cAMP in response to acute stimulation, while the letter increases the basal level of intracellular cAMP much more than that in wild type. However, both mutations cause defects in synaptic plasticity and memory, strongly indicating the importance of cAMP homeostasis. Proper regulation of intracellular cAMP appears to be critical for neural plasticity and memory in fly. Thus it is of interest to understand the role of these two enzymes maintaining homeostasis of cAMP in *Drosophila* neurons.

The *Drosophila* MB is comprised of several subdivisions (e.g., α, β, γ lobe, etc) (Keene and Waddell, 2007; Guven-Ozkan and Davis, 2014). Different MB neurons are involved in distinct types and/or phases of associative learning and memory in the fly. Further details can be found in several excellent reviews on MB structure and function (Fahrbach, 2006; Waddell, 2013; Guven-Ozkan and Davis, 2014). The synaptic inputs to MB are mainly coming from excitatory cholinergic and GABAergic neurons from the centers of sensory system (e.g., antennal lobes for olfaction) as they primarily mediate sensory information. For example, projection neurons (PNs) in antennal lobes relay sensory signals to MB neurons for olfactory associative learning. PN neurons are cholinergic and thus excitatory synaptic inputs. MB neurons also receive synaptic inputs from GABAergic neurons mainly from the region called lateral horn (LH; Busto et al., 2010). Since *rutabaga* and *dunce* are preferentially expressed in MB, cAMP signaling is important for synaptic plasticity in MB neurons and also essential for learning and memory.

Learning and memory defects in *rutabaga* can be easily explained as its basal cAMP level is too low so that no neural input signal can induce proper cAMP-dependent synaptic plasticity mediating learning memory. Interestingly, *dunce* mutants with high levels of cAMP in MB neurons also show defects in short-term memory (Gervasi et al., 2010). Further, the *dunce* MB neurons show an increase in PKA levels. These findings suggest that cAMP-mediated potentiation of cholinergic synaptic transmission and inhibition of GABA receptor should be greater in *dunce* neurons. However, the *dunce* and *rutabaga* mutants, despite having opposing effects on cellular cAMP levels, showed very similar defects in synaptic plasticity at both excitatory and inhibitory synapses (Lee and O'Dowd, 2000; Ganguly and Lee, 2013). Several other studies have also shown that *dunce* and *rutabaga* have similar defects in growth cone motility, neural plasticity and more importantly, short-term memory (Kim and Wu, 1996; Gasque et al., 2006). Although the effects of cAMP on cholinergic and GABAergic synaptic plasticity in *dunce* and *rutabaga* mutants are similar, it is very likely that the molecular mechanisms underlying these responses differ in the two mutants. It has been shown that increased PKA activity in mouse hippocampus hyper-phosphorylates several downstream molecular targets including a tyrosine phosphatase, correlates with decreased PDE protein levels and results in memory defects (Giralt et al., 2011). Therefore, it is expected that high basal levels of cAMP due to the *dunce* mutation leads to the activation of phosphatase(s) and thus reduces the effects of cAMP. However, this remains to be explored in *Drosophila* nervous system. Taken together, all these findings strongly suggest that disruption of cellular cAMP homeostasis can alter excitatory cholinergic and inhibitory GABAergic synaptic plasticity and hence cause defects

in associative learning, although the underlying mechanisms leading to this effect can be different (e.g., reduced PKA activity in *rutabaga* versus increased phosphatase activity in *dunce*).

Drug Targets in cAMP Signaling

Cyclic AMP is the most abundant and important second messenger in the nervous system. Therefore, it makes sense that its signaling involves a variety of physiological and pathological processes such as learning and memory, pain, drug addiction and neurodegeneration. Molecular components in cAMP-PKA signaling pathway should be excellent targets for the development of new therapeutic strategies. These components in cAMP signaling are very well conserved throughout animal groups and, therefore, findings from *Drosophila* can be directly implicated in mammalian systems including human.

Memory is an important physiological process for survival and better quality of life. Thus any compromise in this behavior is likely a problem as seen in Alzheimer's disease (AD) and mental retardation. Given the importance of cAMP signaling in neural plasticity and cognition, any molecular component in this signaling pathway can be a potential target for drug development to enhance cognition. Particularly two enzymes have drawn more attention. The first target is a PDE which down-regulates cAMP-PKA signaling. Therefore, PDE inhibitors will prolong cAMP signaling and produce higher levels potentially enhancing cognition. Indeed, a PDE4 inhibitor HT-0712 has been shown to improve hippocampus-mediated memory in mice (Peters et al., 2014). HT-0712 also increases CRE-mediated gene expression and ameliorates spatial memory impairment in aged mice, therefore, it could be used to treat age-associated memory impairment (AAMI) in humans. In fact, clinical studies with this drug have been carried out and show significant effects on long-term memory in AAMI patients (refer to: www.dartneuroscience.com). Since an increase of cAMP-specific PDE mRNAs was observed in early stages of AD (Bollen and Prickaerts, 2012), PDE inhibitors can be used to slow/treat this disease. However, it should be kept in mind that the chronic increase of basal cAMP by PDE inhibitors can give negative impacts on learning and memory as seen in the *dunce* mutants.

The second promising drug target for cAMP signaling is AC. AC1/AC8 double knockout mice showed loss of LTP as well as memory (Wong et al., 1999). Further, beta amyloid peptides interfere with AC-dependent LTP in hippocampus (Yamamoto et al., 1997). Findings with *rutabaga* fly mutants are consistent as these flies showed defects in synaptic plasticity and short-term memory. Therefore, drugs that stimulate AC activities can be useful to treat memory deficits although no drug is on a clinical test yet. AC is an attractive drug target to be further explored.

Additional potential targets in the cAMP signaling pathway are PKA and CREB-binding protein (CBP). Rubinstein-Taybi syndrome (RTS), a genetic disorder showing mental retardation and physical abnormalities (Bourtchouladze et al., 2003) is known to be caused by mutations of CBP. Regarding the role of CREB and CBP in the nervous system, it is noteworthy to mention that some addictive drugs (e.g., amphetamine, opiates) also alter CREB expression in several brain areas (e.g., nucleus accumbens and hippocampus; Robbins et al., 2007; Nestler, 2013) indicating involvement of cAMP signaling in drug addiction. Interestingly, *Drosophila* has been used as a favorable model animal to study actions of addictive drugs such as cocaine and alcohol (Kaun et al., 2012). A recent study showed that *Drosophila rutabaga* in MB neurons is necessary for robust ethanol self-administration (Xu et al., 2012) demonstrating the role of cAMP signaling in reinforced behaviors. Therefore, PKA and CREB-related proteins can be excellent drug targets to treat not only cognitive deficits but also drug addiction.

Given the suitability of multiple drug targets in cAMP signaling, one important question is the therapeutic safety in addition to drug potency. In other words, how can we deliver a drug to a specific target in order to minimize toxic side effects? Chemogenetic tools have been developed to enhance specific drug delivery spatially and temporally (Sternson and Roth, 2014). One such tool is designer receptors exclusively activated by designer drugs (DREADDs), which can increase or decrease intracellular cAMP levels depending on the receptor type by a biologically inert chemical (Becnel et al., 2013). Using this tool, cAMP signaling can be regulated in a specific set of neurons (e.g., hippocampus, MB) as well as duration of action (e.g., acute versus chronic). Therefore, CREB-mediated long-term effects can be also induced. In addition, DREADD can be an excellent research tool to uncover more specific roles of cAMP signaling in a variety of neuronal processes including learning and memory.

Conclusion

In this commentary, I focused on the role of cAMP signaling in neural excitability, synaptic plasticity, learning and memory. On the basis of work from *Drosophila*, dual regulatory roles (global versus local) of cAMP signaling are to increase the strength of excitatory neural circuits on one hand, but to act locally on postsynaptic GABA receptors to decrease inhibitory synaptic plasticity on the other. Thus the action of cAMP could result in a global increase in the neural circuit excitability and memory. The cAMP signaling is also implicated in pain processing, AD and drug addiction. All these normal and disease-related behaviors are mediated through interaction between specific neural circuits comprised of excitatory and inhibitory synapses. Therefore, knowledge gained from the studies of cAMP signaling can contribute to the development of new or more effective drugs. Since molecular components and functions of cAMP signaling pathway have been well conserved in *Drosophila*, it could be a useful animal model to study mechanisms underlying behaviors mediated by cAMP signaling at the molecular, physiological and circuit levels. The fly can also be an excellent drug discovery platform for diseases with defects in cAMP signaling.

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Spatiotemporal regulation of cAMP signaling controls the human trophoblast fusion

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During human placentation, mononuclear cytotrophoblasts fuse to form multinucleated syncytia ensuring hormonal production and nutrient exchanges between the maternal and fetal circulation. Syncytial formation is essential for the maintenance of pregnancy and for fetal growth. The cAMP signaling pathway is the major route to trigger trophoblast fusion and its activation results in phosphorylation of specific intracellular target proteins, in transcription of fusogenic genes and assembly of macromolecular protein complexes constituting the fusogenic machinery at the plasma membrane. Specificity in cAMP signaling is ensured by generation of localized pools of cAMP controlled by cAMP phosphodiesterases (PDEs) and by discrete spatial and temporal activation of protein kinase A (PKA) in supramolecular signaling clusters inside the cell organized by A-kinase-anchoring proteins (AKAPs) and by organization of signal termination by protein phosphatases (PPs). Here we present original observations on the available components of the cAMP signaling pathway in the human placenta including PKA, PDE, and PP isoforms as well as AKAPs. We continue to discuss the current knowledge of the spatiotemporal regulation of cAMP signaling triggering trophoblast fusion.

Keywords: protein kinase A, cAMP, AKAPs, phosphodiesterases, phosphatases, placenta, trophoblast fusion

Introduction

Cell fusion processes are essential for fertilization, fetal and placental development, skeletal muscle formation, bone homeostasis and appears to play a role in metastasis (Midgley et al., 1963; Zambonin Zallone et al., 1984; Wakelam, 1985; Oren-Suissa and Podbilewicz, 2007; Lu and Kang, 2009). Cell fusion and syncytia formation involve the mixing of plasma membrane components and cell contents between two or more cells. Although occurring in a variety of biological contexts, different fusion processes share many of the same steps that are tightly regulated in space and time (Pérot et al., 2011).

Human embryo implantation requires placentation, a process in which fetal cytotrophoblasts (CT) invade the maternal endometrium to form an interphase with the maternal circulation, ensuring effective exchange of gases, nutrients, and the secretion of pregnancy-specific hormones

(i.e., hCG: human chorionic gonadotropin and hPL: human placental lactogen) (Eaton and Contractor, 1993; Ogren and Talamentes, 1994; Benirschke and Kaufmann, 2000). Throughout pregnancy CTs fuse to form a multinucleated syncytia on chorionic villi extending into the maternal placental blood circulation. Due to their capacity to differentiate into syncytia allowing essential placental exchange between mother and child necessary for fetal growth, the CT plays an essential role during human pregnancy. The *in vivo* fusion process in the placenta is reproducible *in vitro* using purified human CTs, which aggregate and then fuse to form non-proliferative, multinucleated, endocrine active syncytiotrophoblasts (STs; **Figure 1**; Kliman et al., 1986). Numerous proteins have been reported to be implicated in cell fusion processes such as tight junction, adherens junction, and gap junction proteins (Coutifaris et al., 1991; Dahl et al., 1995; Mbalaviele et al., 1995; Ilvesaro et al., 2000; Frendo et al., 2003; Charrasse et al., 2007; Pidoux et al., 2010).

The cAMP signaling pathway is known to play a critical role in induction of CT and myoblast cell fusion reviewed in (Gerbaud and Pidoux, 2015). For instance, in human placentation, hCG stimulates cytrophoblast fusion in an autocrine or paracrine fashion through binding to the LH/CG receptor (LH/CG-R), activating a specific adenylyl cyclase (AC) and the synthesis of intracellular cAMP as second messenger (**Figure 2**). The nine transmembrane AC isoforms have been identified with various expression levels in human CT and on the plasma and microvillous membrane of the ST (Sato and Ryan, 1971; Matsubara et al., 1987; Bernatchez et al., 2003).

Next, the cAMP increase leads to activation of downstream effectors such as cAMP-dependent protein kinase A (PKA) and the phosphorylation of specific targets (Tasken and Aandahl, 2004). Whereas, PKA is known to be the main cAMP effector, other intracellular effectors exist such as exchange proteins activated by cAMP (Epac) and the cyclic nucleotide-gated ion channels (Walsh et al., 1968; Nakamura and Gold, 1987; de Rooij et al., 1998; Kawasaki et al., 1998) (**Figure 2**). The cAMP signaling pathway is one of the best-characterized signal transduction pathways and requires a high level of spatial and temporal regulation to convey the appropriate inputs. The temporal regulation is achieve by the cAMP synthesis through AC and metabolized by cAMP-phosphodiesterase (PDE) activity. Both AC and PDE establish in cAMP microdomains within the cell (Zaccolo and Pozzan, 2002). Furthermore, A-kinase anchoring proteins (AKAPs) provide through PKA anchoring a spatial regulation of the cAMP/PKA signaling by placing the kinase in the vicinity substrates.

Little is known about AKAP proteins underlying the spatio-temporal regulation of the cAMP-induced human cytrophoblast fusion and regulation of endocrine functions in the placenta. However, we have examined the functional role of AKAP-anchored signaling complexes human primary CTs in a recent report (Pidoux et al., 2014). Here we provide for the first time original data and on the repertoire of cAMP signal components in the human placenta and compile the current knowledge about the role of the AKAP proteins underlying cell fusion process.

Materials and Methods

Cell Culture

Villous CTs were isolated from term placentas and cultured as described previously (Pidoux et al., 2014). BeWo cells were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured as described by manufacturer.

Immunolocalization Studies

Immunocytofluorescence was performed as described previously (Pidoux et al., 2014).

Fusion Assay

Cell fusion was quantified by trophoblast fusion assays as described previously (Pidoux et al., 2014). Briefly, fusion indices were calculated as the ratio of the number of nuclei in STs divided by the number of total nuclei. A ST was defined as at least

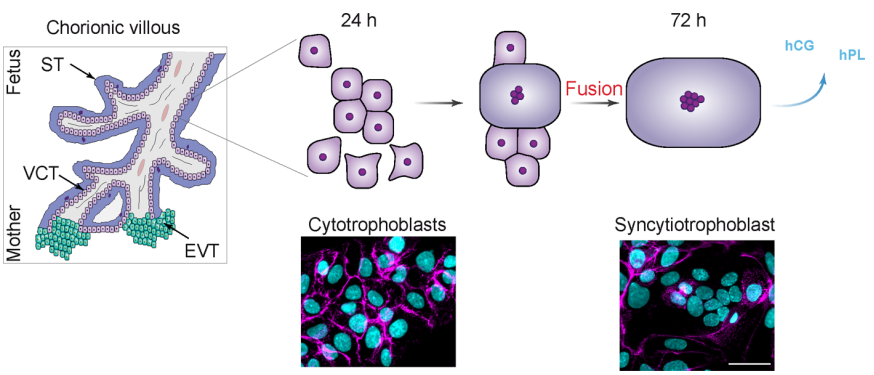

FIGURE 1 | Model of cultured villous trophobasts purified from human placenta. (Left) Schematic view of human chorionic villi. VCT for villous cytotrophoblast, ST for syncytiotrophoblast, and EVT for extravillous trophoblast. (**Right**, upper) Model of trophoblast fusion. Cytotrophoblasts (CT) aggregate after 24 to 48 h of culture, and fuse into a ST after 72 h, which secretes human chorionic gonadotropin (hCG) and human placental lactogen (hPL). (**Right**, Lower) Human trophoblast stained at 24 and 72 h of culture for desmoplakin (magenta) and nuclei (DAPI, cyan). Scale bar: 15 μm.

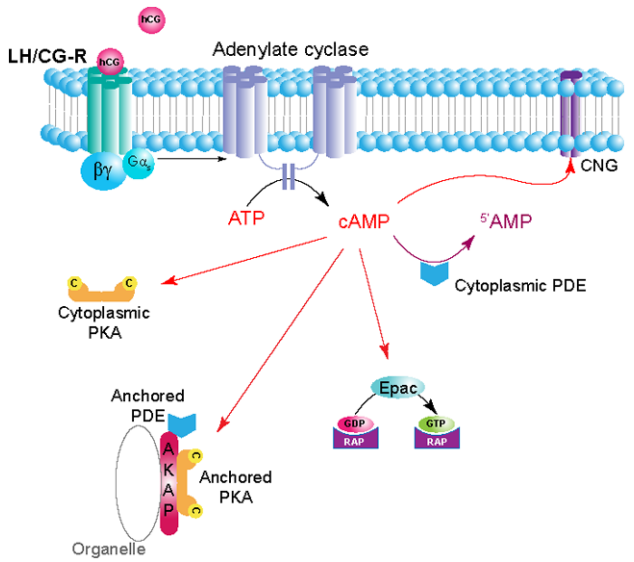

FIGURE 2 | Schematic depiction of hCG/cAMP signaling pathway in trophoblasts. Ligand (hCG) binding to the LH/CG-R receptor (LH/CG-R), a G protein-coupled receptor (GPCR), activates adenylyl cyclase (AC) in its proximity and generates pools of cAMP. The local concentration and distribution of the cAMP gradient is limited by phosphodiesterases (PDEs), that hydrolyse cAMP to 5′-AMP. Subcellular structures may harbor specific isozymes of protein kinase A (PKA) that, through anchoring to A-kinase-anchoring proteins (AKAPs), are localized in the vicinity of the receptor and the cyclase. PDEs are also anchored and serve to limit the extension and duration of cAMP gradients. These mechanisms serve to localize and limit the assembly and triggering of specific pathways to a defined area of the cell close to the substrate. Cyclic AMP has effects on a range of effector molecules encompassing PKA, PDEs, Epacs (exchange proteins activated by cAMP) and cyclic nucleotide-gated ion channels (CNGs).

three nuclei surrounded by a cell membrane as identified by discontinuous desmoplakin immunostaining.

Protein Sample Preparation and Immunoblot Analysis

Cell extracts were prepared as described previously (Pidoux et al., 2014). Protein samples were resolved by SDS-PAGE and immunoblotted with antibodies (catalog numbers and supplier are indicated unless stated above) against PKA RIα (0.25 μg/ml; 4D7; BD Biosciences), PKA RIIα (0.25 μg/ml; 612243; BD Biosciences), PKA RIIβ (0.25 μg/ml; 610626; BD Biosciences); PKA Cα/β (0.25 μg/ml; 610980; BD Biosciences); AKAP18 (0.5 μg/ml; WH0009465M1; Sigma–Aldrich); GAPDH (1 μg/ml; G8795; Sigma–Aldrich). After incubation with the appropriate HRP-conjugated secondary antibody, blots were developed by using Supersignal West Pico substrate (Thermo Scientific, Illkirch, France).

R-Overlays

R-overlays were performed as described previously (Hausken et al., 1998) by using ^{32}P-labeled recombinant murine RIIα. Membranes with immobilized proteins were blocked in Blotto (5% (w/v) non-fat dry milk plus 0.1% BSA in TBS). Purified recombinant R (4 μg) was radiolabeled with purified catalytic subunit (C) (0.02 μg/μl) and [γ-^{32}P]ATP (1.4 μCi/μl) in a buffer containing 50 mM MOPS pH 6.8 with 50 mM NaCl, 2 mM $MgCl_2$, and 1 mM DTT, and separated from free ^{32}P-ATP by gel filtration in G-50 sepharose. Membranes were incubated with 1×10^6 cpm/ml of ^{32}P-labeled recombinant R in TBS-T. For competition assays, Ht31 peptide was added to the radiolabeled R at a concentration of 500 nM and incubated for 2 h before the membranes were added. The membranes were washed in TBS-T and subjected to autoradiography.

Peptide Synthesis and Loading

Peptides used in trophoblast fusion assay (PKI: R9-TYADFIASGRTGRRNAI and scrambled PKI: R11-ANITSGYFDTIAAGR) were synthesized on an Intavis MultiPep robot (Intavis Bioanalytical Instruments AG), uncoupled and verified by high performance liquid chromatography (HPLC). The concentrations of the peptides were determined by amino acid analysis using an amino acid analyzer from Thermo Scientific Dionex. PKI peptide and its respective control were used at 10 μM.

Pull-Down Assays

cAMP pulldown assays (8-AHA-cAMP-agarose beads) were performed as described previously (Pidoux et al., 2014). The lysate-bead suspensions were subjected to LC-MS/MS.

Protein Identification by LC–MS/MS

Protein identifications were performed as described previously (Pidoux et al., 2014).

siRNA and Mammalian Expression Vector Transfection

Transfections of siRNA or plasmid were performed using Lipofectamine 2000 CD reagent (Life Technologies). Silencing RNA transfections [performed as described previously (Pidoux et al., 2014)] were performed with AKAP18 stealth siRNA (HSS145157) and control (Life Technologies). Mammalian expression vector (2 μg) was incubated with the cells for 48 h at 37°C. ^{T}Epac^{VV} clone was described previously (Klarenbeek et al., 2011). The construct was verified by sequencing. Transfection efficiency was determined to be >45%.

Optical Biosensor Recordings

Optical recording with biosensor was performed as previously described (Polito et al., 2013). Wide-field images were obtained with Olympus BX50WI upright microscope with a 20× 0.5 NA or a 40× 0.8 NA water-immersion objective and an ORCA-AG camera (Hamamatsu). Images were acquired with iVision (Biovision). The excitation and dichroic filters were D436/20 and 455dcxt. Signals were acquired by alternating the emission filters, HQ480/40 for CFP and D535/40 for YFP, with a filter wheel (Sutter Instruments). Image acquisition was triggered manually, except for kinetics measurement where images were acquired automatically within 3–5 s intervals. Pseudocolor images display the ratio value coded in hue and the fluorescence intensity coded in intensity. A calibration square indicates the intensity values

from left to right and the ratio values from bottom to top. The size of the square indicates the scale of the image in microns.

RNA Extraction

Total RNA was extracted from primary trophoblast cells after 24 or 72 h of culture by using the Trizol reagent (Life Technologies). The yield of extracted RNA was determined by measuring optical density at 260 nm. The purity and quality of extracted RNA were subsequently assessed by electrophoresis on 1% agarose gel with ethidium bromide staining. Only high-integrity RNA samples were used for PCR analysis.

RT-Polymerase Chain Reaction

Reverse transcription-polymerase chain reaction (RT-PCR) was performed with the TaqMan®Array Human Phosphodiesterase (4414075, Applied Biosystem) and Human PrimePCR Phosphatases (H384, Biorad) or primers ordered from Santa-Cruz Biotechnology according to the manufacturers' protocols for analysis of PDEs, PPs, and AKAPs, respectively. GAPDH or ACTB mRNA and 18S RNA levels were used as endogenous RNA controls and were tested to remain constant during trophoblast fusion. Relative gene expression (ΔC_T) was calculated by subtracting the signal threshold cycle (C_T) of 18S or GAPDH from the C_T value of each studied gene. Subsequently, $\Delta\Delta C_T$ values were calculated by subtracting 18S or GAPDH ΔC_T (set as calibrator) from the ΔC_T of each individual gene and transformed and presented as $2^{-\Delta\Delta CT}$ in order to obtain the relative gene expression (fold) of genes of interest.

Phosphodiesterase Activity

LANCE Ultra cAMP assay was modified and adapted to total cell lysate to measure the cAMP-PDE activity. The europium chelate (Eu)-cAMP tracer molecule is captured by an ULight-labeled anti-cAMP monoclonal antibody (mAb), which brings donor and acceptor dye molecules into close proximity. Following irradiation of the samples at 340 nm, the excited energy of the Eu chelate donor is transferred by FRET to the ULight acceptor dye. Thus ULight molecules emit a signal detectable at 665 nm in TR-FRET mode. Residual energy from the Eu chelate produce light at 615 nm and remains constant. In the absence of free cAMP, maximal TR-FRET signal is achieved. Free cAMP produced by stimulated cells competes with the Eu-cAMP tracer for the binding to the ULight mAb, causing a decrease in TR-FRET signal proportional to the concentration of cAMP produced. LANCE Ultra cAMP provides an assay with S/B ratio of 44.6 and an IC50 of approximately 1 nM.

Proteins from trophoblast cell lysate (20 μg) were incubated in presence of 6 nM cAMP with PDE antagonists or DMSO as vehicle, for 90 min at 24°C and under shaking. Subsequently (Eu)-cAMP tracer molecule and ULight-labeled anti-cAMP antibody were added to the solution prior to analysis. Cyclic AMP hydrolysis was below 20%. Histograms represented the profile of PDE activity in trophoblasts.

Statistical Analysis

The GraphPad Prism 6 software package was used for statistical analysis. Differences between groups were evaluated with ANOVA. *Post hoc* analysis (Tukey) was used for individual comparisons and to obtain *p*-values shown in the figure legends. The sample size and significance level is shown in the figure legends for each graph. All data are presented as means ± SEM. $P < 0.05$ was considered statistically significant.

Results

Protein Kinase A Triggers Human Trophoblasts Fusion

Human placental trophoblasts express RIα, RIIα regulatory subunits and the Cα, Cβ catalytic subunits as evident from immunoblots (**Figures 3A,B**) which support earlier reports (Keryer et al., 1998a,b). RIα is in the cytosol and decreases significantly during the cell fusion process and differentiation to STs while RIIα remains constant and relocates from the cytosol to the Golgi apparatus and to the plasma membrane (Keryer et al., 1998a,b). Interestingly the loss of PKA RIα protein expression during cell fusion process was not associated with a decrease in mRNA level (data not shown), but is probably due to protein destabilization. Indeed, hCG-mediated cAMP increase during syncytialization dissociates and activates PKA holoenzyme and the free RIα and C subunits have been shown in other cell types to be more rapidly degraded to prevent overshoot of catalytic activity in response to signal transduction by cAMP (Tasken et al., 1993). A chemical proteomics approach where we did cAMP affinity chromatography followed by mass spectrometry analysis allowed us to identify PKA RIα and RIIα in human CT and STs (**Figure 3B**), which supported previous reports and our observations by immunoblot analysis. Furthermore, we also observed expression of PKA RIβ isoform in CT and ST at a constant level during cell fusion (**Figure 3A**; Pidoux et al., 2014).

In vitro, the addition of cAMP analogs or hCG promote CT fusion to ST, whereas PKA inhibitors (H89) or a cell permeable version of protein kinase inhibitor (PKI) impair cell fusion (**Figure 3C**; Keryer et al., 1998b; Pidoux et al., 2014). It has been proposed that type I PKA is the major PKA holoenzyme that control the cell fusion process and in a less extend type II PKA (Keryer et al., 1998a). Moreover the redistribution of type II PKA during cell fusion is suggested to control the transport of vesicles from *trans*-Golgi network leading to hormone secretion as well as in the reorganization of the subcortical cytoskeleton occurring during the plasmalemma membrane fusion between CT (Keryer et al., 1998a). Interestingly, transient overexpression of the PKA catalytic subunit led to an increase fusion of the trophoblast-derived choriocarcinoma cell line BeWo cells (Knerr et al., 2005).

A Kinase Anchoring Proteins Control Placental Functions

A kinase anchoring proteins are structurally diverse family of functionally related proteins that include more than 50 members (including space variants) (Pidoux and Tasken, 2010). They are defined on the basis of their ability to bind to PKA and co-precipitate catalytic activity. This binding ensures

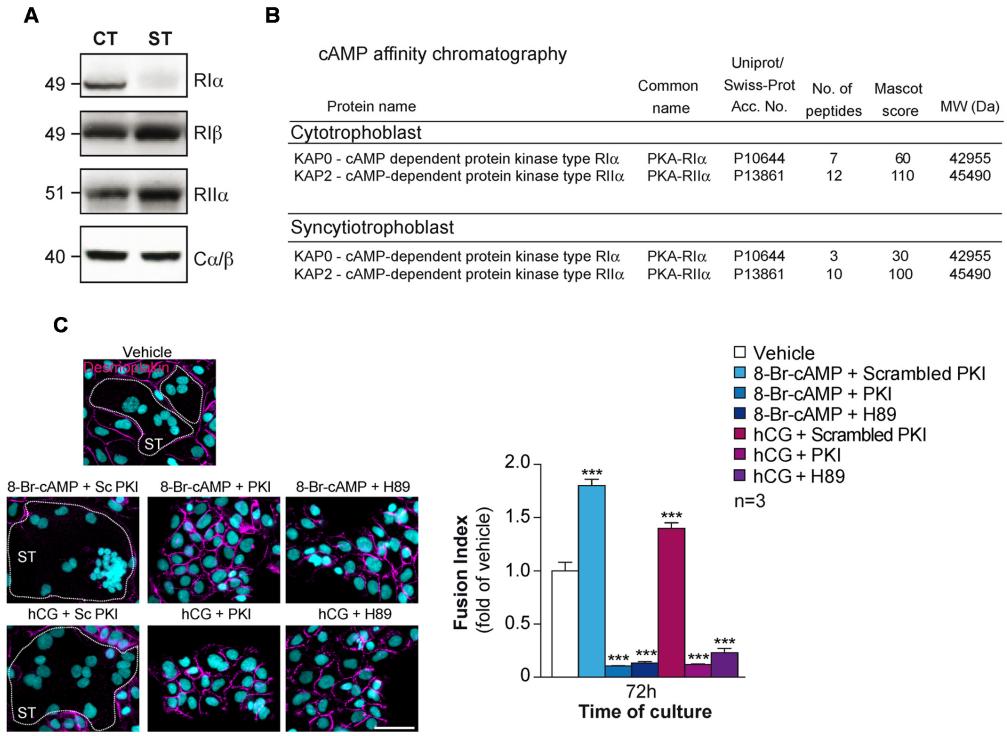

FIGURE 3 | Characterization of PKA in human trophoblasts. (A) Immunoblot analysis of RIα, RIβ, RIIα, and Cα/β in lysates of human primary trophoblasts at 24 and 72 h of culture. CT, ST (formed after 72 h of culture). **(B)** PKA R subunits identified by cAMP pulldown in trophoblasts. Proteins from CTs and STs were purified by cAMP affinity chromatography and identified by nanoLC-LTQ Orbitrap Mass Spectrometry analysis of tryptic digests of bands excised from SDS-PAGE. **(C)** Effect of 8-Br-cAMP (100 μM), hCG (1 μM), scrambled PKI or PKI peptide (10 μM each) and H89 (3 μM) on trophoblast fusion at 72 h of culture. Cells were immunostained for desmoplakin (magenta) and nuclei were counterstained with DAPI (left). Syncytia (ST) boundaries are indicated by dashed lines. Effect of 8-Br-cAMP, hCG in combination with PKI peptide or H89 on cell fusion represented as fusion indices histograms (upper right). Results are expressed as the mean ± SEM of $n = 3$ independent experiments (***$p < 0.001$). Scale bar: 30 μm.

specific subcellular compartmentalization of the enzyme thereby providing spatial and temporal regulation of the PKA-signaling events. All AKAPs contain a PKA regulatory-binding domain that consists of an amphipatic helix of about 14–18 residues (Carr et al., 1991). Moreover, all AKAPs present a unique targeting domain directing the PKA–AKAP complex to defined subcellular structures, membranes, or organelles (**Figure 2**). In addition to these two domains, several AKAPs are also able to form multivalent signal transduction complexes by interaction with phosphoprotein phosphatases (PPs), kinases, PDEs and other proteins involved in signal transduction (Coghlan et al., 1995; Schillace and Scott, 1999; Feliciello et al., 2001; Tasken et al., 2001; Scott and Pawson, 2009). This scaffolding property of AKAPs functions to coordinate cell-signaling events in space and time and enhance cross talk between signaling pathways. Type II PKA is typically particulate and confined to subcellular structures and compartments anchored by cell- and tissue-specific AKAPs (Colledge and Scott, 1999; Dodge and Scott, 2000; Diviani and Scott, 2001). However, several RI-specific AKAPs have also been characterized (i.e., SKIP, smAKAP), although the type I PKA, which is classically known to be biochemically soluble, has been assumed to be mainly cytoplasmic (Means et al., 2011; Burgers et al., 2012). In addition, dual-specific AKAPs (D-AKAPs) are capable of anchoring both types of R subunits (Huang et al., 1997a,b).

Several AKAPs have been identified in placenta or placental trophoblast cell lines, for instance the AKAP PAP7 is highly expressed in placenta (Li et al., 2001; Liu et al., 2003; Weedon-Fekjær and Taskén, 2012). A number of other AKAPs are expressed in varying amounts in placenta such as AKAP18γ, AKAP350, and AKAP-Lbc 10 kb transcript (Lin et al., 1998; Trotter et al., 1999).

In search for AKAPs in primary CTs, proteins from subcellular fractionation or eluates from pull-down of cAMP-binding proteins using 8-AHA-cAMP-agarose beads were separated by SDS-PAGE, blotted to nitrocellulose membranes which were overlaid with radiolabelled RII in the absence or presence of Ht31 anchoring disruptor peptide (**Figure 4A**). Several bands with molecular masses in range of ∼160 and ∼15 kDA were detected in cytosol, membrane, nucleus, and cytoskeleton fraction by RII-overlay and competed by Ht31 peptide (**Figure 4A**). In the same manner, bands with similar molecular masses were detected by RII-overlay following pull-down of cAMP-binding proteins (**Figure 4A**). Our original observations are in agreement with previous studies looking at mRNA expression of AKAPs

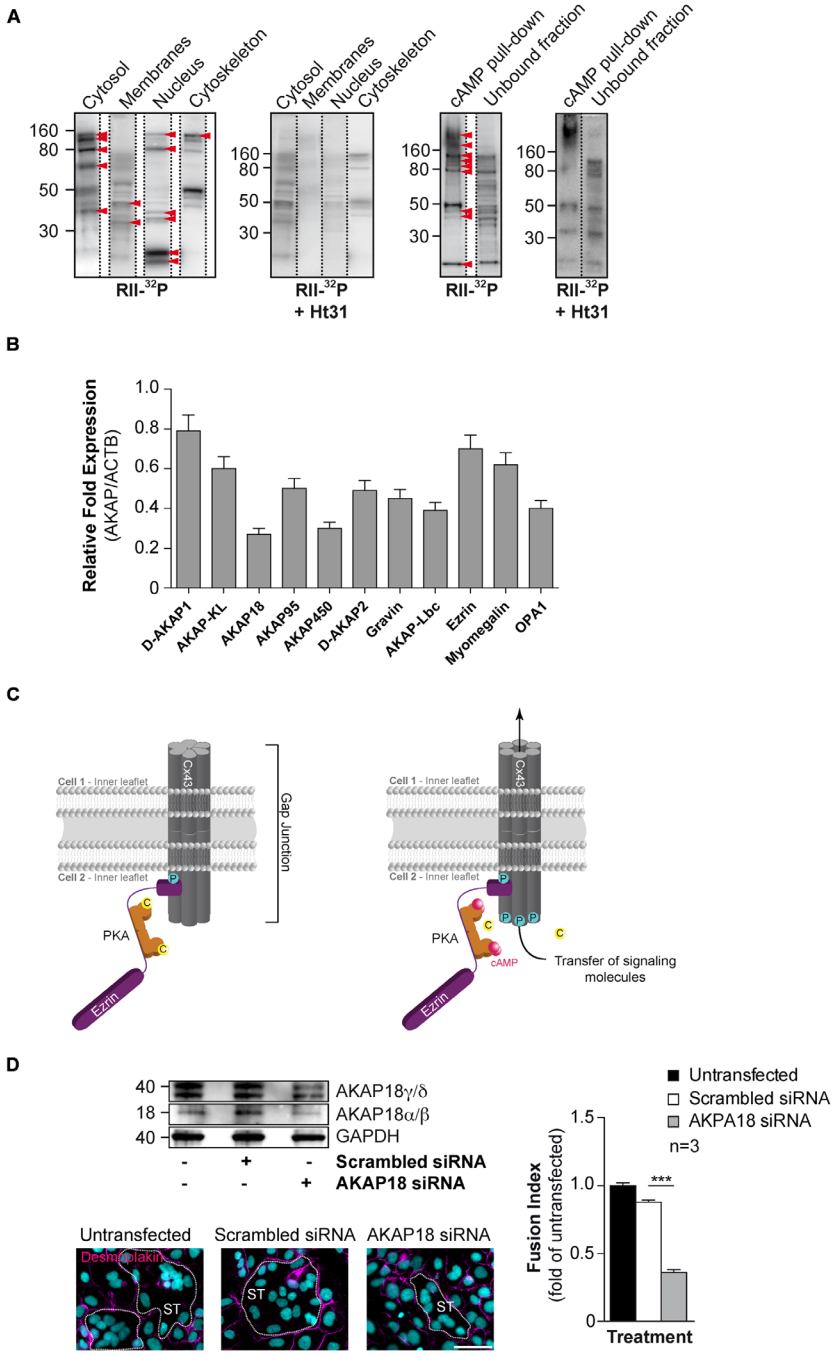

FIGURE 4 | Characterization of AKAPs in human CTs. (A) Proteins purified by sub-cellular fractionation (left) or by cAMP affinity chromatography (right) from cytrophoblasts were subjected to a solid phase binding assay using ^{32}P-radiolabeled RII (RII-overlay) as a probe in the absence or presence of the Ht31 anchoring disruptor peptide (500 nM). Red arrowheads indicate putative AKAPs expressed in CTs. **(B)** Total RNA was purified from CTs ($n = 3$ cultures each) and subjected to RT-PCR with specific AKAP primers (identified in **Table 1**). Histograms represent mRNA expression of AKAPs in CTs normalized to beta-actin mRNA expression. **(C)** Cx43 gap junction communication is controlled by PKA anchoring through ezrin (Pidoux et al., 2014). Schematic depiction of a resting state gap junction in primary human trophoblast with Cx43 and a compartmentalized pool of PKA anchored to ezrin thus bound to Cx43 (left). Elevated intracellular cAMP levels lead to activation of PKA and subsequent spatiotemporally controlled phosphorylation of Cx43, which promotes the communication through gap junctions. This communication triggers trophoblast cell fusion. C, catalytic subunit of PKA; P for phosphorylation; pink dots, molecules of cAMP. **(D)** BeWo cells were transfected with AKAP18 siRNA (Invitrogen, Cat. # 1299001) or scrambled control, cultured for 48 h, stimulated for fusion with FSK (15 μM) for 24 h and subjected to immunoblot analysis with the indicated antibodies (upper left). Cells with AKAP18 knockdown or controls were stained for desmoplakin (magenta) and nuclei (DAPI). Syncytia (ST) boundaries are indicated by dashed lines. Scale bar: 30 μm. (Lower left) The effects of AKAP18 siRNA, scrambled control on fusion were assessed after 24 h treatment with FSK (15 μM) and summarized in histograms (right). Results are expressed as the mean ± SEM of $n = 3$ independent experiments (***$p < 0.001$). Scale bar: 30 μm.

and suggesting existence of several AKAPs in human primary CTs. To identify AKAPs involved in the cell fusion process, we isolated cAMP-signaling complexes from cultures CTs and ST, either by pull down of cAMP-binding proteins or by FLAG-affinity chromatography after incubation with purified FLAG-tagged regulatory RI and RII subunits, and we subjected bands excised and eluted from SDS-PAGE to tryptic digestion and mass spectrometry analysis. By this approach we identified 11 AKAPs, which are summarized in **Table 1**. An RT-PCR screening strategy was applied to characterize expression of AKAP mRNAs in CTs (**Figure 4B**). We found that these cells expressed AKAP mRNAs with various levels that supports our mass spectrometry analysis. A screen by siRNA-mediated knockdown of various identified-AKAPs allowed us to characterize the involvement of five AKAPs (D-AKAP1, AKAP18, AKAP450, Ezrin, and myomegalin) in the regulation of cell fusion (**Table 1**; Pidoux et al., 2014). This suggests specific roles for pools of AKAP-anchored type I and/or type II PKA in trophoblast fusion.

We recently demonstrated that trophoblast fusion is regulated by ezrin, a known AKAP, which binds to connexin-43 and delivers PKA in the vicinity gap junctions. We found that disruption of the ezrin-Cx43 interaction abolished PKA-dependent phosphorylation of Cx43 as well as gap junction communication and subsequently hCG-induced cell fusion in human primary trophoblasts (**Figure 4C**; Pidoux et al., 2014). This appears to be a general mechanism for gating of Cx43 gap junctions and a dominant mechanism in controlling CT fusion, although not the only mechanism.

Looking at other AKAPs that could be involved in hCG action and cell fusion, AKAP79, AKAP95, and AKAP250 (gravin) were also detected in the trophoblast cell line BeWo (Delidaki et al., 2011). In the same study, the authors demonstrated cross talk between the cAMP/PKA and MAPK pathways involved in secretion of hCG, which acts as an auto-paracrine loop to induce trophoblasts fusion. The authors found that this cross talk was dependent on PKA and PKA–AKAP interaction as a specific PKA inhibitor (myr-PKI) and a PKA anchoring disruptor peptide (Ht31) inhibited the forskolin (FSK)-induced MAPK stimulation (Delidaki et al., 2011). Based on our original observations, siRNA-mediated knockdown of AKAP18 in BeWo cells reduced all isoforms of AKAP18 expression after normalization to GAPDH levels compared with cells transfected with scrambled

TABLE 1 | A-kinase-anchoring proteins (AKAPs) identified in trophoblasts.

AKAP *(gene nomenclature committee name)*	Method of identification	Subcellular localization	Properties/function	CT/ST	Cell-fusion
D-AKAP1 (*AKAP1*)	RII affinity chromatography	Outer mitochondrial membrane Endoplasmic reticulum Nuclear envelope	Dual-specific AKAP (D-AKAPs) Binds lamin, PP1 and PDE7A Multiple splice variants	CT	Decrease – T
AKAP-KL (*AKAP2*)	RII affinity chromatography	Actin cytoskeleton Apical membrane	Multiple splice variants	CT/ST	No effect – T
AKAP18α, β, γ, δ (*AKAP7*)	cAMP chromatography	Basolateral (α) Apical (β) Plasma membrane (γ) Cytoplasm (γ) Secretory vesicles (δ)	Targeted to plasma membrane Modulation of Na+ Associate to L-type channels (α)	CT	Decrease – BW
AKAP95 (*AKAP8*)	RII affinity chromatography	Nuclear matrix	Chromosome condensation Binds Eg7, condensin and PDE7A	CT/ST	No effect – T
AKAP450 (*AKAP9*)	RII affinity chromatography	Centrosome Golgi	Binds PDE4D3, PP1, PP2A PKN and PKC_{ε} Targets PKA and PP1 to NMDA-R Multiple splice variants	CT/ST	Decrease – T
D-AKAP2 (*AKAP10*)	cAMP chromatography	Vesicles Peroxisomes Centrosome	D-AKAPs Binds PP1, PP2A	CT	No effect – T
Gravin (*AKAP12*)	RII affinity chromatography	Actin cytoskeleton Cytoplasm	Binds PKC and β-AR	CT	? – T
AKAP-Lbc (*AKAP13*)	RII affinity chromatography	Cytoplasm	Binds Rho-GEF Couples $G_{\alpha 12}$ to Rho	CT/ST	? – T
Ezrin	RI/II and cAMP chromatography	Actin cytoskeleton Plasma membrane	D-AKAPs Binds Cx43 Linked to CFTR via EBP50 RISR domain	CT/ST	Decrease – T
Myomegalin	RII affinity chromatography	Cytoskeleton Centrosome Cytoplasm	Binds PDE4D	CT	Decrease – T
OPA1	RI affinity chromatography	Inner mitochondrial membrane Mitochondrial intermembrane Lipid droplets	D-AKAPs	CT	? –T

Proteins from cytotrophoblasts and syncytiotrophoblast were purified by cAMP or RI-flag and RII-flag affinity chromatography and identified by nanoLC-LTQ Orbitrap Mass Spectrometry analysis of tryptic digests of bands excised from SDS-PAGE. For each AKAP, the subcellular localization, the properties described in literature and the cellular origin of the identification (CT for cytotrophoblast and/or ST for syncytiotrophoblast) were specified. The effect of tested siRNA mediated knockdown on trophoblast fusion was presented from in vitro experiments using primary culture of human trophoblasts (T) or BeWo cells (BW). Silencing of AKAPs not tested on cell fusion was indicated by "?".

siRNA (**Figure 4D**). SiRNA mediated depletion of AKAP18 decreased BeWo cell fusion after treatment with FSK (a potent cAMP activator) by approximately 60% ($p < 0.001$) compared to scrambled control transfected cells. It is known that AKAP18 isoforms are involved in the regulation of intracellular calcium fluxes. AKAP18α/β is associated with the plasma membrane and is necessary for PKA-dependent phosphorylation of the L-type Ca^{2+} channel under β-adrenergic stimulation in cardiomyocytes. This phosphorylation by PKA increases the opening probability and conductance of this channel (Fraser et al., 1998). AKAP18γ/δ located to the sarcoplasmic reticulum membrane facilitates adrenergic regulation of calcium reabsorption from cytosol by regulating phospholamban that controls the sarcoplasmic reticulum calcium ATPase Serca2 (Lygren et al., 2007). It is noteworthy that trophoblastic syncytialization requires extracellular calcium (Dimitrov et al., 1993; Rote, 2005). Furthermore, calcium entry through L-type Ca^{2+} channel controls hCG released, which could indirectly triggers cell fusion in an auto-paracrine manner (Meuris et al., 1994). However, more experiments are needed to fully decipher the putative regulation of a PKA–AKAP18 complex in cAMP signaling-induced calcium homeostasis in BeWo cell fusion. Another AKAP, AKAP-121 co-immunoprecipitates with PKA and protein tyrosine phosphatase (PTP) D1 in ST mitochondrial membrane that could be involved in progesterone synthesis (Gomez-Concha et al., 2011).

Cyclic AMP PDE Control Human Trophoblasts Fusion

Cyclic AMP-PDE contribute to the regulation of local cAMP gradients by specifically hydrolyzing the phosphodiester bond of cyclic nucleotides thereby controlling the cAMP cellular level, mediating its return to the basal state concomitantly forming intracellular AMP (**Figure 2**). The termination of cAMP signaling is conferred by a large enzyme superfamily that includes over 40 different PDE isoforms (Baillie et al., 2005; Lugnier, 2006). In mammals, 3 of the 11 PDE families selectively hydrolyze cAMP (PDEs 4, 7, and 8), three families are selective for cGMP (PDEs 5, 6, and 9), and five families hydrolyze both cyclic nucleotides with varying efficiencies (PDEs 1, 2, 3, 10, and 11) (Lugnier, 2006; Conti et al., 2007). An explanation of the complexity of PDEs and the existence of numerous splicing variants is that the divergent domains specify protein–protein interactions. These interactions engender a catalytic domain with new regulatory properties and integrate the function of the holoenzymes in macromolecular complexes strategically located within the cell. Although PKA may interact directly with some PDEs, most interactions with kinases and phosphatases are mediated by AKAPs and participate to intracellular signaling compartmentation (**Figure 2**; Dell'Acqua and Scott, 1997).

The presence of cAMP-PDE activity in human placenta was first reported (Ferre et al., 1975). Since then PDE3 and PDE4 were characterized in cytosolic fraction of human placenta biopsies (Xiong et al., 1990). To further examine the role of PDEs in human primary trophoblast, we performed live-cell imaging and fluorescence resonance energy transfer using $^{T}Epac^{VV}$ biosensor (**Figure 5A**) (Klarenbeek et al., 2011). Trophoblasts were transfected with a plasmid directing the expression of $^{T}Epac^{VV}$ biosensor to visualize the dynamic of cAMP accumulation and PDE activity under hCG stimulation (**Figure 5A**). Activation of the LH/CG-R with a supraphysiological concentration of hCG (1 μM) increased the F480/F535 emission ratio (**Figure 5A**). The maximal response was obtained by the addition of the AC activator FSK (15 μM) in combination with IBMX (pan-PDE inhibitor; 200 μM). These original findings indicate that hCG promotes cAMP synthesis and suggest that PDEs could regulate the cAMP signaling in human CTs. Thus an RT-qPCR screening strategy was applied to characterize PDE genes expression in CTs and ST (**Figure 5B**). Interestingly, the fusion process did not induce changes in the PDE gene expression profile (unpublished data). However, we found that trophoblasts expressed mRNA of PDE7A, PDE8A, and PDE10A isoforms at high levels and PDE4A mRNA to a lesser extent whereas trophoblasts displayed low levels of expression of PDE1A, PDE1C, PDE2A, PDE4B, and PDE4D mRNA. Interestingly, no mRNA expression was observed for PDE1B, PDE3A, PDE3B, and PDE4C isoforms in human trophoblasts. It is noteworthy that PDE3 activity identified by Xiong et al. (1990) was assessed on human placental biopsies comprising other cell types than trophoblasts that may express PDE3. To support these data, we also examined cAMP-PDE activity in human CT cell extracts by using a TR-FRET strategy (**Figure 5C**). As evident from the data, IBMX significantly reduced the PDE activity in trophoblast cell lysates compared to vehicle. Furthermore, using specific antagonists we found mainly PDE4 and PDE7 activity and to a less extent PDE2 activity. Interestingly, no PDE8 and PDE10 activity were detected in CT lysate, whereas mRNA of these two PDE isoforms were characterized. Moreover, no PDE3 activity was found, which is in agreement with our RT-qPCR analysis and the absence of PDE3 mRNA. In order to establish the temporal effect of PDE antagonists on cell fusion of primary trophoblasts from human placenta, cells were cultured for 24 h in the presence of hCG to induce cell fusion with or without IBMX, Cilostamide (PDE3 antagonist), and Rolipram (PDE4 antagonist) (**Figure 5D**). As evident from immunostaining and activity data, the combination of hCG and pan-PDE or PDE4 antagonists potentiated human trophoblast fusion in the same manner compared to cells treated with hCG alone. PDE3 antagonists displayed no effects on cell fusion, which is in agreement with the absence of PDE3 mRNA and activity in human CTs. We demonstrate for the first time that various cAMP-PDEs are expressed in human CT and PDE4 isoform regulates the CT cell fusion. However, more experiments need to be performed to identify the specific PDE4 isoforms that regulates the human trophoblast fusion and in which subcellular compartment this occurs.

Serine/Threonine PPs Expression Profile in the Primary Human Trophoblasts

To our knowledge, there are no reported studies of PPs in trophoblasts and their putative role in the regulation of cell fusion. An RT-qPCR screening strategy was applied to characterize expression of PP mRNAs in CTs and ST

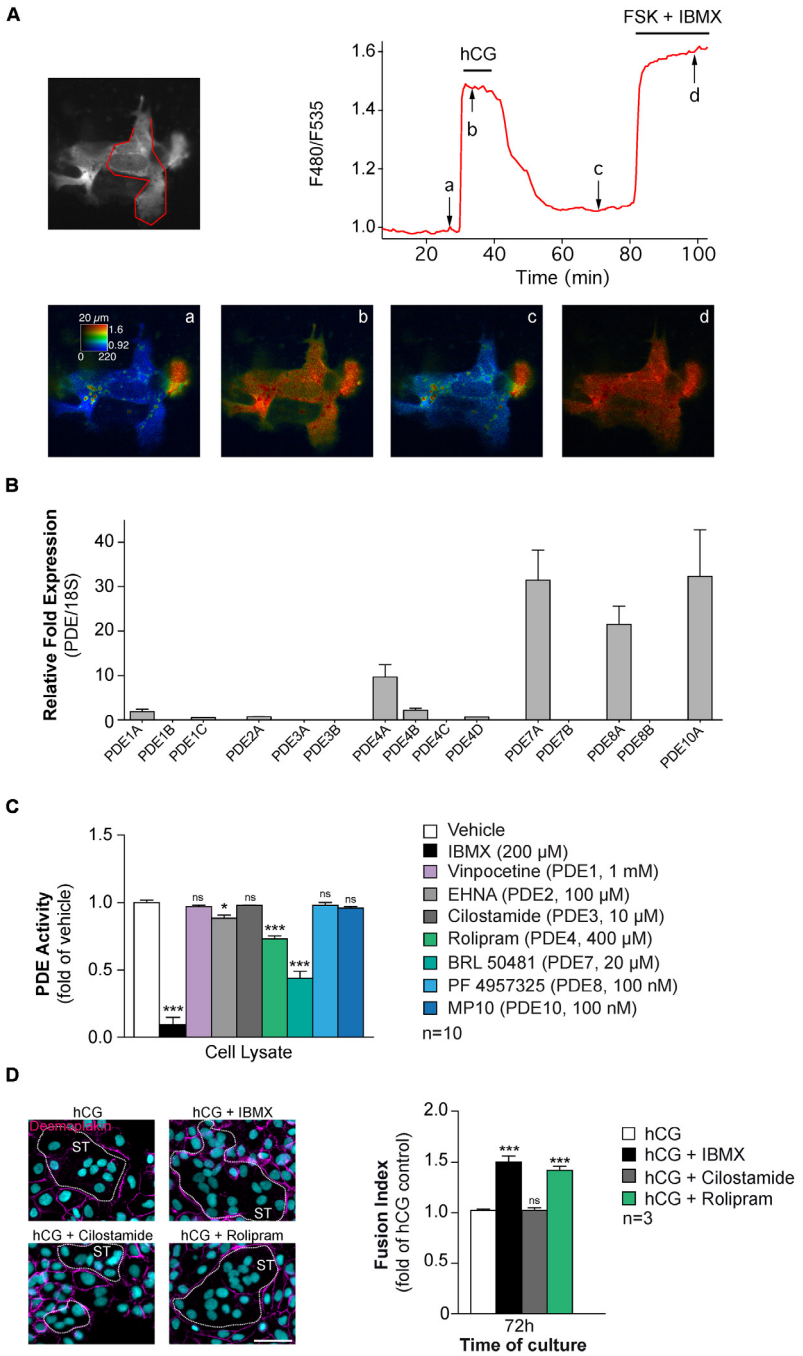

FIGURE 5 | Characterization of cAMP-PDEs in human CTs. (A) CTs purified from human placenta were transfected with a DNA construct directing the expression of the ^{T}Epac^{VV} biosensor and imaged with wide field microscopy. Images show the fluorescence at 535 nm (upper left, in gray scale) and the ratio (in pseudocolor; lower row) indicating a ratiometric change of ^{T}Epac^{VV}, reporting the binding of cAMP at the times indicated by arrows on the graph (upper right panel). The trace on the graph indicates the F480/F535 emission ratio measurement on region indicated in red on the gray scale image. Human CG (1 µM) induced a robust ratio increase in CTs. FSK + IBMX incubation were used to induced the maximal response. Data are representative of three separate experiments **(B)** cAMP-PDE mRNA expression profile in CTs. Total RNA was purified from CTs ($n = 5$ cultures) and subjected to RT-qPCR, using Human PDE TaqMan Gene Expression Assays chip (Applied Biosystems). Histograms represent mRNA expression of cAMP-PDEs in trophoblast normalized to 18S rRNA expression. **(C)** Cyclic AMP-PDE activity in human CTs. LANCE Ultra cAMP assay (Perkin Elmer) was adapted to quantify PDE activity in total CT lysates. Trophoblast cell lysates (20 µg) were incubated in presence of 6 nM cAMP with or without PDE antagonists, subsequently (Eu)-cAMP tracer and ULight-labeled anti-cAMP antibody were added to the solution prior to analysis. Histograms represented the profile of PDE activity in trophoblasts. **(D)** Effect of PDE inhibitor on CT fusion. Cytrophoblasts were stimulated with hCG (1 µM) and treated with pan-PDE (IBMX, 200 µM), PDE4 (Rolipram, 4 µM) or PDE3 (Cilostamide, 100 nM) antagonists. Cells were stained for desmoplakin (magenta) and nuclei (DAPI, left), Syncytia (ST) boundaries are indicated by dashed lines and fusion indices were measured (right histograms). Results are expressed as the mean ± SEM of indicated independent experiments (n.s., non significant, $^{*}p < 0.05$, $^{***}p < 0.001$). Scale bar: 30 µm.

A

PPPs (Gene - Protein name)	Mean C_T	RQ
PPP1CA - Serine/threonine-protein phosphatase PP1-α catalytic subunit	19.25 ± 0.14	0.550 ± 0.056
PPP1CB - Serine/threonine-protein phosphatase PP1-β catalytic subunit	19.15 ± 0.35	0.600 ± 0.143
PPP1CC - Serine/threonine-protein phosphatase PP1-γ catalytic subunit	20.55 ± 0,15	0.220 ± 0.023
PPP1R15B - Protein phosphatase 1 regulatory subunit 15B	18.70 ± 0,01	0.800 ± 0.001
PPP1R3B - Protein phosphatase 1 regulatory subunit 3B	20.20 ± 0,30	0.290 ± 0.059
PPP2CA - Serine/threonine-protein phosphatase 2A catalytic subunit α isoform	18.95 ± 0.25	0.680 ± 0.110
PPP2CB - Serine/threonine-protein phosphatase 2A catalytic subunit β isoform	19.40 ± 0.10	0.490 ± 0.030
PPP2R2A - Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B α isoform	20.25 ± 0.05	0.270 ± 0.009
PPP2R2B - Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B β isoform	25.45 ± 0,15	0.007 ± 0.001
PPP2R2C - Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B γ isoform	33.35 ± 0.85	ns
PPP2R4 - Serine/threonine-protein phosphatase 2A activator	28.70 ± 0.30	0.001 ± 2e-004
PPP2R5A - Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit α isoform	22.40 ± 0.30	0.062 ± 0.012
PPP2R5B - Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit β isoform	24.40 ± 0.60	0.017 ± 0.006
PPP2R5C - Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit γ isoform	21.82 ± 0.07	0.091 ± 0.005
PPP2R5D - Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit δ isoform	22.70 ± 0.50	0.053 ± 0.017
PPP2R5E - Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit ε isoform	20.80 ± 0.10	0.186 ± 0.013
PPP3CA - Serine/threonine-protein phosphatase 2B catalytic subunit α isoform	20.65 ± 0.05	0.207 ± 0.007
PPP3CB - Serine/threonine-protein phosphatase 2B catalytic subunit β isoform	21.20 ± 0.01	0.141 ± 0,001
PPP3CC - Serine/threonine-protein phosphatase 2B catalytic subunit γ isoform	22.40 ± 0.30	0.062 ± 0.013
PPP4C - Serine/threonine-protein phosphatase 4 catalytic subunit	19.85 ± 0.05	0.360 ± 0.012
PPP5C - Serine/threonine-protein phosphatase 5	22.65 ± 0.05	0.051 ± 0.002
PPP6C - Serine/threonine-protein phosphatase 6 catalytic subunit	20.25 ± 0.04	0.273 ± 0.009
GAPDH - Glyceraldehyde-3-phosphate dehydrogenase	17.38 ± 0.12	1

B

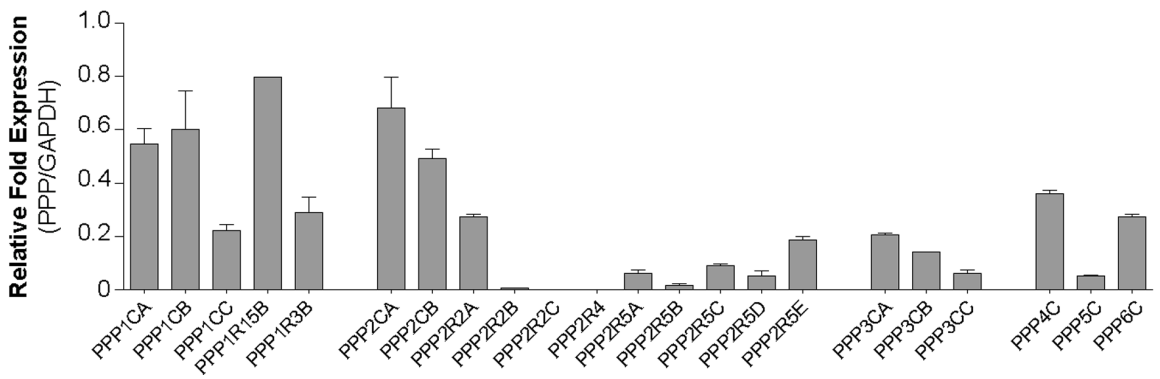

FIGURE 6 | Characterization of PPs in human trophoblasts. Messenger RNA expression profile of serine/threonine protein phosphatases (PPs) in trophoblasts. Total RNA was purified from CTs and ST ($n = 5$ cultures each), pooled and subjected to RT-qPCR, using Human Phosphatase Collection Panel chip (Prime PCR, Biorad). **(A)** Table shows mean ± SEM of C_T and associated-relative quantification (RQ). **(B)** Histograms represent mRNA expression of PPPs in trophoblast normalized to GAPDH mRNA expression.

(**Figure 6**). We found that trophoblasts expressed mRNA of PP1, PP2A catalytic subunits, and PP1 regulatory subunit 15B and 3B isoforms and PP2A regulatory subunit alpha isoform (**Figure 6**), whereas expression of calcineurin (PP2B) mRNA was weaker. Interestingly, the fusion process did not induce modifications in PP genes expression profiles (unpublished data). By immunoblotting we characterized expression of PP1-Cα, PP2A-Cα/β, and Calcineurin A subunits in primary human trophoblasts (data not shown). Protein phospho- and dephosphorylation, controlled by kinases and phosphatases, respectively, affect many cellular processes and their regulations must be specific to act on a defined subset of cellular targets to ensure signal fidelity. More experiments need to be done here to identify subcellular targets of PPs and may characterize in detail their functions in the regulation of human trophoblasts fusion.

Discussion

Trophoblast fusion and ST formation is a complex biological process essential for the maintenance of pregnancy and for fetal growth. For more than two decades, it have been recognized that the cAMP/PKA signaling pathway is the major signaling pathway involved in this process, which modulates the expression of proteins that trigger human trophoblast fusion (Gerbaud and Pidoux, 2015). However, cAMP-induced trophoblast fusion remains a field where a number of molecular details remain to be elucidated. To this end, the characterization of components of the cAMP signaling pathway (PDEs, PPs, and AKAPs) as reported here and may help understand how cAMP signaling as propagated intracellularly to regulate this complex biological process.

In an autocrine–paracrine loop, hCG binds to LH/CG-R at the membrane of human trophoblasts and induces intracellular cAMP production (Shi et al., 1993; Keryer et al., 1998b; Pidoux et al., 2007a,b, 2014). Subsequently, cell fusion is triggered by activation of PKA that phosphorylates the CREB transcription factor (cAMP Response Element-Binding Protein), which associates with CBP (CREB-binding protein) and P300 to increase specific gene expression of fusogenic proteins (i.e., hCG, GCM1, Cx43, and syncytins; Knerr et al., 2005; Chen et al., 2008; Chen and Cheong, 2011; Ul Hussain, 2014). Moreover, induction of cAMP/PKA signaling promotes association between the transcription factors GCM1 and CBP, thereby enhancing the transcription of fusogenic genes such as those encoding Cx43 and syncytins (Chen and Cheong, 2011; Dunk et al., 2012; Gerbaud and Pidoux, 2015). Despite all of these characterized events, specific roles of individual PKA isoforms in the regulation of the trophoblast fusion process remains to be elucidated. Furthermore, precise spatial and temporal activation of PKA inside the cell is accomplished by sequestering PKA in specific locations through interaction with AKAPs and by generation of localized pools of cAMP.

Protein kinase A is considered as the major effector of the cAMP-signaling pathway. However, other intracellular effectors for this signaling exist such as exchange proteins activated by cAMP (Epac) and the cyclic nucleotide-gated ion channels (CNG) (**Figure 2**). CNGs are heterotetrameric complexes that are composed by different types of subunits. CNG channels are non-selective cation channels allowing passage of alkali ions as well as divalent cations (i.e., Ca^{2+}) (Kaupp and Seifert, 2002). The opening of CNG channels is under dependence of cyclic nucleotide binding (either cAMP and/or cGMP). To our knowledge, the role and full characterization of CNG channels in trophoblasts and during cell-fusion has not been assessed. Epac have been identified less than two decades ago and characterized as an important effector of the cAMP signaling cascade that act differently that PKA (de Rooij et al., 1998; Kawasaki et al., 1998). Epac signaling is involved in cell differentiation, secretion of vesicles, cell adhesion, and cell–cell adhesion (Bos, 2006). The Epac family comprises Epac1 and Epac2. Epac1 is ubiquitously expressed while Epac2 has been originally identified in brain and adrenal glands (Kawasaki et al., 1998). Epac binds cAMP on a CNB domain (cyclic nucleotide-binding) and subsequently function as a guanine nucleotide exchange factors (GEFs) for Rap1 and Rap2, which belongs to Ras family of small G protein. In the guanosine diphosphate (GDP)-bound state Epac is inactive and becomes active once GDP is exchanged for guanosine triphosphate (GTP). GEFs induce the transfer of GDP for GTP and thereby the activation of the small G protein, whereas GTPase-activating proteins (GAPs) hydrolyze GTP (for review, Gloerich and Bos, 2010). Recent studies undertook to characterize the role of Epac in trophoblast cell fusion. Yoshie et al. (2010) found the expression of Epac1 and Epac2 in CT and STs. In the same report, the authors showed that Epac activation promotes BeWo cell fusion. However, the molecular mechanisms underlying this process were not clear. In another recent report, it has been shown that an Epac/CaMKI signaling cascade works in synergy with PKA signaling to trigger BeWo cell fusion. This cascade activates GCM1 transcription factor, which enhances the transcription of fusogenic genes (Chang et al., 2011). More studies are needed to fully elucidate the molecular mechanisms underlying trophoblast cell fusion and ST regeneration, and thus the pathophysiology of human placental development. Any alteration of syncytial formation and regeneration during pregnancy will affect fetal growth and the outcome of the pregnancy. Anomalies of villous trophoblast differentiation and cell fusion can lead to severe placental abnormalities that could lead to intrauterine growth restriction (IUGR) and preeclampsia (Huppertz and Kingdom, 2004). A better understanding in the cAMP signaling pathway and its regulation in space and time that induce cell fusion could lead to the development of therapeutic tools that may counteract pathologies of the pregnancy with a defect in syncytial formation.

Author Contributions

GP and KT wrote the paper. GP did artistic work. PG did experiments and commented on the text and figures; all the authors read and commented on the drafts and approved the final version.

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PERSPECTIVE
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The Spatiotemporal Regulation of cAMP Signaling in Blood Platelets—Old Friends and New Players

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Atherothrombosis, the pathology underlying numerous cardiovascular diseases, is a major cause of death globally. Hyperactive blood platelets play a key role in the atherothrombotic process through the release of inflammatory mediators and formation of thrombi. In healthy blood vessels, excessive platelet activation is restricted by endothelial-derived prostacyclin (PGI_2) through cyclic adenosine-5′-monophosphate (cAMP) and protein kinase A (PKA)-dependent mechanisms. Elevation in intracellular cAMP is associated with the control of a number of distinct platelet functions including actin polymerisation, granule secretion, calcium mobilization and integrin activation. Unfortunately, in atherosclerotic disease the protective effects of cAMP are compromised, which may contribute to pathological thrombosis. The cAMP signaling network in platelets is highly complex with the presence of multiple isoforms of adenylyl cyclase (AC), PKA, and phosphodiesterases (PDEs). However, a precise understanding of the relationship between specific AC, PKA, and PDE isoforms, and how individual signaling substrates are targeted to control distinct platelet functions is still lacking. In other cells types, compartmentalisation of cAMP signaling has emerged as a key mechanism to allow precise control of specific cell functions. A-kinase anchoring proteins (AKAPs) play an important role in this spatiotemporal regulation of cAMP signaling networks. Evidence of AKAP-mediated compartmentalisation of cAMP signaling in blood platelets has begun to emerge and is providing new insights into the regulation of platelet function. Dissecting the mechanisms that allow cAMP to control excessive platelet activity without preventing effective haemostasis may unleash the possibility of therapeutic targeting of the pathway to control unwanted platelet activity.

Keywords: platelets, adenylyl cyclase, cAMP, A-kinase anchoring proteins, protein kinase A

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INTRODUCTION

Blood platelets play a key role in haemostasis through binding to sites of vascular injury to form a primary haemostatic plug. However, uncontrolled platelet activation is intimately linked to thrombotic events associated with the rupture of atherosclerotic plaques. Controlling inappropriate platelet activation is critical to protecting against thrombotic episodes. Primary inhibitory mechanisms are mediated by endothelial derived-nitric oxide (NO) and prostacyclin (PGI_2), which stimulate soluble guanylyl cyclase (sGC) and adenylyl cyclase (AC) leading to activation of cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP)-mediated signaling pathways, respectively (Smolenski, 2012). cAMP/cGMP-dependent protein

kinases phosphorylate target proteins that are associated with controlling platelet activation while multiple phosphodiesterases (PDEs) hydrolyse cyclic nucleotides to terminate signaling. The cAMP and cGMP signaling pathways have overlapping target specificity, but also synergise indicating diversity in target selection. In many cell types the enzymes that generate, propagate and terminate cAMP signaling are organized into restricted microdomains that focus the activity of the cAMP signaling cascade to specific substrates (Tasken and Aandahl, 2004; Scott and Pawson, 2009; Stangherlin and Zaccolo, 2012). This compartmentalisation of cAMP signaling allows stimulus-specific control of distinct cell functions. Given the central importance of cAMP signaling to the control of platelet function, surprisingly little is known about the molecular mechanisms that coordinate the synthesis and hydrolysis of cAMP, the precise molecular targets of cAMP signaling and how these targets contribute to the control of specific platelet functions. In this perspective article, we provide an overview of cAMP signaling and examine emerging concepts of compartmentalization in platelets.

THE cAMP SIGNALING SYSTEM IN PLATELETS

The binding of PGI_2, prostaglandin E_1 and adenosine to their receptors on platelets leads to the elevation of intracellular cAMP through the activation of AC. While there are nine membrane bound isoforms of AC, human platelets express only AC3 and AC5/6 (Rowley et al., 2011; Burkhart et al., 2012; Cooper and Tabbasum, 2014). The roles of these individual AC isoforms and whether they are linked to specific receptors or stimuli are unknown. Increased cAMP leads to the activation of protein kinase A (PKA), the foremost effector of cAMP signaling in platelets. The holoenzyme of PKA is an inactive heterotetramer composed of two regulatory (R) and two catalytic (C) subunits. Platelets express all the known isoforms of the regulatory and catalytic subunits (RIα, RIβ, RIIα, RIIβ, Cα, Cβ, and Cγ) (Pidoux and Taskén, 2010; Burkhart et al., 2012), indicating that platelets likely possess multiple variations of the two PKA subtypes, PKA-I and PKA-II, which have distinct biochemical properties. The binding of four cAMP molecules to their binding sites on PKA, result in a conformational change unleashing the catalytic subunit and consequently the phosphorylation of adjacent protein substrates. To date sixteen substrates have been characterized in platelets, although a recent proteomic study suggests that over 100 substrates may be present (Beck et al., 2014). These substrates can be categorized into proteins involved in regulating: (i) shape change (actin polymerization), (ii) calcium signaling, or (iii) integrin activation (Schwarz et al., 2001; Raslan and Naseem, 2014). The phosphorylation of these proteins by PKA is proposed to be the primary mechanism by which prostacyclin modulate platelet activation. As platelet pass through the circulation they are marginalized to the periphery of the vessel facilitating constant exposure to endothelial-derived PGI_2. Therefore it is likely that the default status of platelets in the circulation is one of elevated cAMP and activated PKA, which maintains the cells in a quiescent state. However, to facilitate platelet activation at sites of vascular injury the inhibitory effects of basal cAMP signaling must be overcome. Upon activation, platelets generate and release soluble factors that act in a paracrine fashion to promote platelet activation through the inhibition of cAMP signaling. Platelet-derived adenosine diphosphate (ADP) inhibits AC activity through binding to Gαi-coupled $P2Y_{12}$ that blocks cAMP synthesis. Simultaneously, thrombin and thrombospondin-1 activate PDE3A, leading to reduced intracellular cAMP (Gachet et al., 2006; Zhang and Colman, 2007; Roberts et al., 2010). Together, these mechanisms act to reduce the threshold for platelet activation and ensure rapid recruitment of platelets to vascular lesions. An imbalance in this dynamic system can have profound effects on platelets function as exemplified by several recently identified congenital disorders that affect cAMP production (Geet et al., 2009). A Gαs hypofunction mutation is associated with platelet hyperactivity (Freson et al., 2009). Conversely, a Gαs gain-of-function mutation is reported to lead to a trauma-related bleeding tendency (Freson et al., 2003). Consistent with these observations, a recently identified mutation in the gene encoding the catalytic subunit of PKA leads to macrothrombocytopenia with the homozygous patients showing a bleeding tendency (Manchev et al., 2014). The control of cAMP signaling and therefore the phosphorylation of key PKA substrates is mediated by both protein phosphatases and several PDEs through the hydrolysis of cAMP into 5′-AMP. Platelets express two cAMP hydrolysing enzymes, PDE2A and PDE3A (Dickinson et al., 1997; Schwarz et al., 2001; Rondina and Weyrich, 2012). However, the phosphatases that dephosphorylate PKA substrates are unknown. Thus, platelets possess a complex cAMP signaling network that includes multiple isoforms of AC, PKA and PDE (**Figure 1A**), but molecular control and integration of this network is required to maintain the balance between haemostasis and thrombosis.

SPATIOTEMPORAL REGULATION OF cAMP SIGNALING—THE ROLE OF AKAPs

The spatiotemporal control of cAMP signaling is influenced by the opposing functions of localized AC and PDE isozymes and the scaffolding properties of A-kinase anchoring proteins (AKAPs). The cAMP signal is conveyed through assembly of macromolecules that constrain cAMP signaling to specific regions in the cell. The formation of multiple cAMP signaling hubs where individual isoforms of PKA interact with anchoring proteins that focus PKA activity on specific substrates, allows the control of specific biological functions in response to distinct stimuli (Tasken and Aandahl, 2004; Scott and Pawson, 2009; Stangherlin and Zaccolo, 2012). Early studies reported that PKA substrates were specifically localized in distinct subcellular compartments of blood platelets (El-Daher et al., 1997, 2000). Our own studies support these observations with distinct phospho-PKA substrates found in cytosolic and membrane fractions of platelets (**Figure 1B**). Consistent with the detection of PKA substrates in multiple cell compartments we found using both subcellular fractionation and immunostaining experiments that PKA-I and PKA-II are differentially localized in platelets. PKA-RI was found primarily at the cell periphery, while PKA-RII was clustered within the cytosol (Raslan et al., 2015). We found that membrane lipid rafts allow for further compartmentalization of cAMP

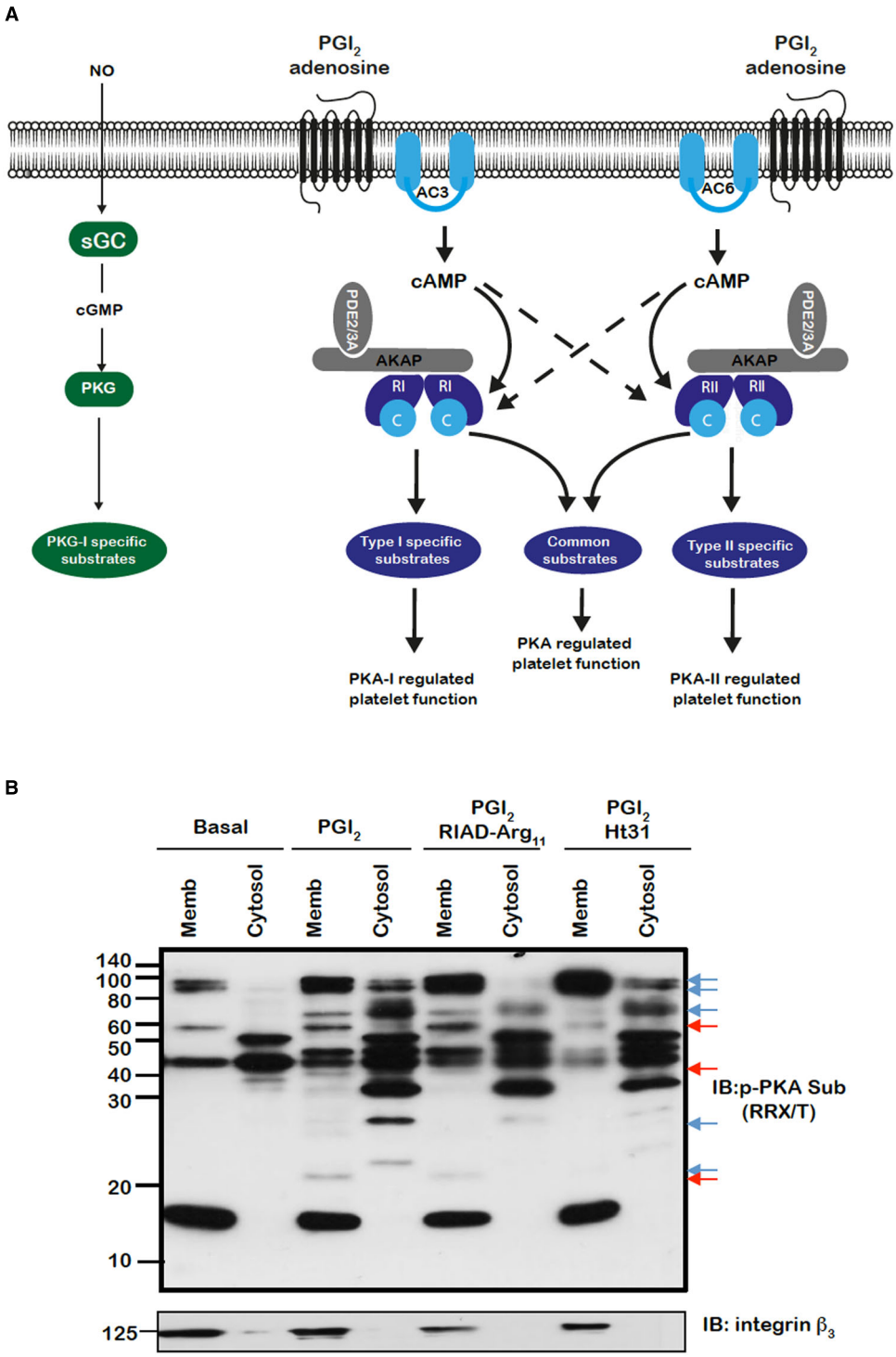

FIGURE 1 | (A) Model for the control of platelet function by cAMP signaling in platelets. Segregated pools of cAMP are formed by the individual isoforms of AC. The cAMP generated may target individual PKA isoforms. AKAPs act to focus isoform specific PKA activity to individual substrates that are linked to distinct platelet functions. The AKAPs may also act to localize PDE isoforms in manner that controls specific signaling events. **(B)** PKA substrates are differentially distributed and targeted by PKA in an AKAP-dependent manner. Washed platelets (5×10^8/mL) were left untreated or pre-treated for 60 min with RIAD-Arg_{11} (2 μM) or stHt31 (2 μM), which target PKA-I and PKA-II specific AKAPs respectively, followed by PGI_2 (50 nM) for 1 min. Platelets were lysed then subjected into subcellular fractionation (20,000 × *g*, 90 min at 4°C) to separate platelet membranes. Membrane and cytosol fractions (20 μg) were analyzed by SDS-PAGE and immunoblotting using phosphoPKA substrate antibody and integrin β as a membrane marker. Blots are representative of three independent experiments. Membranes were visualized using enhanced chemiluminescence. Red and blue arrows represent AKAP sensitive bands in membrane and cytosolic bands respectively.

signaling in platelets. For example, a pool of AC isoform 5/6 and PKA-I, but not PKA-II, are localized to these membrane microdomains in platelets where the rafts act to constrain PKA activity (Raslan and Naseem, 2015). Thus, PKA signaling in platelets, like T-cells, dendritic cells and cardiomyocytes is compartmentalized to membrane fractions in close proximity to the site of cAMP generation (Ruppelt et al., 2007; Delint-Ramirez et al., 2011; Schillace et al., 2011; Burgers et al., 2012). The question then arises as to how these cAMP signaling compartments are organized and configured in platelets. An elegant study using a chemical proteomic approach indicated the presence of localized and active cAMP signaling nodes, which were dynamic in nature with PKA activity changing within distinct signaling complexes in response to platelet activation.The formation of these complexes was attributed to the presence of several AKAPs, although the precise composition of these complexes or how they were convened was not extrapolated (Margarucci et al., 2011). AKAPs are structurally diverse scaffolding proteins that interact with PKA and target it to a specific subcellular compartment through a unique targeting domain. All AKAPs possess a highly conserved PKA binding domain. This is an amphipathic helix, which can bind to the docking and dimerization domain (D/D domain) of the PKA regulatory homodimer (Carr et al., 1991; Kinderman et al., 2006; Sarma et al., 2010). While AKAPs specifically bind PKA, they can act as hubs that coalesce other signaling components including kinases, phosphatases, PDEs, ACs, and even receptors. The assembly of such macromolecules act as signal switches that are intricately regulated spatially and temporally by signal initiators and terminators. Over fifty AKAPs have now been identified and of these fifteen are potentially expressed in platelets (Margarucci et al., 2011; Rowley et al., 2011; Burkhart et al., 2012). Given the potential complexity within the cAMP signaling system, it is likely that AKAPs play a key role in the control of platelet function by cAMP (**Figure 1A**). Unfortunately, the study of AKAPs in platelets has been limited by an inability to apply standard molecular biology approaches used to characterize AKAPs in many other cells. However, he development of cell permeable disruptor peptides that mimic the sequence of the amphipathic helix of the PKA binding domain and target PKA-AKAP interactions in an isoform-specific manner has allowed us to begin examine this hypothesis (Carlson et al., 2006; Gold et al., 2006; Zhang et al., 2014). These peptides can displace PKA from multi-protein complexes formed by AKAPs allowing the functional relevance of these AKAPs to be evaluated. Focusing on PKA-I we used RI-anchoring disruptor peptide or RIAD-Arg_{11} to demonstrate that a number of cAMP signaling events in platelets were dependent on PKA-I-AKAP interactions (Carlson et al., 2006).Examining PKA signaling events using immunoblotting following subcellular fractionation, we were able to observe a decrease in the intensity of PKA phosphorylation events after stimulating with PGI_2 in the presence of RIAD-Arg_{11} in both membrane and cytosolic fractions when compared with PGI_2 alone suggesting that PGI_2 trigger PKA-I-specific signaling events that are AKAP-dependent in platelets (**Figure 1B**). This was followed by a number of specialized assays, including aggregation, secretion and spreading to evaluate the role of PKA-I-AKAPs interactions in the inhibitory effect of prostacyclin on platelet function (**Figure 2**). As expected collagen-induced platelet aggregation was inhibited by PGI_2, but the inhibitory action was diminished significantly by the presence of RIAD-Arg_{11} (**Figure 2A**). The secretion of dense granules is a major factor in potentiating platelet aggregation in response to collagen.Luminescence assays demonstrated that PGI_2 abolished the secretion of ATP from these granules in response to collagen, but again the presence of RIAD-Arg_{11} reversed its inhibitory effects (**Figure 2B**). Finally platelets were adhered to immobilized collagen to induce a physiological spreading response. In response to collagen, platelets extended filopodia and lamellipodium consistent with spreading (**Figure 2C**). The presence of PGI_2 prevented the spreading response without influencing the ability of platelets to adhere. However, pre-treating platelets with RIAD-Arg_{11}, allowed platelets to adhere and spread on collagen. Interestingly, in all assays, RIAD-Arg_{11} failed to cause a full recovery from the inhibitory effects, with the remaining effects of cAMP potentially caused by PKA-II, which would be insensitive to the effects of RIAD. While the data produced using the peptides should be interpreted with caution, our observations suggest that PKA-I-AKAP interactions are required for optimal PGI_2 inhibition of collagen-induced platelet activation and that PKA-I and PKA-II have non-redundant roles in human platelets.

The signaling events driven by compartmentalisation of PKA isoforms are finessed by the PDEs (Rondina and Weyrich, 2012). Using FRET, Lissandron et al. (2006) demonstrated that distinct pools of cAMP could be shaped by specifically localized PDE isoforms (Lissandron et al., 2006; Stangherlin and Zaccolo, 2012). While the subcellular localisation of platelet PDEs is not clear there is evidence for potential non-redundant roles in controlling cAMP signaling. Pharmacological inhibition of PDE2A resulted in increased basal cAMP, while inhibition of PDE3A blocked platelet aggregation, reduced Ca^{2+} mobilization and increased vasodilator stimulated phosphoprotein (VASP) phosphorylation (Feijge et al., 2004). These data suggest PDE2A and PDE3A play non-redundant roles, whereby the former controls elevated cAMP levels and the latter maintains a threshold of cAMP levels in pools that control integrin activation and Ca^{2+} mobilization. Interestingly, PKA has been shown to phosphorylate and activate of PDE3A in platelets (Macphee et al., 1988; Hunter et al., 2009), suggesting a feedback regulatory mechanism within platelets to control cAMP signaling. The molecular composition of this feedback regulatory complex is ill-defined, although preliminary data in our laboratory suggests that phosphorylation of PDE3A is performed by PKA-II in an AKAP-dependent manner (Law and Naseem, unpublished). The localisation of PDE isoforms to specific signaling complexes and PKA substrates is a key area for further characterisation of cAMP signaling in platelets.

EVIDENCE OF FUNCTIONAL AKAPs IN PLATELETS

The concept of isoform-specific PKA substrates in platelets that are regulated by an AKAP is an attractive one, although evidence for their functional roles is still lacking. Platelet AKAPs as reported in proteomic and transcriptome studies include AKAP1, AKAP2, AKAP7(γ), AKAP9, AKAP10, AKAP11, AKAP13, moesin, ezrin, Rab32, BIG2 (brefeldin-A-inhibited guanine-nucleotideexchange

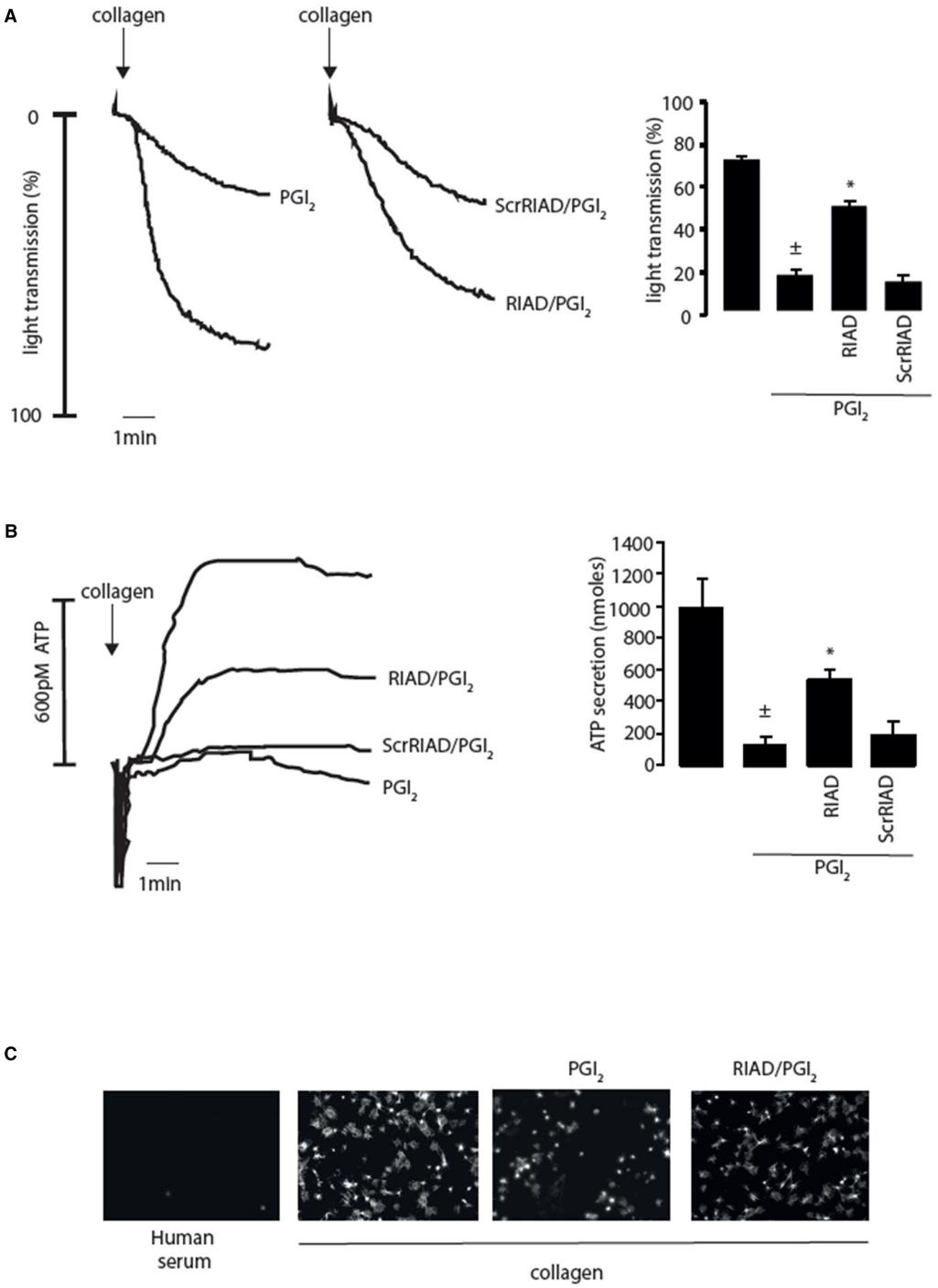

FIGURE 2 | Inhibition of platelet activity by prostacyclin (PGI_2) requires PKA-I/AKAP interactions. (A) Washed platelets (2.5×10^8/ml) were left untreated or pre-incubated with RIAD-Arg_{11} (2 μM) or scrRIAD-Arg_{11} (2 μM) for 1 h followed by addition of PGI_2 (50 nM) for 1 mine and then stimulated with collagen (5 μg/ml) for 4 min under stirring conditions. (Ai) representative aggregation traces. (Aii) Collated data of four independent experiments expressed as mean ± SEM. ± $p < 0.01$ compared to absence of PGI_2; *$p < 0.05$ compared to PGI_2 alone. **(B)** As in (A) except platelet ATP secretion was measured by the signal released by a Luciferin-Luciferase reaction using a Chrono-log Lumi-aggregometer. (Bi) representative ATP secretion traces. (Bii) Collated data of four independent experiments expressed as mean ± SEM. ±$p < 0.01$ compared to absence of PGI_2; *$p < 0.05$ compared to PGI_2 alone. **(C)** Washed platelets (5×10^7/ml) were placed on human serum (i) or collagen-coated coverslips (50 μg/ml) (ii–iv) for 60 min at 37°C in the absence (ii) or presence (ii) PGI_2 (100 nM). In (iv) washed platelets were first pre-treated with RIAD-Arg_{11} (2 μM) for 1 h followed by addition of PGI_2 (100 nM). At the end of the incubation period, non-adherent platelets were removed by washing with PBS. The remaining adherent platelets were fixed, permeabilized with 0.1% Triton, and stained for F-action with TRITC-conjugated phalloidin then visualized with fluorescence microscope. Scale bar is 5 μm.

protein 2), WAVE-1 (Wiskott–Aldrich syndrome protein verprolin homologous 1), MAP2 (microtubuleassociated protein 2), smAKAP (small-membrane AKAP) and neurobeachin. Of those fifteen AKAPs we were able to verify the presence of several by immunoblotting techniques. To the best of our knowledge only two functional AKAPs have been identified in platelets, small membrane AKAP (smAKAP) and moesin. SmAKAP was identified in platelets and cardiomyocytes by using chemical proteomics. This AKAP specifically targets PKA-I to cellular membrane through myristoylation and palmitoylation anchors (Burgers et al., 2012). Verification of the presence of smAKAP by immunoblotting and its functional role is yet to be determined in platelets. Using a combination of cAMP pull-down, immunoblotting and kinase assays we reported recently that moesin targets PKA-I into platelet lipid rafts where it can phosphorylate its physiological substrate GPIbβ (Raslan et al., 2015). This phosphorylation event contributes to the inhibition of platelet-driven thrombus formation to the adhesive protein von Willebrand factor. This data identifies moesin as the first functionally validated AKAP in platelets, but also indicate GPIbβ as the first PKA-I-specific substrate in platelets. Interestingly, we observed multiple phosphoproteins in platelet lipid rafts that were sensitive to the localisation of PKA-I suggesting that these microdomains may focus PKA-I on several targets required for regulation of platelet function by cAMP. These two studies provide an insight into the potential compartmentalization of cAMP signaling by AKAPs, although more work is required to understand the composition and dynamics of AKAP-regulated macromolecules in platelets.

PERSPECTIVE

The cAMP signaling pathway represents the most potent endogenous system for the control of platelet activation through its ability to modulate multiple platelet functions. However, the molecular mechanisms by which cAMP/PKA signaling controls individual platelet functions are unclear. Fundamental to elucidating this issue is an improved understanding of the organization and spatial resolution of the downstream effectors of cAMP in platelets. Emerging data suggests that compartmentalization of key cyclic nucleotide signaling complexes may control aspects of platelet function as evident by the identification of a localized PKG1β-IP_3R1-IRAG-PDE5 complex that regulate calcium release in response to NO (Wilson et al., 2008). The challenge for platelet biologists is to determine whether this multi-protein complex model can be extended to the cAMP pathway. The advances in both transcriptomics and proteomics have now identified a number of AKAPs that potentially provide spatial resolution and specificity to PKA-mediated phosphorylation events. Our data demonstrating differential localization of PKA-I and PKA-II and compartmentalization of PKA-I by the AKAP moesin to target a specific substrate provide proof of principle that the AKAP hypothesis can be translated to platelets. Interestingly, we have found that the catalytic subunit of PKA is constitutively associated with RhoA (Aburima et al., 2013), PDE3A and IP_3R1 through AKAP-dependent mechanisms (unpublished), suggesting the presence of numerous individual PKA-AKAP complexes. Unfortunately, platelet research in this area is hampered by an inability to apply conventional gene silencing or overexpression technologies and therefore relies on the identification of native complexes through protein purification. As protein purification techniques become more sensitive, the ability to identify and characterize native complexes increases and may allow functional relevance to be established. Our data with cell permeable disruptor peptides provide one approach to address this issue. However, these peptides are limited in their specificity and only allow resolution of isoform-specific signaling and functional events. Future lines of investigation could involve the identification of individual PKA-AKAP complexes and the application of a new generation of peptide disruptors that discriminate between individual PKA-AKAP complexes (Gold et al., 2013). An important resource for the functional assessment of AKAPs in platelets is genetically modified mice. In the absence of standard molecular biology approaches, mice offer a strategy for examining the role of specific PKA-AKAP complexes on haemostasis and thrombosis *in vivo*. For example, mice heterozygous for Neurobeachin, an established AKAP (Wang et al., 2001), demonstrated altered PKA phosphorylation profile and abnormal platelet dense granules (Nuytens et al., 2013). Detailed examination of platelet sensitivity to cAMP of such mice and their use in adoptive transfer protocols are likely to be key tools for future evaluation of specific PKA-AKAP complexes in the physiological control of platelet function.

The original AKAP hypothesis has now evolved beyond simple anchoring of PKA signaling, with identification of numerous other signaling moieties including phosphatases, PDEs and other kinases coalescing into the complexes (Zaccolo, 2011; Scott et al., 2013). Moreover, these multi-protein signaling hubs are extremely dynamic in nature and have been shown to be both tissue and substrate specific. This particular concept has a specific relevance for platelets where cAMP signaling needs to respond to physiological stimuli that promote platelet activation. Indeed, it may be that the dynamic adjustment of these complexes, in response to agents that promote cAMP generation and platelet agonists, is what determines the threshold for platelet activation and allows effective haemostasis while preventing thrombosis. We believe that the identification of platelet AKAPs through proteomics provides the platform for new lines of investigation in establishing the spatial and temporal organization of cAMP signaling in platelets and how this intersects with platelet activatory signaling.

AUTHOR CONTRIBUTIONS

ZR, AA, and KN designed the experimental work, planned and edited the manuscript; ZR and AA performed the experiments; ZR and KN analyzed the data and wrote the manuscript.

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frontiers in Pharmacology

REVIEW
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Studying GPCR/cAMP pharmacology from the perspective of cellular structure

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Signal transduction via G-protein coupled receptors (GPCRs) relies upon the production of cAMP and other signaling cascades. A given receptor and agonist pair, produce multiple effects upon cellular physiology which can be opposite in different cell types. The production of variable cellular effects via the signaling of the same GPCR in different cell types is a result of signal organization in space and time (compartmentation). This organization is usually based upon the physical and chemical properties of the membranes in which the GPCRs reside and the repertoire of downstream effectors and co-factors that are available at that location. In this review we explore mechanisms of GPCR signal compartmentation and broadly review the state-of-the-art methodologies which can be utilized to study them. We provide a clear rationale for a "localized" approach to the study of the pharmacology and physiology of GPCRs and particularly the secondary messenger cAMP.

Keywords: GPCRs, cAMP, compartmentation, caveolae, T-tubules, lipid rafts, scanning ion conductance microscopy, FRET sensors

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Introduction

The members of the G-protein coupled receptor (GPCR) family act through multiple pathways upon activation as they possess the ability to bind a panel of G-proteins and β-arrestins. As a result ligand binding can potentially activate multiple effector pathways with differential effects upon cellular physiology. The array of these effectors is vast and outside of the scope of both this article and this review series, readers are therefore encouraged to consult previous reviews (Neves et al., 2002; Kenakin, 2011; Shukla et al., 2011). However, in the setting of specialized tissues and cells GPCRs are located into specific compartments with defined molecular profiles, which strictly determine the potential physiological outcomes of signaling. The consequence is that although GPCR signaling is potentially "omnidirectional," in reality signaling outcomes are restricted by the accessibility of secondary signaling molecules such as cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) and the presence of their modulators such as phosphodiesterases or protein kinases activated by the cyclic nucleotides. For a comprehensive and exhaustive review on the cyclic nucleotides and their modulators in cardiac cells the reader is encouraged to refer to the works of the Zaccolo and Movsesian (2007) or the Conti group (Conti and Beavo, 2007).

In addition to the diversity of signaling partners, the GPCRs' status as transmembrane proteins ensures that local plasma membrane properties perform a central role in the production of downstream signaling effects. If we set aside differences in the expression profiles of specific GPCRs, three major mechanisms contribute to shape GPCR signaling; these are the biased

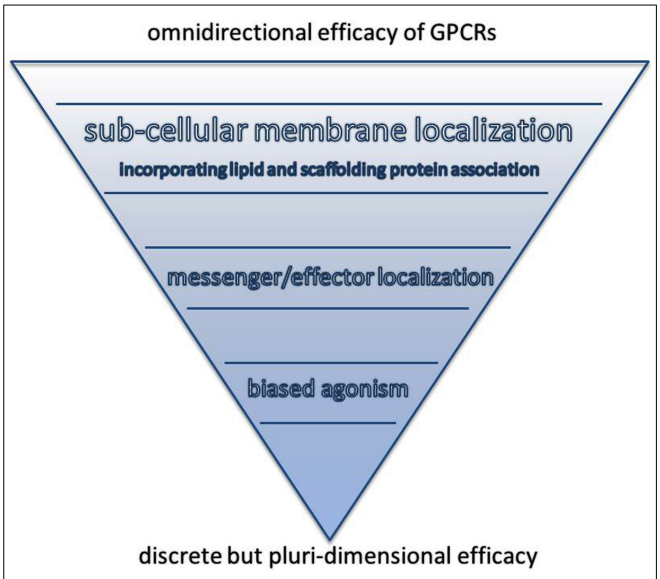

FIGURE 1 | Schematic representation of the mechanisms shaping the control of GPCR signaling. The efficacy of a ligand binding to a GPCR is potentially omnidirectional. Meaning that in theory any physiological outcome can be produced following the signal transduction event. However, through various levels of intermolecular and, ultimately, cellular organization the efficacies of ligands and GPCR function become essentially discrete.

agonism of GPCRs (Violin et al., 2014), secondary messenger compartmentation (Perera and Nikolaev, 2013) and modulation by lipid raft association (Escribá et al., 2007). All of these phenomena will be discussed later in this review (See **Figure 1**).

Over the last decades numerous methodological and technological advances have enabled researchers to better determine the localized pharmacology of GPCRs from the perspective of cell membrane structure. However, the advancement of methods for studying the mechanisms of local modulation of GPCR signaling to ever greater resolution remains a necessity. Equally, deeper investigation of the central organizing principles of this signaling compartmentation at a subcellular level is required. These advances will allow a better understanding of the ways in which GPCRs shape cellular physiology. There is a desire to create drugs based upon improved ligands (Shonberg et al., 2014) for GPCRs producing very specific (ultra-biased) mono-directional signaling to reduce off-target effects. This will only be realized after rigorous assessment of the ways in which, sub-cellular micro-domains modulate GPCR signaling, in health and within the context of pathologies. This current is gaining momentum, particularly within the cardiovascular field; cardiac muscle has been shown to display examples of highly defined GPCR signal regulation. In this paper the pharmacology of multiple GPCRs will be discussed but the adrenoceptors (ARs) are frequently used as examples, due to them being seen as a prototypical GPCR by many researchers. This has led to a large amount of work being focused upon their physiology and pharmacology. They are also of special interest to the authors.

The Concept of the Structural GPCR Microdomain

The effect of a specific ligand on a GPCR is in theory, identical in all tissues, however, these signaling events often produce different effects in different cells. For example the binding of an agonist such as adrenaline to a βAR will increase the contractility of cardiac muscle but reduce the contractility of airway smooth muscle (Brodde, 1993; Barnes, 1995). To allow for cell type-specific divergence, in the downstream effectors of signaling must be locally organized in space and time (Bethani et al., 2010). This allows a single stimulus to result in a biologically relevant whole organ/organism response. The difference mentioned above is due to the altered targeting of protein kinase A-regulatory type 2 (PKA-RII) by the secondary messenger cAMP within the two different cell types. In cardiac muscle PKA phosphorylates L-Type Ca^{2+} channels (LTCC) and phospholamban (PLB), the effect of which is to increase the amplitude and speed of the cellular Ca^{2+} transient (Brodde, 1993). In smooth muscle cells an increase in cAMP activates PKA but with the effect that the myosin light chain kinase (MLCK) is phosphorylated thereby reducing its affinity for the Ca^{2+}/calmodulin complex and lowering contractility (Barnes, 1995). Glucagon receptors (GLU-R) activate glycogen phosphorylase and cause positively inotropic and lusitropic effects in cardiomyocytes (Farah, 1983). G_s-linked receptors like GLU-R may cause divergent effects, for example Glucagon-like peptide-1 receptors (GLP1R) cause negatively inotropic effects (Vila Petroff et al., 2001). β_2AR is also G_s-linked but its effects upon cardiomyocyte inotropy are somewhat equivocal. Some studies report it to exert no effect upon relaxation and it cannot activate glycogen phosphorylase like Glu-R (Xiao and Lakatta, 1993; Kuznetsov et al., 1995). However, other studies report changes in inotropy (Bartel et al., 2003). We discovered that β_2AR stimulation affected inotropy to a different degree depending on which region of the heart the cells were isolated from (Paur et al., 2012). Equally, we discovered that the cAMP responses of G_s-linked β_1AR and β_2AR were quantitatively different at the sub-cellular level (Nikolaev et al., 2010). In these cases different receptors are signaling via the same G-protein and the outcome is the same in terms of signal transduction, i.e., increases in cAMP, but due to differing cellular organizations the signal is interpreted differently and the contractile outcome is divergent.

Cyclic Nucleotide Compartmentation

A large and increasing body of work has described biological mechanisms which serve to shape intracellular cAMP pools (Mika et al., 2012; Perera and Nikolaev, 2013; Guellich et al., 2014). This secondary messenger is produced upon the activation of adenylate cyclase (AC) following the dissociation of the stimulatory-G (G_s) protein from specific classes of GPCRs. Compartmentation of cAMP regulatory mechanisms appears to be a structural phenomenon specific to cell type. This area of study began to accrete following the pioneering work of Buxton and Brunton (1983) who asked how it was that two agents (Prostaglandins and Isoprenaline), which serve to increase cellular cAMP concentrations via different receptor pathways produce differing effects upon cellular physiology.

cAMP produces its effects within cells by causing the activation of PKA (Zaccolo, 2009), exchange protein activated by cAMP (Epac; Métrich et al., 2008) or cyclic nucleotide gated channels (CNGCs; Rochais et al., 2004). Furthermore the cAMP signaling domains are at once both physical and virtual compartments. They rely upon the formation of molecular networks which involve the close apposition of plasma membrane and cellular organelles such as sarcoplasmic reticulum; in addition, the sub-cellular localization of phosphodiesterases, phosphatases and tertiary effectors as well as important protein associations. The central principle of cAMP compartmentation is that cAMP must be present in the vicinity of cAMP-dependent effectors (PKA, Epac, or CNGCs) to cause the transduction of signaling into physiological changes within the cell (Di Benedetto et al., 2008). However, it must be prevented from diffusing from the effector compartment. Therefore cAMP is either degraded or actively extruded from cells through an energy consuming ATP anion pump (Wiemer et al., 1982). Stringent control of cAMP levels assures that a discrete panel of effectors is being activated as a result of a particular signaling event. The differential activities of Prostaglandin and Isoprenaline are due to the activation of different pools of PKA within the cardiomyocyte. Due to stringent control of its localization, cAMP produced as a result of the activation of Prostaglandin receptors cannot cause the cAMP-dependent PKA mediated phosphorylation of members of the excitation-contraction coupling pathway within cardiomyocytes in the manner of the AR.

Cells organize their effector molecules on the basis of the specific needs, and as a result the efficacy of a given agonist in a cell type effectively becomes discrete. Consequently gross changes in cellular structure or gene expression in response to pathology which involve this secondary messenger physiology must always be viewed through this prism. Due to the fact that physiological effects represent the sum of many stringently controlled local events it is of great importance to study localized cell signaling.

Membrane Organization

Cyclic AMP compartmentation is heavily controlled by membrane structures. The following paragraphs will illustrate the involvement of membranous subcellular domains in the regulation of signaling compartmentation.

Lipid Rafts

The structure of the lipid bi-layer is essential for maintaining GPCRs' defined molecular structure, and therefore, their function. The lipid make-up of the membrane is not homogeneous. Indeed, for the past three decades researchers have been aware of detergent insoluble components of the lipid bilayer (Simons and Ikonen, 1997). Many fundamental studies have been carried out to establish the effect of membrane composition upon GPCR function. Many of these have utilized rhodopsin due to this molecules status as an archetypal GPCR for structural studies (Botelho et al., 2002). It appears that a flexible membrane composed of a greater amount of lipids with phosphatidylethanolamine (PE) head groups and docosahexaenoyl chains (DHA), shift rhodopsin toward its active state. The presence of greater quantities of cholesterol increases the rigidity of membranes and as a result has been demonstrated to drive rhodopsin toward its inactive state. The same study demonstrated this was also true for the depletion of DHA (Feller and Gawrisch, 2005). The Singer and Nicolson (1972) fluid mosaic model was postulated and states that the cell membrane is a fluid bilayer through which protein constituents are able to float freely. This "lamellar" structure has been observed to be a basic state of the cell membrane upon which more complex states are superimposed. The basic state described above is defined as being liquid crystalline (Yeagle, 2004). Other states observed are known as gel, pseudo-crystalline, rippled and liquid ordered (Brown and London, 1997). The liquid ordered phase is the most interesting of these states from the perspective of GPCR biology. This configuration is also frequently described as being a "lipid raft," as these phases represent sub-regions of the liquid membrane (Simons and Vaz, 2004). These structures are produced by concentrating the acyl chains of lipids which were in a gel phase. The result of which is the preservation of a degree of lateral mobility. Lipid rafts are enriched in cholesterol and glycosphingolipid and represent about 30% of cellular membranes. They appear to be intrinsically important in modulating GPCR function (Oates and Watts, 2011). Scaffolding molecules such as the A-kinase anchoring proteins (AKAPs), which organize PKA effectors and stabilize the interaction of phosphodiesterases within their domains, are also thought to be localized to lipid rafts where they cluster with the effectors (Kritzer et al., 2012). Caveolae are by far the most well studied lipid raft domains within the context of GPCR and cAMP signaling. The role of these domains in controlling GPCR function is detailed below.

Caveolae

Caveolae are specialized lipid raft domains found in the plasma membrane of many cell types. They are classed as a distinct type of lipid raft as they contain specialized scaffolding proteins such as caveolins and cavins. In two-dimensional transmission electron microscopy (TEM) studies caveolae appear as 50–100 nm in diameter flask-shaped regions of the lipid bilayer. Thus in the three dimensional environment of the cell membrane they are bulb-like invaginations with restricted mouths open to the extracellular environment (Razani et al., 2002). They appear to be formed via the concentration of cholesterol and the aforementioned scaffolding peptides. Caveolae are responsible for cell signaling, lipid storage and endocytosis. The majority of cellular studies have historically relied on TEM to visualize these domains and the cholesterol chelating agent methyl-β-cyclodextrin (MβCD) to disrupt them (Harvey and Calaghan, 2012). As a pharmacological or physiological tool TEM is limited in efficacy requiring cells to be fixed and stained although it still offers the gold standard in spatial resolution. MβCD is extremely efficacious in removing caveolar domains (as confirmed by TEM studies) but also results in the non-specific depletion of plasma membrane cholesterols. Thus non-specific effects may arise and data obtained from MβCD-based studies should be treated with some caution. Caveolae also represent mechanosensitive regions of the cell, in cardiomyocytes they act as reservoirs of membrane

allowing the cell to increase its surface area in response to osmotic or mechanical stress. In the case of both stresses caveolae are observed to disappear in TEM studies (Kohl et al., 2003; Kozera et al., 2009).

Physiological investigations have revealed that caveolar depletion results in the loss of compartmentation of cAMP signaling following β_2AR stimulation, thereby altering its effects on cardiomyocytes function. This appears to be due to the removal of protein phosphatase (PP) activity suggesting a role for caveolar localization in controlling the β_2AR's signaling characteristics (MacDougall et al., 2012; Wright et al., 2014). Interestingly, β_2AR is pleiotropic and may signal via G_s, G_i, or β-arrestins. Removal of caveolar localization alters this capacity; caveolar localization appears to be necessary for β_2AR to bind Gi (Xiang et al., 2002). Heart failure has been shown to significantly alter β_2AR-cAMP compartmentation as well as caveolae number and expression of caveolae scaffolding molecules (Nikolaev et al., 2010; Feiner et al., 2011). Given the important role of the β_2AR as a cardio-protective molecule this situation may exacerbate heart failure. The opioid receptor μOR localizes to lipid rafts in various cell types, including cardiomyocytes where it specifically localizes in caveolae. Its chronic activation leads to receptor internalization and can directly influence cAMP levels by "super-activating" AC. Methyl-β-cyclodextrin disruption of caveolae completely abolishes this "super-activation" (Zhao et al., 2006). Equally, transforming growth factor-β (TGF-β) receptors TβRI and TβRII are assumed to sit inside caveolae. There they regulate the endothelial nitric oxide synthase (eNOS; Schwartz et al., 2005) and additional TGF-β signaling downstream effectors which play a role in various physiological processes such as cell apoptosis and proliferation (Razani et al., 2001). The activation of TβRI and TβRII does not change cAMP level; at the same time otherwise increased cAMP can suppress the TGF-β-dependent signaling pathways (Schiller et al., 2010). Another receptor assumed to be situated inside the caveolae is the bradykinin type 2 receptor (Haasemann et al., 1998; Calizo and Scarlata, 2012). Activation of this receptor subtype has been shown, at least in vascular smooth muscle cells, to increase cAMP level (Webb et al., 2010). In contrast, in adult rat cardiomyocytes bradykinin type receptors appear to activate their downstream effectors without raising cAMP level, and this leads to dephosphorylation of the proteins PLB and troponin I, which reduces cardiomyocyte contractility (Ke et al., 2010). Though α_1 adrenergic receptors (α_1AR) appear to have no effect on cAMP levels (Bogoyevitch et al., 1993), they can elicit increased contractility and are thought to interact with the βAR signaling pathway (Brodde and Michel, 1999). Signaling of the α_1AR, i.e., via specialized pools of phosphatidylinositol (4, 5) bisphosphate (PIP2) is localized to caveolae together with $G_{\alpha q}$ and PLCβ1 (Morris et al., 2006). It is not clear if the α_1AR themselves are actually inside the caveolae. Instead they might be localized exclusively at the nuclear membrane. However, their downstream targets, extracellular signal-regulated kinases (ERKs) and protein kinase C (PKC) are seen to be located in caveolae (Petrashevskaya et al., 2004; Wright et al., 2008). It is generally hypothesized that caveolar localization of receptors within caveolae leads to their control by compartmentalizing these molecules with effectors which serve to inhibit their activity (Head et al., 2005). This control can be exerted by molecules which act directly upon the receptor, molecules which serve to produce (Head et al., 2006) cAMP responses or control downstream effectors of GPCR signaling, such as phosphatases (MacDougall et al., 2012).

T-tubules

The T-tubules are specialized domains within muscle cell types which allow efficient transmission of action potentials into the cell interior. They can be thought of as a further specialization of the plasma membrane, in a similar fashion to caveolae, as they represent modified membrane domains with specific scaffolding proteins (Louch et al., 2010). These include T-cap and Bin-1 as well as an appreciable amount of caveolin, although the presence of true caveolae in these structures remains controversial, in cardiac tissue (Wong et al., 2013). As a result, T-tubules serve as scaffolds to assemble components of ion channels and receptor cascades to exert tight control ionic fluxes in response to the respective extracellular stimuli (Bers, 2002). Certain GPCRs are found within the T-tubular regions, which appear to exert a degree of control over their signaling properties. The T-tubules of cardiomyocytes have been shown to be important organizing factors for βAR signaling and the disruption of T-tubules during pathologies alters the physiological outcome of βAR signaling (Nikolaev et al., 2010). In the adult myocardium the disruption of the T-tubular system is germane in situations of pathology (Louch et al., 2010). The general mechanism suggested for t-tubular control of GPCR signaling is compartmentation with molecular inhibitors/effectors, much like the situation in caveolae. However, the large structural aspect of T-tubules, especially those found in cardiomyocytes means a more physical role is also mooted. In our study it appeared that the T-tubular domains were able to cause a tight coupling between membrane domains rich in AKAP and PKA. The disruption of these domains leads to a β_2AR-cAMP response which was no longer spatially localized. We suggest this alteration may lead to an altered panel of effectors for β_2AR within diseased cardiomyocytes.

Techniques to Study Localized cAMP Pharmacology

As the first part of the review has described, overall alterations to cellular and organ physiology by biochemical stimuli, mediated by GPCRs, are increasingly understood to be the product of many structurally defined signaling events where GPCRs, their secondary messengers and downstream effectors are tightly regulated at the sub-cellular level. Therefore to understand the true nature of a given pathway or the intrinsic efficacy of a ligand, researchers must use techniques with sub-cellular resolution.

Historically, researchers have not been able to determine the localized pharmacology of GPCRs and instead have relied on physiological or pharmacological studies of isolated cells or tissues. Given the current state of knowledge it seems that only by studying the outcomes at the level of signaling structures/domains can we move forward in our understanding of these events. We need to know how the GPCRs, which modify cellular function, are themselves guided by micro-domains in specific cell types and

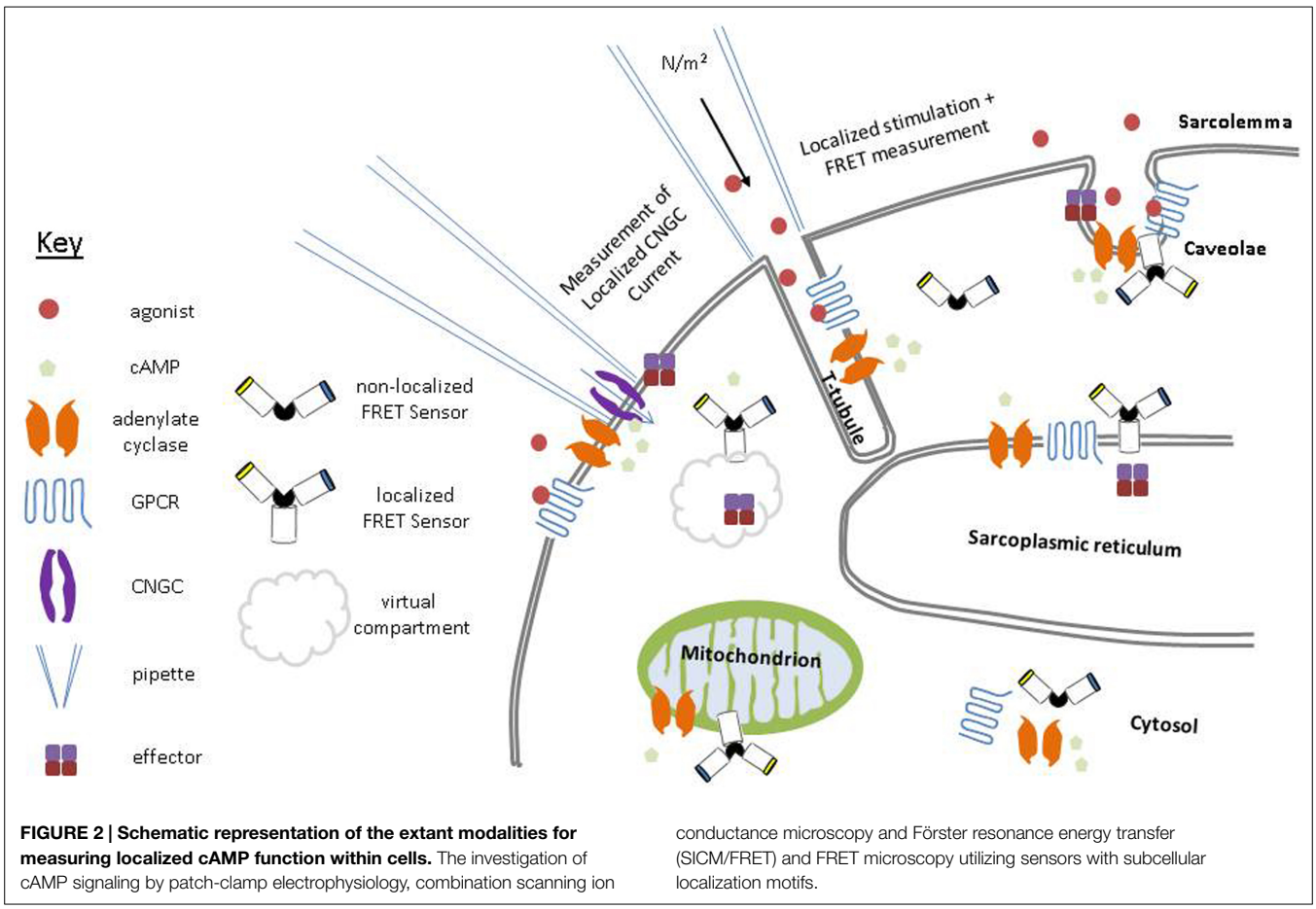

FIGURE 2 | Schematic representation of the extant modalities for measuring localized cAMP function within cells. The investigation of cAMP signaling by patch-clamp electrophysiology, combination scanning ion conductance microscopy and Förster resonance energy transfer (SICM/FRET) and FRET microscopy utilizing sensors with subcellular localization motifs.

how this regulation is altered by pathologies. In cardiac physiology it remains unclear whether increased aberrant signaling by GPCRs in pathologies and derangements of T-tubules are an initiator of, or a response to, abnormal cellular function. The poor spatial and temporal resolution of traditional biochemical techniques was what initially led Buxton and Brunton (1983) to question how it could be that cAMP could cause opposing biological effects. It has become evident that the localized nature of GPCR physiology demands localized measurements of secondary messengers. However, studies of the localized physiology of signaling events produced by GPCRs in domains of a radius smaller than 500 nm are rare, as a consequence of the diffraction limit of light, curtailing the ability of light microscopy methods to operate at such small scales. In recent years new methods have emerged which deal with the limitations described above. The next section will describe the current state of the art in techniques to study the localized physiology/pharmacology of GPCRs. (See **Figure 2**).

Patch Clamp

The patch clamp technique was the first method to effectively study localized cellular physiology and localized receptor pharmacology (Auerbach and Sachs, 1984). This technique exploits the phenomena of ion exchange between the intra and extracellular regions of impermeable plasma membrane, via voltage sensitive and ion-selective channels. This technique has a special utility in the study of electronically excitable cells such as neurons and muscle cells (myocytes). By placing a glass micropipette, machined to provide a pore with a radius of less than a micron onto the surface of a cell and generating a high resistance seal, the holding voltage of the membrane can be set. This allows the current and therefore the activity of ion channels to be recorded. A number of studies have used a modified patch clamp technique to indirectly investigate GPCR dependent cAMP production (Rochais et al., 2004; Abi-Gerges et al., 2009; Ghigo et al., 2012). The focus of many of these studies was the adenylyl cyclase family. Most utilized the properties of certain Ca^{2+}-transporting channels which are modulated by cyclic nucleotides (CNGCs) and which co-localize with adenylyl cyclases in cell membranes (Finn et al., 1996). Although the wild-type channels do not differentiate very effectively between cAMP and cGMP, mutations causing increased selectivity have been produced. Site-directed mutagenesis has been used to alter a single glutamic acid residue to a methionine (E583M) and a further compound mutation of a cysteine to a tryptophan (C460W/E583M) (Rochais et al., 2004). This produced two separate cAMP sensing channels; these have been expressed in various primary cell types (Rich et al., 2001). Consecutive patching reveals channels which give responses on the basis of local cAMP levels. The modulation of CNGCs current as a result

of the application of receptor agonists or external stimuli can be obtained by standard electrophysiological means. Further work looked at the hyperpolarizing cation channels (HCN) which are between 10 and 1000 times more sensitive to cAMP than CNGCs (Rich et al., 2001). These channels are related in that they both contain an evolutionarily conserved cyclic nucleotide binding cassette domain (CNBD). Their development built upon the work of Trivedi and Kramer (1998) who pioneered what was the first method to give truly localized real-time measurement of GPCR activity, "patch-cramming." The patch-cramming technique is based upon the activity cyclic nucleotide sensitive ion channels and involves excision of patches from the lipid membrane bi-layer of cell types which express a high density of CNGCs, for example of oocytes or retinal rod cells, with a patch clamp pipette. Through this operation an inside-out patch of cell membrane will be removed and the CNGCs within can be used to sense external cAMP levels. Calibration of these isolated channels is possible with external solutions containing predefined concentrations of cyclic nucleotides. The CNGC based pipette sensor can then be "crammed" into a cell of interest for which the level of the secondary messenger is to be determined.

Localized Förster Resonance Energy Transfer (FRET) Sensors

A further way to study the localized pharmacology of GPCRs would be to use FRET sensors which are localized to a sub-cellular region of interest. The general approach of this technique is to produce genetically encoded peptide constructs comprising a targeting motif, a signaling molecule binding region and a fluorescent sensor component. The sensor component is usually two fluorophores which are designed, so as to be held in a specific conformation upon the folding of the peptide. One fluorophore acts as an energy donor and the other is an energy acceptor. These fluorophores are either brought into or moved out of proximity upon the binding of the messenger molecule of interest, the result is an alteration in the fluorescence of the donor fluorophore due to the phenomenon of Förster resonance energy transfer. These alterations can be measured by either ratiometric or fluorescence life-time protocols to give an indication of the relative concentration of the secondary messenger molecule of interest in the proximity of the sensor (Sprenger and Nikolaev, 2013). The location targeting motif ensures that the measurement of FRET responses after secondary, messenger activity occurs within a specific locality.

FRET-based investigation of secondary messenger activity is a somewhat immature field, but localized FRET sensor technology has been a critical component of this field since its inception. The seminal studies of Zaccolo and collaborators describe the production of sensors based upon the regulatory I and II (RI/RII) regions of PKA (Zaccolo and Pozzan, 2002). This resulted in sensors which were localized to either the PKA_RI or RII regions. These are regions which, within the cardiomyocytes, control different aspects of cellular physiology. Thereby it was made possible to demonstrate that cAMP pools activated upon Isoprenaline (βAR) or Prostacyclin (EPR) were indeed discrete (Di Benedetto et al., 2008). Interestingly, non-localized sensors were developed later than localized sensors as a solution to the problem of the high sensitivity of PKA-based constructs. As cAMP is often present at high concentrations within cells the initial class of FRET sensors were easily saturated, meaning experiments lost resolution at physiological levels of cAMP production. Non-localized sensors have most often been based upon the cAMP binding domains of EPAC1 or EPAC2 (Nikolaev et al., 2004). These sensors have shown efficacy in studies assessing the diffusion of cAMP throughout the cell (Nikolaev et al., 2006). Transgenic technology has allowed the HCN2 and Epac1 sensors to be incorporated into mouse DNA, creating strains of animal which express these sensors within every cell of their body (Nikolaev et al., 2006; Calebiro et al., 2009). cGMP sensors have been created by using the cAMP binding domains of phosphodiesterases or PKG as their detector region. There is a transgenic mouse strain with the RED DE5 cAMP sensor (Sprenger et al., 2015).

On the basis of these general sensors with lower sensitivity, localized sensors have been created by fusing various localization domains. Many if not all of the applications of these localized sensors have been focused upon investigating the molecular actors involved in modulating excitation-contraction coupling within the cardiomyocyte. This is the result of a relatively small number of groups being involved in this process and their general interest in the cardiac field. Fusions of the PKA-RI and RII regulatory domains with the non-localized Epac-1 sensor were introduced as an update of the original genetically encoded FRET sensors by the Zaccolo group and measure cAMP in PKA microdomains (Di Benedetto et al., 2008). Fusions of cGMP and cAMP sensing domains have been made with the N-termini of phosphodiesterases to investigate the dynamics of secondary messengers within the vicinity of the molecules charged with controlling their levels (Herget et al., 2008). Fascinating studies have been conducted looking at specific membrane microdomains such as the sarcoplasmic reticulum, which are beyond the reach of the pipette based approaches discussed in this review. These have investigated cAMP levels and the activity of PKA in these regions (Dyachok et al., 2006; Liu et al., 2011). Equally, the plasma membrane itself has been investigated with a fusion peptide targeted toward caveolar membranes by fusion with a motif from Lyn kinase (Wachten et al., 2010; Mohamed et al., 2011). As well as this the domains of specific adenyl cyclases have been probed by fusions of Epac2 and AC8. The membranes of mitochondria and the nucleus have been probed by fusion of mitochondrial sequences and nuclear targeting motifs to the ICUE (indicator of cAMP using Epac) class of FRET sensors (DiPilato et al., 2004; Sample et al., 2012). This area and other biophysical techniques for cyclic nucleotide measurement have been reviewed in exhaustive detail by Sprenger and Nikolaev (2013).

Scanning Ion Conductance Microscopy/FRET (SICM/FRET)

Scanning ion conductance microscopy (SICM) was developed by Paul Hansma who realized that ion fluxes, present when performing patch clamp experiments, could also be used to image cellular topography (Hansma et al., 1989). The glass capillary

pipettes of a type similar to that is utilized in patch clamping, but of higher resistance, are able to function as a scanning probe. The ion flux present between the negative electrode inside the pipette and the positive one in the bath is reduced when the pipette tip is moved in close proximity to cellular structures. This results in a drop in conductance and a measurable drop in current. By scanning the pipette across the surface of interest a 3D map of the relative variance in conductance can be built up. The system is run in a feedback mode meaning that conductance may not drop beneath a pre-defined value; this prevents the pipette from coming into contact with the sample surface. The topographical images acquired by this imaging modality give sub-optical resolution, which is defined and only limited by the radius of the pipette tip. SICM scans bear a striking resemblance to scanning electron micrograph images only cells and other biological objects are live and non-prepared, unlike the aforementioned imaging modality (Miragoli et al., 2011). Novak et al pioneered the "Hopping Mode" approach which removed image artifacts caused by large structures obstructing the scanning pipette in the x/y directions (Novak et al., 2009).

Clearly the pipette itself cannot monitor GPCRs function; this imaging modality must be multiplexed with other techniques. In our group SICM has been used in combination with FRET microscopy to study GPCR function. SICM is able to resolve complex membrane topography and the nanopipette allows application of picolitres of solution to cells. FRET microscopy can then offer a "read-out" upon the relative presence of a secondary messenger response (Nikolaev et al., 2010; Wright et al., 2014). The resolution of SICM utilized in the two studies using this hybrid technique has been around 200 nm, allowing T-tubule openings to be observed in adult cardiomyocytes. This means that the openings of individual T-tubular regions can be targeted for agonist application. The presence of cAMP responses can then be investigated at points both near and far from the region of application to assess the relative diffusion/ propagation of the cAMP response upon agonist application. The measurement of the propagation of cAMP within the cell cytosol is only possible with a non-localized FRET sensor. This makes this class of sensor important for this modality. In one of the extant studies a modified high-resolution SICM setup was able to resolve structures on the scale of 50 nm which may be caveolae, meaning that these structures could be targeted at some point in the future.

In normal cardiomyocytes transfected with a cAMP-sensing FRET construct SICM/FRET has demonstrated that β_2AR are strictly localized to T-tubules (Nikolaev et al., 2010). Application of Isoprenaline from the nanopipette into T-tubules, but not to the areas between T-tubules (cellular sarcolemma crests) gives rise to stringently localized sub-cellular cAMP responses. This situation is subverted in failing cardiomyocytes, T-tubules are disrupted and the application of Isoprenaline to cellular crests begins to elicit β_2AR-cAMP responses. The cyclic AMP response following β_2AR stimulation also loses its stringent localization in space. The pathological consequences of this alteration are not clear, but it may contribute to the loss of contractile function observed in the failing myocardium and the apparent desensitization of the myocardium to sympathetic input. If this is the case then it is clear that a disruption of the sub-cellular environment in the setting of pathology, even if not modifying the intrinsic properties of receptors, can modify its extrinsic pharmacological function. A follow up study assessed the treatment of rats with an experimental gene therapy technique (Lyon et al., 2012), which caused the overexpression of the Ca^{2+} pump SERCA2a in failing cardiomyocytes. This treatment restored the T-tubular structure of the failing cardiomyocytes and the β_2AR response was observed in the T-tubule whilst a response which was inducible at the crest was no longer present. This demonstrated the importance of maintaining discrete sub-cellular structures to enable the proper control of cAMP compartmentation.

In parallel to the disruption of T-tubular structures the caveolar domains also appeared to heavily modify β_2AR-cAMP compartmentation (Wright et al., 2014). MβCD caused the β_2AR-cAMP response to appear upon cellular sarcolemmal crests and to propagate throughout the cell. Further to that, the importance of cAMP compartmentation by caveolar structures was exposed by knocking down Caveolin-3 (cav-3) (Wright et al., 2014). This displacement caused the β_2AR-cAMP response to remain localized to T-tubular domains but caused the cAMP response to propagate throughout the cell. Over-expression of cav-3 in failing cardiomyocytes was able to restore localized β_2AR-cAMP response which was previously deranged. A novel computer model has been produced which accurately predicted the displacement of cav-3 to be more difficult at the sarcolemma in relation to the crest due to the differences in the formation of caveolae in the different regions of the cardiomyocytes (Wright et al., 2014). The latter prediction was confirmed by an entirely separate work using super-resolution confocal microscopy techniques (Wong et al., 2013).

Conclusion

As this review demonstrates the function of GPCRs and the control of cAMP and other secondary messengers cannot be divorced from the membrane environment that these molecules are localized within. As a result only through understanding how these domains affect the function of GPCRs and control cAMP responses can one begin to understand how to rationally manipulate intracellular cAMP responses to provide benefit within the contexts of human pathology. The studies reviewed have exclusively been performed in animal models and many, as discussed, have been performed by groups with a special interest in the cardiac field. The cardiomyocyte is a singular cell type and presents a degree of structural complexity second perhaps only to that of neurons. As a result the apparent stringency of secondary messenger compartmentation by structural means may not be as essential in other cell types.

The techniques above present avenues toward the assessment of GPCR function at the level of membrane localization. The techniques are not prohibitively sophisticated, with SICM being, at least in theory, within reach of laboratories utilizing patch-clamp technology. Equally, simple ratiometric FRET is a straightforward microscopy technique and the combination of SICM/FRET requires only the co-ordination of both techniques. Adenoviral and plasmid constructs encoding various localized and general FRET sensors targeted at different second messengers

are becoming widely available to the research community. The emergence of a greater number of manufacturers on the market for this instrumentation will drive more researchers to adopt what has proven to be very powerful experimental approaches.

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REVIEW
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Targeting protein–protein interactions in complexes organized by A kinase anchoring proteins

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Cyclic AMP is a ubiquitous intracellular second messenger involved in the regulation of a wide variety of cellular processes, a majority of which act through the cAMP – protein kinase A (PKA) signaling pathway and involve PKA phosphorylation of specific substrates. PKA phosphorylation events are typically spatially restricted and temporally well controlled. A-kinase anchoring proteins (AKAPs) directly bind PKA and recruit it to specific subcellular loci targeting the kinase activity toward particular substrates, and thereby provide discrete spatiotemporal control of downstream phosphorylation events. AKAPs also scaffold other signaling molecules into multi-protein complexes that function as crossroads between different signaling pathways. Targeting AKAP coordinated protein complexes with high-affinity peptidomimetics or small molecules to tease apart distinct protein–protein interactions (PPIs) therefore offers important means to disrupt binding of specific components of the complex to better understand the molecular mechanisms involved in the function of individual signalosomes and their pathophysiological role. Furthermore, development of novel classes of small molecules involved in displacement of AKAP-bound signal molecules is now emerging. Here, we will focus on mechanisms for targeting PPI, disruptors that modulate downstream cAMP signaling and their role, especially in the heart.

Keywords: cAMP, AKAP, protein–protein interaction, heart, disruptor peptide, small molecule

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Introduction

Intracellular 3′-5′-cyclic adenosine monophosphate (cAMP) is an important second messenger that regulates a number of biological processes. Even though cAMP is diffusible, its concentration and signaling are tightly controlled and coordinated through the involvement of a molecular machinery coordinating the spatial and temporal processes of localized cAMP signaling events. Signal transduction through the cAMP pathway starts by stimulation of G-protein-coupled-receptors (GPCRs), via specific extracellular ligands leading to activation of adenylyl cyclase (AC), which converts ATP into cAMP. The rise in intracellular cAMP levels leads to a set of events mediated by specific effector molecules, hereunder protein kinase A (PKA; Walsh et al., 1968), cyclic nucleotide gated ion channels (Brown et al., 1979) and exchange protein directly activated by cAMP (Epac; de Rooij et al., 1998; Kawasaki et al., 1998). To terminate the signal, intracellular cAMP levels must be brought back to basal levels; this is attained by cyclic nucleotide phosphodiesterases (PDEs), which hydrolyse cAMP and/or cGMP. Additionally, cAMP signalosomes targeted to specific subcellular locales by A-kinase anchoring proteins

(AKAPs) bring together signal initiators, effector and terminators in supramolecular signaling complexes. The existence of these specific complexes (illustrated in **Figure 1**) governed by protein–protein interactions (PPIs) creates an opportunity for new therapeutic strategies to control cAMP dependent signaling that is out of tune or involved in pathologies. In this review we will first focus on signaling through AKAP-coordinated complexes, next on targeting PPIs as a possible strategy to control and regulate cAMP signaling events and finally mention a few examples of possible PPIs that could be targeted. We will discuss cAMP/PKA/AKAP signaling in general terms but will particularly focus on the heart, where cAMP signaling pathways are involved in different stages of the cardiac cycle and in several pathologies.

cAMP Compartmentation

Cyclic AMP is a ubiquitous intracellular second messenger involved in the regulation of a wide variety of cellular processes. For this reason there are molecular mechanisms that rigorously control cAMP signal responses, intimately linked with spatial and temporal fine tuning. The first indication that cAMP could be organized in specific microdomains came in the early 1980s, when different GPCR ligands were observed to trigger very different cardiomyocytes responses, even if there was a similar increase in cAMP levels (Hayes et al., 1980). Meanwhile, the development of new methodologies to study compartmentation (for example, fluorescent resonance energy transfer FRET, reviewed in Zaccolo and Pozzan, 2002; Smith et al., 2006) provided solid evidence of cAMP dynamics and of the mechanisms and proteins involved.

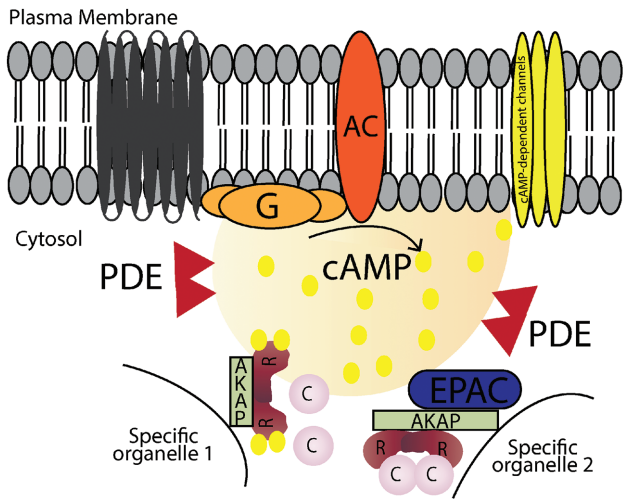

FIGURE 1 | Schematic illustration of cAMP signaling pathways. Stimulation of G-protein-coupled receptors leads to activation of adenylyl cyclase (AC), which converts ATP into cAMP. cAMP increases in local microdomains and binds to different effectors such as protein kinase A (PKA), cyclic nucleotide gated ion channels and exchange protein directly activated by cAMP (Epac) leading to specific downstream effects. Cyclic nucleotide phosphodiesterases (PDEs) hydrolyse cAMP into AMP and terminates the signal. A-kinase anchoring proteins (AKAPs) anchor the signaling molecules involved in the pathway and target them to specific organelles in the cell.

First, an important starting point for generation of well-defined local gradients of cAMP is the organization and diverse expression of receptors, G proteins and associated cyclases at the plasma membrane. The G proteins are heterotrimeric, guanosine triphosphate-binding (GTP-binding) proteins, assembled from three subunits: α-, β- and γ-. There are more than 20 different Gα-subunits that can activate or inhibit different effectors. For example, Gαs activates and Gαi inhibits ACs, respectively. Like G proteins, AC exist in different isoforms in mammals, most of them are associated with the plasma membrane (AC1-9) whereas AC10 is soluble (Steegborn, 2014). Both G proteins and AC isoforms have also been reported in lipid rafts and caveolae, and implicated in the generation of local cAMP microdomains at the membrane (Ostrom and Insel, 2004; Patel et al., 2008). Additionally, it was recently shown that cAMP production does not exclusively occur at the plasma membrane and is not terminated when receptors are internalized (Calebiro et al., 2009; Ferrandon et al., 2009). Moreover, Irannejad et al. (2013) have shown GPCR signaling both in the plasma membrane and after internalization in living cells using a biosensor. In summary, both G proteins and ACs have a distribution that contributes to generation of local gradients of cAMP.

Second, the expression and availability of various effector molecules in cAMP microdomains also contribute to discretely controlling how the signal is propagated (Mei et al., 2002; Bresnnesvik et al., 2005; Bacallao and Monje, 2013; Vitali et al., 2014). The specificity introduced by different cAMP effector molecules is illustrated, for example in the heart where it was shown that Epac modulates cardiac sarcomeric contraction despite a decrease in Ca^{2+} levels, while PKA modulates contractility via an increase in intracellular Ca^{2+} (Cazorla et al., 2009). Here, we will not go into details with respect to cAMP-gated ion channels and Epac, however, cAMP effectors can perform both synergistically and antagonistically in the regulation of specific cellular functions and coordinated action may have biological significance (reviewed in Craven and Zagotta, 2006; Cheng et al., 2008). The PKA holoenzyme is a tetramer composed of regulatory (R) subunit dimer and two catalytic (C) subunits, which are associated in the inactive state when cAMP intracellular levels are low. When cAMP levels increase PKA becomes activated; this proceeds by a concerted reaction where cAMP molecules bind cooperatively to the two cyclic nucleotide binding domains (CNBD) in each R-subunit of PKA leading to a conformational change by releasing the C-subunit. The free C-subunit becomes active and can then phosphorylate specific serine/threonine residues in target proteins, usually in the sequence Arg-Arg-X-Ser/Thr, where X is a hydrophobic amino acid. There are two types of PKA holoenzymes, type I and type II, that mainly differ in their localization and affinity for cAMP and with different R-subunit composition (RI and RII) (Reimann et al., 1971; Corbin et al., 1975; Cadd et al., 1990; Dostmann and Taylor, 1991; Gamm et al., 1996). Both R-subunits are very similar concerning their domain organization which includes the N-terminal docking

and dimerization (D/D) domain important for localization inside the cell, a substrate/auto-inhibitor region that binds to the C-subunit in the holoenzyme and in the C-terminal two highly conserved CNBDs (Corbin et al., 1978; Doskeland, 1978). Although, both RI and RII share the same organization their substrate/auto-inhibitor region is significantly different, in that RII can be autophosphorylated whereas RI contains a pseudo-phosphorylation site. Another difference between PKA-RI and PKA-RII is their localization, where PKA type I is primarily found in the cytosol while PKA type II is predominantly localized to specific cellular organelles (Tasken and Aandahl, 2004; Wong and Scott, 2004). Additionally, PKA type I and type II also differ from each other in their cAMP activation constants which is lower for type I than for type II (typically 50–100 nM versus 200–400 nM; Dostmann and Taylor, 1991). PKA exist in different isoform combinations encoded by different genes, four C-subunit isoforms (in human Cα, Cβ, Cγ, and PRKX) and four R-subunit isoforms (RIα, RIβ, RIIα, and RIIβ). In combination these different R- and C-subunits isoforms can form different PKA holoenzymes that can be present in diverse signalosomes with distinct expression patterns in different tissues and cell types.

Third, intracellular gradients of cAMP and consequently their signaling pathways are highly controlled by PDEs. They are key players in controlling intracellular cAMP levels due to the fact that PDEs are the only cAMP degrading enzymes. PDEs catalyze the degradation of cAMP by breaking the phosphodiesterase bound resulting in adenosine-5-monophosphate (AMP). PDEs are highly conserved between species, with around 50 different isoforms that are part of 11 families (PDE1-11) with different enzymatic and regulatory characteristics (Houslay and Milligan, 1997; Conti and Jin, 1999). They all share similarities in their structure, mainly in the C-terminal catalytic domain, while the N-terminal regulatory and targeting domains differ (Bender and Beavo, 2006; Conti and Beavo, 2007). PDEs can be cAMP-specific, cGMP-specific or hydrolyse both. The great variety in isoforms, their specific tissue and subcellular localization and the fact that PDEs have different enzymatic profiles, makes PDEs key players in spatial and temporal control of intracellular cAMP levels. In the heart, the cAMP-specific PDE4 and cAMP/cGMP PDE3 are responsible for the majority of cAMP hydrolysed in cardiomyocytes (Mongillo et al., 2004; Rochais et al., 2004; Fischmeister et al., 2006; Mika et al., 2012).

Fourth, scaffold proteins coordinate the physical assembly of components of a signaling pathway. In cAMP signaling pathways AKAPs are responsible for the assembly of specific signalosomes. They form complexes between PKAs and their specific targets in localized subcellular domains, consequently they are important contributors to compartmentation, which will be further discussed in the next chapter.

AKAPs – Scaffold Proteins Involved Assembly of Supramolecular Signaling Complexes

The spatial and temporal organization of cAMP/PKA signaling is attained by a carefully tuned balance between local activation of the signal effector and signal termination machinery assembled and targeted by AKAPs. There are more than 50 AKAPs identified, and even though they belong to a structurally diverse family they all share the ability to enable tightly regulated phosphorylation of substrates that are anchored to or localized in the vicinity of AKAPs together with PKA (Tasken and Aandahl, 2004; Wong and Scott, 2004). The four main features that characterize the AKAP complexes formed (**Figure 2**) are:

(1) Anchoring of the dimer R-subunit D/D-domain through interaction between the hydrophobic pocket of PKA and the

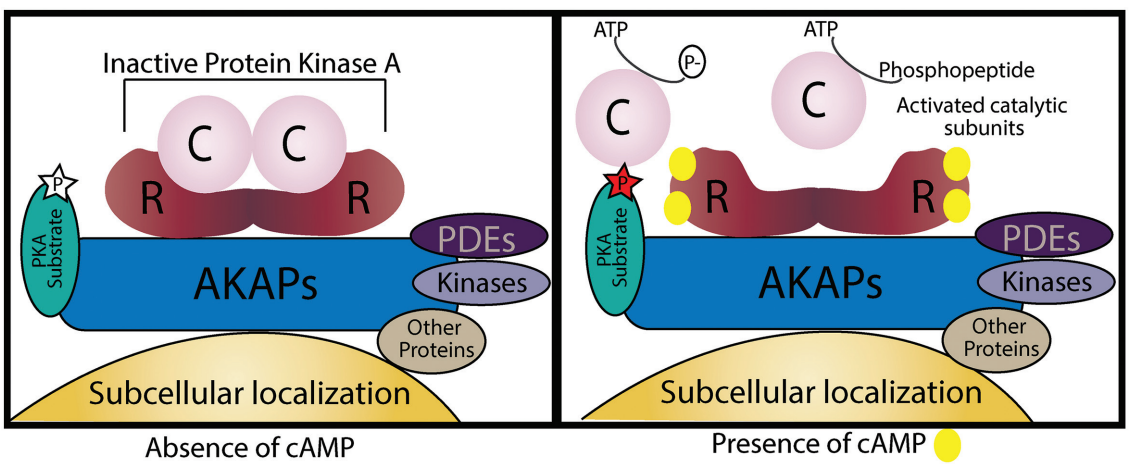

FIGURE 2 | Schematic illustration of an A kinase anchoring protein (AKAP). AKAPs are categorized by four different characteristics. First, an amphipathic α-helical region of the AKAP interacts with D/D-domain of the PKA R-subunit dimer. Second, they target the supramolecular complex to specific subcellular localizations. Third, AKAPs may also hold PKA substrates by direct binding or by targeting in their vicinity. Lastly, AKAPs can also function as signaling scaffolds for other signaling enzymes. In the absence of cAMP, PKA is inactive and its substrates are not phosphorylated, when cAMP levels increase it binds to the R-subunits and the active catalytic subunits are free to phosphorylate their targets.

14–18 amino acid amphipathic helix region of AKAPs (Carr et al., 1991). AKAPs can bind both RI- and RII-subunits of PKA, the majority binds to RII-subunits;

(2) Targeting to specific subcellular locations. AKAPs typically contain a targeting domain that localizes the entire AKAP/PKA complex inside the cell. This can be a defined organelle, membrane or structure and the binding may occur by protein–protein or protein–lipid interactions.

(3) Directly binding to or co-localizing with specific substrates that will be phosphorylated by PKA.

(4) Assembly of multi-protein complexes with additional signaling enzymes such as PDEs, phosphatases (PPs), and other kinases;

AKAPs can be found in a number of tissues and are involved in the composition of a wide variety of complexes implicated in different signaling cascades (reviewed in Pidoux and Taskén, 2010; Tröger et al., 2012; Esseltine and Scott, 2013; Scott et al., 2013). Most of these AKAP complexes preferentially anchor the PKA type II holoenzyme with higher affinity. There are, however, some exceptions where AKAPs anchor PKA-RI. For example, the cardiomyocyte sphingosine kinase interacting protein (SKIP) exclusively binds PKA type I and facilitates phosphorylation of ChChd3 inside mitochondria (Means et al., 2011). Recently a small membrane AKAP (smAKAP) was found to target RI to the plasma membrane (Burgers et al., 2012). There are also some cases where AKAPs can have dual specificity, for example D-AKAP1 and D-AKAP2 (Huang et al., 1997a,b), ezrin (Ruppelt et al., 2007) and Opa1 (Pidoux et al., 2011) can bind both RI and RII at physiological concentrations. There is also a number of cellular contexts where AKAPs contribute to the spatial organization of other effectors such as Epac (Dodge-Kafka et al., 2005). AKAPs complexes have been shown to directly interact with PPs and PDEs which provides tight control of signal termination, since PPs that remove phosphorylation or PDEs that degrade cAMP are found in the same complex (Coghlan et al., 1995; Schillace and Scott, 1999; Dodge et al., 2001; Tasken et al., 2001).

In the heart the existence of a supramolecular complex with PKA/mAKAP/PDE4D3 creates a negative feedback loop mechanism under stimulation, where PDE4D3 phosphorylation increases cAMP hydrolysis and turns off PKA activity (Dodge-Kafka et al., 2005). Several AKAPs occur in different isoforms and spliced variants that are targeted to different subcellular localizations. For example, AKAP18 has several isoforms (α, β, γ, and δ) that are localized to distinct subcellular structures in specific cell types. Both AKAP18α and AKAP18β are mainly found in plasma membrane, while AKAP18α is associated with L-type Ca^{2+} channels in the skeletal muscle and pancreatic cells less is known about AKAP18β function (Fraser et al., 1998; Gray et al., 1998). When overexpressed in polarized epithelial kidney cells, however, AKAP18α and AKAP18β preferentially localize to the basolateral and apical membrane, respectively (Trotter et al., 1999). Overexpressed AKAP18γ cloned from pancreas and lung is mainly localized in the cytosol but it was also found in the nucleus of mouse oocytes (Trotter et al., 1999; Brown et al., 2003). AKAP18δ was first found in the kidneys anchored to vesicles (Henn et al., 2004) and later AKAP18γ/δ was found in cardiomyocytes anchored to the sarcoplasmic reticulum (SR; Lygren et al., 2007). Furthermore, AKAPs can target complexes to other localizations like mitochondria, the Golgi complex, centrosomes, cytoskeleton and many other loci (Wong and Scott, 2004; Tröger et al., 2012).

Several AKAPs have been found to be expressed in cardiac tissue involved in different processes: calcium-induced calcium release in depolarization and plateau phase, cardiac repolarization and cardiac remodeling due to stress responses (Scott et al., 2013; Soni et al., 2014). It has been shown that different pathologies are associated with AKAPs, due to polymorphisms and mutations in members of this family of proteins, in heart diseases (Chen et al., 2007) and cancer development (Frank et al., 2008). In the heart, AKAPs have been implicated in several cardiac diseases such as rhythm disorder, long-QT syndrome, cardiac hypertrophy and heart failure (reviewed in Soni et al., 2014).

This review will focus on the potential targeting of AKAPs as a therapeutic strategy.

Targeting Protein–Protein Interactions

There are a number of approaches to target signal effector and signal termination enzymes in specific signalosomes. One strategy is the development of inhibitors against specific enzymes and receptors. However, as signaling enzymes may be components of several different signalosomes, specificity may not be at the level of the individual type of complex. Another possibility would be to target scaffold protein such as AKAPs using RNA interference (RNAi). Small interfering RNAs (siRNAs) can be specifically designed to target any gene and can silence target mRNA expression to overcome different pathologies. Even though siRNA is been increasingly used and some siRNA based therapies are in human clinical trials, siRNA therapeutics still need to overcome the obstacles of efficient drug delivery to be a fully viable drug development strategy (for more details about this approach see de Fougerolles et al., 2007). In this review we will mainly focus on another approach, targeting AKAPs by disrupting the binding between two proteins, i.e., interfering with PPIs.

Hundreds of thousands of PPIs probably occur in human cells and are involved in assembly of supramolecular signaling complexes and signalosomes as well as in target-depend signaling by docking and adaptors. PPIs represent an exciting group of potential therapeutic targets implicated in a wide array of diseases. The fact that they are intrinsically associated with specific signalosomes offers potential for high specificity and PPIs may therefore constitute valuable targets in new therapeutic strategies. For this reason designing and developing PPI disruptor peptides represents an area of increasing interest in target validation and drug discovery. Peptides can be highly selective and specific and their affinity to the target makes them appealing candidates in vivo due to minimal off-target effects. Peptides and peptidomimetics offer several benefits such as ease of synthesis, optimization and evaluation, high affinity, minimal

immune responses and low toxicity. However, peptides can be metabolically cleaved and rapidly cleared from body and non-natural peptides or peptidomimetics that abandon the amino acid backbone may be necessary to avoid excessive degradation. Furthermore, intracellular delivery may be an issue (reviewed in Cochran, 2000). Nevertheless, the development of disruptor peptides for PPIs can be valuable research tools to perturb supramolecular signaling complexes and retrieving information regarding the role of the proteins involved.

During this time also disruptor peptides for complexes involving AKAPs have been progressing, mainly to prevent the interaction between AKAP and PKA. These peptides were primarily designed to serve as tools to study AKAPs and interaction with different PKA R-subunits, both *in vitro* and *in situ* inside cells. As mentioned above, AKAPs bind through a conserved amphipathic helixes domain to the hydrophobic dimerization domain of the PKA R-subunit (Carr et al., 1991). These small disruptor peptides mainly mimic the amphipathic helices domain of AKAP, like in the case of Ht31, the first and most commonly used disruptor peptide. This was derived from the PKA binding domain in AKAP-Lbc (Carr et al., 1992) and it was later shown to be non-selective, with the ability to disrupt both RI and RII from AKAPs (Herberg et al., 2000). More than 20 years later Ht31 is still being used as a tool to describe new AKAPs, for example neurochondrin (Hermann et al., 2015). In 2003, a disruptor peptide was developed by determining the minimal binding domains of several AKAPs that had high binding affinity to RII, called AKAP-in silico (AKAP-IS; Alto et al., 2003). A later version was developed to have almost no RI-binding, SuperAKAP-IS (Gold et al., 2006). At the same time, peptides designed to disrupt AKAP/RI binding, like PV38 (Burns-Hamuro et al., 2003) and RIAD (Carlson et al., 2006) were put in use. Besides the improvements regarding binding affinity to distinguish between AKAP binding to RI or/and RII, a big limitation is the cell permeability of the designed peptides. Several approaches have been used to address this problem by attaching a cell-permeable poly-basic sequence to the disruptor peptide, like the TAT or *antennapedia* sequences or a poly-arginine peptide. For example, a peptide derived from the TAT protein of the human immunodeficiency virus (HIV-1) can, when linked to the disruptor peptide, easily facilitate its transport into the cell. TAT-AKAP-IS could at micromolar concentrations disrupt endogenous AKAP/PKA interaction and affect PKA subcellular localization in insulin-secreting pancreatic B-cells (Faruque et al., 2009). In perfused hearts, TAT-conjugated A-kinase-anchoring disruptor (TAT-AKAD) affected heart rate and contractility after β-adrenergic stimulation and disrupt PKA localization in cardiomyocytes (Patel et al., 2010). Another approach that has been developed to improve peptide permeability is to use all-hydrocarbon-stapled α-helical peptides, where non-natural amino acids are incorporated into the peptide resulting in a stapled peptide that is locked in a α-helical conformation. Additionally, it has been shown that stapled peptides have increased binding affinity, less susceptibility to proteolytic degradation, improved pharmacologic performance and serum half-life (Verdine and Hilinski, 2012). Using this technique, Wang and co-workers developed disruptor peptides that are highly cell permeable to different cell-lines and that could efficiently prevent the interaction between AKAP and PKA, which they called Stapled Anchoring Disruptors (STADS). They designed STAD-2 and STAD-3 (Wang et al., 2014) and RI-STAD-1 and RI-STAD-2 (Wang et al., 2015) that are highly selective for disrupting the interaction between AKAPs/PKA-RII and AKAP/PKA-RI, respectively. Interestingly, STAD-2 has been used in a very different cell model system as a potential strategy to study and develop new antimalarials targets (Flaherty et al., 2015).

Even if these are highly efficient peptides, the fact that they disrupt the AKAP/PKA interaction diminishes their specificity since this interaction is common to all PKA-AKAP complexes. By disrupting the AKAP/PKA interaction, these peptides will affect several AKAP/PKA complexes inside the cell regardless of the AKAP present. A much more precise strategy would be to disrupt individual AKAP complexes, which would be possible by preventing the interaction between a specific AKAP and an attached substrate protein that will be phosphorylated by PKA. There are several examples of such peptides, which were also designed mainly as tools to study and confirm AKAP-interactions. One example is a short peptide derived from the phospholamban (PLB) domain that binds to AKAP18δ and competes with and displaces the AKAP18δ/PLB interaction (Lygren et al., 2007). This PLB peptide with a poly-arginine attached to the C- or N-terminus for penetrance in neonatal cardiomyocytes, blocked noradrenalin-induced increase in Ca^{2+} reabsorption. Another possibility would be to disrupt the interaction between the AKAP targeting domain and its interaction partner providing subcellular localization. For example Dodge-Kafka et al. (2005) designed a fragment encoding residues 585–1286 of mAKAP that displaced mAKAP from the perinuclear membrane.

Recently, a new approach was established that uses structure-based phage selection to design new RII D/D domain fragments that can selectively distinguish between different AKAPs, which were designated R_{Select} (Gold et al., 2013). Using Western blot and amplified luminescence proximity homogenous assay (AlphaScreen) assays R_{Select}AKAP2 and R_{Select}AKAP18 were shown to preferably interact with AKAP2 and AKAP18 *in vitro*. Additionally, *in vivo* experiments showed that the same mutants have a similar subcellular distribution as their AKAP partners and they recognize and interact with them.

Even though disruptor peptides are of great importance for interfering with PPIs and an invaluable asset to understand cAMP/AKAP/PKA signaling effectors and effects, they have limited use as therapeutics. Peptides are designed and derived from one of the binding proteins involved. When compared to peptidomimetics and small molecules, low permeability and poor per-oral bioavailability are big drawbacks in peptides. Peptidomimetics has been used also as a possible strategy for the modulation and regulation of AKAPs by interfering with PPIs. For example, RIAD peptidomimetics have been developed by adding unnatural amino acids at different positions, leading to increased stability in serum though still retaining specificity to disruption of the AKAP/PKA-RI interaction (Torheim et al., 2009). In addition, a RIAD peptidomimetic (RIAD-P3) has been

shown to limit HIV-1 viral replication and stabilize CD4 levels by disrupting AKAP/PKA-RI *in vivo* (Singh et al., 2014).

When compared to peptides, small molecules offer several advantages in drug discovery: due to their smaller size they can be synthesized easily and at a lower price, get faster to their targets, have potential for higher oral bioavailability, can offer better stability and can be used to allosterically target quite large protein interaction surfaces. However, one downside to this approach is the fact that it requires detailed and profound knowledge about the interaction between the two proteins involved and PPI targeting can be quite challenging and make small molecule approaches less attractive from a drug discovery point-of-view.

Several techniques have been contributing to identifying PPIs and increasing the information regarding their structure such as X-ray crystallography, nuclear magnetic resonance (NMR), thermal shift assay, surface plasmon resonance (SPR), immunoprecipitation and other biochemical assays as well as in silico modeling of interactions. However, there are still challenges when screening for small molecule PPI disruptors: the PPI interfaces may be big, discontinuous or flat and hydrophobic with an absence of pockets and typically a screen yields PPI disruptors with micromolar affinity. Peptides do not have these limitations, but have issues with stability and permeability. In order for small molecule targeting of PPIs to be successful, a topology of the interacting proteins with small pockets or the identification of key residues that contribute to the binding energy, so called "hot spots" are necessary requirements (reviewed in Turnbull et al., 2014). Furthermore, increasing the size and molecular weight of the small molecule or assembling new compounds by a fragment-based approach may overcome some of these problems. Small molecules can also allosterically bind to one of the two interacting proteins outside the binding interface inducing conformational changes affecting the PPI (Arkin and Wells, 2004; Fry, 2006; Wells and McClendon, 2007; Turnbull et al., 2014).

The first study reporting the use of small molecules as disruptors of PPIs in AKAP complexes was in Christian et al. (2011), when it was reported that a group of structurally similar small molecules could prevent the interaction between an AKAP and PKA-RII. After screening a library of 20,000 compounds, the authors found that FMP-API-1 and its derivatives disrupted the interaction between AKAP18δ and both RIIα and RIIβ by allosteric binding to RII outside its D/D domain instead of binding to the AKAP interacting surface. Using cardiomyocytes they showed both *in vitro* and *in vivo* evidence that these small molecules disrupt AKAP and RII binding and at the same time activate PKA, indicating a dual effect.

Increasing interest in PPI disruptors together with improvements in high-throughput screening (HTS) for compounds targeting PPIs has resulted in an increasing number of projects in this area. Combinations of primary and secondary assays in HTS can create an attractive and very useful setting to screen fast and easily large compounds libraries. These are normally miniaturized assays performed in a robotics workstation, where different instruments and liquid handling systems are used. There are several published assays to screen both for peptides and small molecules that were specifically developed to target PPIs in complexes involving AKAPs such as AlphaScreen; SPR; enzyme-linked immunosorbent assay (ELISA); and homogenous time-resolved fluorescence (HTRF) (Stokka et al., 2006; Jarnaess et al., 2008; Christian et al., 2011; Gold et al., 2013; Schächterle et al., 2015).

Certainly targeting PPIs is a very appealing and promising therapeutic strategy as it is possible to specifically target interactions in single molecules complexes at defined subcellular places that until now has not been fully address and exploited.

Therapeutic Targets in the Heart in cAMP Signaling Pathways

Heart pathologies are a leading cause of hospitalization and mortality in the Western World. Moreover, population growth and increase in life expectancy accelerates the number of heart incidents. Currently there are several treatment choices that interfere with cAMP signaling in the heart. Beta-blockers block adrenergic signaling and have a negative inotropic and chronotropic effect. In contrast dopamine has a positive inotropic effect and paces the heart. Beta-blockers are, however, accompanied by significant side effects, because they affect all downstream signaling. As an alternative to beta-blockers, the recently described biased agonists and antagonists can provide functional selectivity, where the biased ligand activates or terminates a specific intracellular signaling pathway downstream of the GPCR. In this case one can achieve a more specific and selective effect rather than the "all or nothing" effect of beta-blockers (Kenakin and Miller, 2010). Particular attention has been given to the importance of ligand bias as a new potential therapeutic strategy for classical GPCR in cardiology targeting angiotensin II type 1 receptors and the β-adrenergic receptors (DeWire and Violin, 2011). With this strategy it might be possible to attain a more precise and specific outcome without the unwanted side effects, making these ligands very interesting drugs for new therapeutic strategies. Additionally, a more effective strategy could be to directly alter a specific AKAP signalosome by affecting a single PPI in complexes where AKAPs have well-established roles in the heart regarding: (i) Ca^{2+}-handling and excitation-contraction coupling; (ii) hypertrophic stress responses; and (iii) controlling electrical signaling. Here we will shortly mention a few of the most relevant AKAPs targets.

(i) Ca^{2+}-Handling and Excitation-Contraction Coupling

In cardiac myocytes AKAP18α (also known as AKAP15) anchors PKA to L-type Ca^{2+} channels in plasma membrane. It was also shown that in skeletal muscle cells AKAP18α directly interacts with both PKA and the channel (Hulme et al., 2002). The authors used a peptide to disrupt the leucine zipper motif interaction between the AKAP and the channel, inhibiting voltage-dependent potentiation of L-type Ca^{2+} channel. The disruptor peptides abolish sympathetically induced, AKAP18α-dependent PKA phosphorylation of L-type Ca^{2+} channels and consequently channel open probability. This in turn prevents Ca^{2+} entry increased in response to local cAMP that increases contractility, resembling β-blockers effect.

More recently, has also been shown that in cardiac myocytes AKAP5 (also known as AKAP79/150) assembles a complex in caveolin 3-associated L-type Ca^{2+} channels together with β-adrenergic receptor, PKA, AC5/6 and calcineurin (CaN), which is important for sympathetic regulation (Nichols et al., 2010). Also, AKAP5 was shown to directly interact with L-type Ca^{2+} channels in HEK293 cells via modified leucine zipper motifs, similar to AKAP18α (Oliveria et al., 2007). Taken together this suggests that both AKAP18α and AKAP5 supra molecular complexes are involved in sympathetically stimulated Ca^{2+} entry trough L-type Ca^{2+} channels, likely associated with different channels subpopulation and cAMP microdomains. Additionally, Makarewich et al. (2012) showed that these caveolin 3-associated L-type Ca^{2+} channels might be an important target for cardiac hypertrophy.

Lygren et al. (2007) found a PKA/AKAP18δ/PLB complex that regulates SR Ca^{2+}-ATPase 2 (SERCA2) in the heart, which was also later shown in human myocardium (Ahmad et al., 2015). The PLB/SERCA2 complex plays a crucial role in calcium homeostasis in cardiomyocytes and is major regulator of cardiac contractility *in vivo* (Koss and Kranias, 1996). Under normal conditions dephosphorylated PLB inhibits SERCA2 mediated Ca^{2+}-reabsorption into the SR, a process that is critical for relaxation of the cardiomyocytes and refilling of the heart before the next contraction. However, AKAP18δ acts as a scaffold protein forming a complex of AKAP18δ and PKA together with PLB/SERCA. Upon β-adrenergic stimulation, PLB is phosphorylated and inhibition of SERCA2 is released leading to an increase in Ca^{2+}-reuptake into the SR allowing for pacing the heart by facilitating faster relaxation and filling. Inhibition of PLB phosphorylation by targeting this complex with PPI disruptors is thought to be cardioprotective (Lygren and Taskén, 2008). Moreover, the AKAP18δ/PLB complex should be relatively heart specific, thus minimizing potential side effects. The binding between AKAP18δ and PKA has already been targeted, both by peptides and small molecules. Here they used HTS to screen libraries of small molecules which inhibit the binding between AKAP-PKA (Christian et al., 2011). Another possibility would be to disrupt the interaction between other proteins in the complex (**Figure 3A**), Lygren et al. (2007) also showed that in neonatal cardiac myocytes the displacement of AKAP18δ/PLB by a short peptide (13–20 aa) affects the phosphorylation of PLB on Ser16 and consequently Ca^{2+}- re-uptake into the SR. Also, removal/reduction of AKAP18δ by siRNA injected in adult cardiomyocytes had the same effect.

In the two previous examples the PPI disruptor approach has been to target the binding between AKAPs and the phosphorylated substrate. Another possibility is to target PPIs between AKAPs and other enzymes, such as PDEs which can potentially be good targets for therapeutic strategies since PDEs are involved in many physiological aspects of cardiomyocyte function, for review see (Mongillo and Zaccolo, 2006). PDEs contribute to the regulation of small cAMP pools by anchoring to AKAPs (Richter et al., 2008; Scott and Santana, 2010). In rat primary cardiomyocytes both PDE3 and PDE4 are likely the major contributors in cAMP signaling (Mongillo et al., 2004; Rochais et al., 2004; Fischmeister et al., 2006; Mika et al., 2012). Additionally, PDE4D are components of the supramolecular complex formed with AKAP18δ/SERCA2 (Lygren et al., 2007; Ahmad et al., 2015). Also in renal principal cells PDE4D is anchored in the AKAP18δ complex (Stefan et al., 2007). Recently, Ahmad et al. (2015) also showed that PDE3A1 also associates with the PLB/AKAP18δ/SERCA2 supramolecular signaling complex in human myocardium SR.

(ii) Hypertrophic Stress Responses

A-kinase anchoring proteins are also present in signalosomes that govern the cellular response known as myocardial hypertrophy,

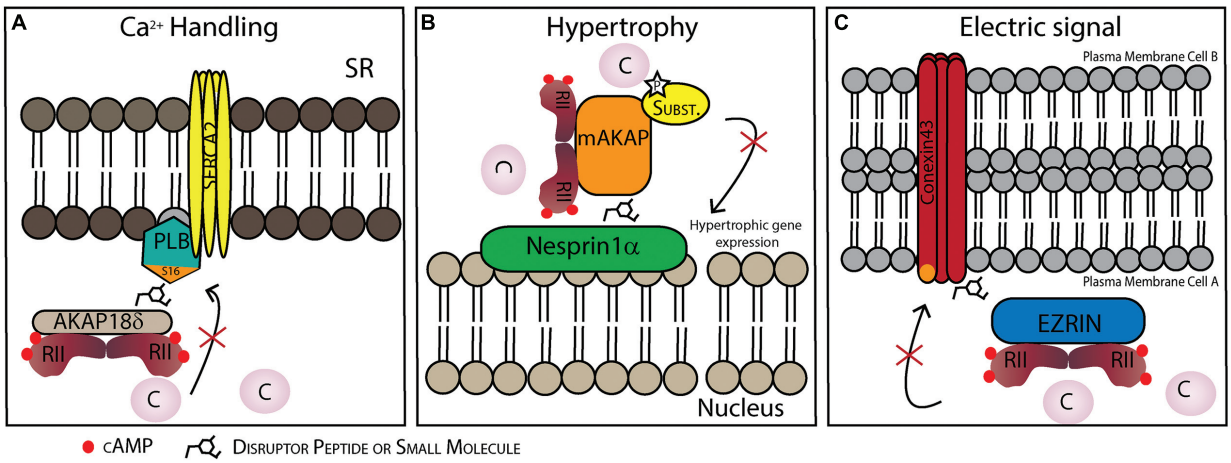

FIGURE 3 | Possible therapeutic strategies to target protein–protein interactions (PPIs) in specific AKAP complexes in the heart. (A) Disruption of the AKAP18γ/δ-PLB interaction prevents PLB phosphorylation on Ser16 and dislocation from SERCA2. This inhibits SERCA2 activation and consequently Ca^{2+} uptake into the sarcoplasmic reticulum (SR). **(B)** Disruption of the nesprin-1α/mAKAP interaction promotes AKAP/PKA complex dissociation from the perinuclear membrane and might be a strategy to reduce hypertrophy. **(C)** Disruption of the connexin 43-ezrin interaction could prevent PKA-mediated phosphorylation increasing inter-cardiomyocyte conductivity which could be cardioprotective following myocardial infarction damage.

which occurs due to different types of cardiac stress where increased levels of catecholamines induce transcriptional activation evoking heart remodeling. These "hypertrophy signalosomes" include a multicity of proteins: protein kinases, PDEs, PPs, calcium channels and others, that all come together in supramolecular complexes coordinated by AKAPs (reviewed in Negro et al., 2008; Carnegie and Burmeister, 2011; Diviani et al., 2013; Soni et al., 2014). At least two AKAPs are involved in cardiomyocyte hypertrophy, an alternatively spliced isoform of muscle AKAPβ (mAKAP, previously called AKAP100; Dodge-Kafka et al., 2005) and AKAP-Lbc (Appert-Collin et al., 2007).

One of the signalosomes that is scaffolded by mAKAP includes PKA, ryanodine receptors (RyR), CaN and transcription factors NFAT and MEF2 (Pare et al., 2005a; Li et al., 2010, 2013). In response to a cAMP increase due to β-adrenergic stimulation, activated PKA phosphorylates RyR channels, the Ca^{2+} release activates CaN, which mediates NFAT and MEF2 transcriptional activity. NFAT and MEF2 transcriptional activity regulated by CaN is dependent and requires mAKAP (Li et al., 2010, 2013). Additionally, Li et al. (2013) showed that cardiomyocytes expressing a mCherry-CaN binding site peptide disrupts mAKAP/CaN complex and inhibits adrenergically induced myocyte hypertrophy, providing a possible therapeutic strategy by targeting mAKAP/CaN. Small molecules might also be potential candidates, since it was already shown that they efficiently disrupt the PPI between CaN and NFAT in T cells (Roehrl et al., 2004).

It has also been shown that mAKAP binds directly to phospholipase C∈ (PLC∈; Zhang et al., 2011), which generates diacylglycerol (DAG) at the nuclear envelope using as substrate phosphatidylinositol 4-phosphate (PI4P) from the Golgi apparatus, leading to activation of protein kinase D (PKD; Zhang et al., 2013). The formation of this complex and its involvement in myocyte hypertrophy has been shown using siRNA based depletion of PLCε and observing that this prevents development of cardiac hypertrophy (Zhang et al., 2011, 2013). Recently, it was shown that histone deacetylase (HDAC) 4 and PKD, which phosphorylates HDAC4 also resides in this AKAP complex (Kritzer et al., 2014). *In vivo* evidence showed that mAKAP knockout mice have better chance of survival when cardiac hypertrophy is induced both by pressure and catecholamine overload. It was also shown that mAKAP knockout decreased apoptosis, fibrosis and pathological gene expression via decreasing activation/phosphorylation of PLC∈/PKD1/HDAC4 complex proteins (Kritzer et al., 2014). Taken together these data provides a novel therapeutic target between mAKAP/PLC∈ for chronic hypertrophy.

Another potential PPI target is that of the AKAP targeting domain and its localization partner. mAKAP is localized in the perinuclear membrane, however, it is not a transmembrane protein, but binds to the outer nuclear membrane protein nesprin-1α (Pare et al., 2005b). Dodge-Kafka et al. (2005) showed that the supramolecular mAKAP/PDE4D3/Epac/ERK5 complex modulates cardiomyocytes hypertrophy. Briefly, mAKAP anchored PKA phosphorylates PDE4D3 that hydrolyses local cAMP forming a negative feedback loop. At the same time PDE4D3 that binds to Epac1and ERK5 can induce cardiomyocyte hypertrophy. Additionally, RNA interference of mAKAP or disruptor peptides competing for the mAKAP/perinuclear membrane binding site blocks the cytokine induced cardiomyocyte growth (**Figure 3B**) (Dodge-Kafka et al., 2005).

AKAP-Lbc acts as a scaffold protein for several protein kinases: PKA, protein kinase C (PKC) and PKD that phosphorylate different substrates leading to hypertrophy, additionally AKAP-Lbc can also acts as a guanine nucleotide exchange factor (GEF) for the small GTPase Rho (reviewed in Carnegie and Burmeister, 2011; Diviani et al., 2013; Soni et al., 2014).

Unlike the mAKAP complex, PKA binding to AKAP-Lbc leads to myocyte cardioprotection. Recently it was demonstrated that PDE4 directly binds to heat-shock protein of 20 kDa (Hsp20) in the heart (Sin et al., 2011). HSPs are chaperone proteins that are important for normal cell function; moreover, their role in protecting against ischemia-reperfusion injury, apoptosis and hypertrophy is well known (Fan et al., 2006; Edwards et al., 2011; Fan and Kranias, 2011). Edwards et al. (2012) showed the involvement of AKAP-Lbc which anchors PKA and Hsp and is responsible for directing PKA phosphorylation of Hsp20, which is cardioprotective (Lee et al., 2013). In contrast, in the absence of cAMP stimulation PDE4 hydrolyses the basal levels of cAMP and prevents activation of PKA resulting in unphosphorylated Hsp20 (Edwards et al., 2012). Furthermore, targeting the Hsp20-PDE4D interaction with a disruptor peptide reduced the development of pressure overload hypertrophic response in aortic-banded mice (Martin et al., 2014). Moreover, small molecules might offer a potential therapeutic avenue since they have already been shown to modulate Hsp20 activity in human airway smooth muscle (An et al., 2011).

As previously mentioned AKAP-Lbc also recruits PKC and PKD1 which enables phosphorylation and activation of PKD1 by PKC (Carnegie et al., 2004), the activated PKD1 is then released from the complex to phosphorylate HDAC5, which has also been shown to be involved in hypertrophy (Zhang et al., 2002; Vega et al., 2004). Additionally, disruption of AKAP-Lbc/PKD1 interaction by truncating AKAP-Lbc in mouse models affects hypertrophy induced by transverse aortic constriction (TAC)-induced pressure overload (Taglieria et al., 2014).

Finally AKAP-Lbc can act as a GEF for the small GTPase Rho and this signalosome can be a possible target for preventing hypertrophy. It was also shown that due to α-adrenergic receptor stimulation, AKAP-Lbc assembles RhoA effectors PKNα, MLTK, MKK3 leading to activation of p38 MAPK (Cariolato et al., 2011) and that this complex regulates hypertrophic responses in the stressed heart (Pérez López et al., 2013). Furthermore, the authors showed by breeding transgenic mice overexpressing a disruptor peptide that inhibition of AKAP-Lbc/p38 complex reduces cardiomyocyte hypertrophy, proving for the first time an *in vivo* role of AKAP in regulating cardiac hypertrophy (Pérez López et al., 2013).

It is also important to mention that cardiomyocyte hypertrophy may sometimes be beneficial and other times harmful (reviewed in Crozatier and Ventura-Clapier, 2015). In some of the studies mentioned targeting AKAPs decreases

compensatory hypertrophy, which can lead to apoptosis and heart failure. Nevertheless, targeting such AKAP complexes may prove useful in combination with other pharmacological approaches.

(iii) Controlling Electrical Signaling

A-kinase anchoring proteins also play a role in promoting electrical cell-to-cell coupling. Gap junctions play a crucial role in cell-to-cell conductance in cardiomyocytes. These channels are composed of connexin 43 (Cx43) hexamers creating pores throught the cell membrane of two adjacent cells allowing passage of the signal. It has recently been shown that an AKAP, ezrin, is involved in the expression and regulation of gap junction conductivity by organizing a PKA/Cx43/ezrin supramolecular complex in other cell types (Pidoux et al., 2014). Moreover, it is known that Cx43 is involved in several pathological conditions in the heart (reviewed in Severs et al., 2008). For example during ischemia, gap junctions are affected mainly by changes in phosphorylation of Cx43 (Beardslee et al., 2000; Axelsen et al., 2006). Targeting the PPIs between the AKAP and Cx43 in the heart may create a possible therapeutic strategy since Cx43 gap junction communication is damaging in the post-infarction heart (**Figure 3C**).

Conclusion

Evidence that cAMP compartmentation is particularly important for transmission of accurate and specific biological information is increasing. In order to respond to local cAMP gradients AKAPs contribute to correct and specific propagation of the cAMP signal by organizing supramolecular complexes where PKA, its substrates and other signaling proteins are assembled. These complexes come together through PPIs, which are fundamental for correct propagation of the response. Until recently targeting AKAP interactions by designing peptides to disrupt such binding interfaces was predominantly used as a tool to study function of components of these signalosomes. Currently, there is growing interest in the possibility to develop small molecule PPI disruptors which creates new opportunities for developing therapeutic strategies by preventing interactions in AKAP-complexes. Here we suggest some possible target complexes in the heart, however, other potential targets were not mentioned and more may still be discovered. Finally, we expect that targeting PPIs in complexes organized by AKAPs will receive increased attention as knowledge in development of small molecule PPI disruptors increases and the benefits of specifically perturbing individual signaling complexes come out.

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REVIEW
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Therapeutic strategies for anchored kinases and phosphatases: exploiting short linear motifs and intrinsic disorder

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Phosphorylation events that occur in response to the second messenger cAMP are controlled spatially and temporally by protein kinase A (PKA) interacting with A-kinase anchoring proteins (AKAPs). Recent advances in understanding the structural basis for this interaction have reinforced the hypothesis that AKAPs create spatially constrained signaling microdomains. This has led to the realization that the PKA/AKAP interface is a potential drug target for modulating a plethora of cell-signaling events. Pharmacological disruption of kinase–AKAP interactions has previously been explored for disease treatment and remains an interesting area of research. However, disrupting or enhancing the association of phosphatases with AKAPs is a therapeutic concept of equal promise, particularly since they oppose the actions of many anchored kinases. Accordingly, numerous AKAPs bind phosphatases such as protein phosphatase 1 (PP1), calcineurin (PP2B), and PP2A. These multimodal signaling hubs are equally able to control the addition of phosphate groups onto target substrates, as well as the removal of these phosphate groups. In this review, we describe recent advances in structural analysis of kinase and phosphatase interactions with AKAPs, and suggest future possibilities for targeting these interactions for therapeutic benefit.

Keywords: protein kinase A (PKA), protein phosphatase 2B (PP2B), calcineurin, A-kinase anchoring protein (AKAP), intrinsic disorder, cAMP signaling, short linear interaction motifs (SLiMs)

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Characterizing Protein Kinase A (PKA) Anchoring

The cAMP-dependent protein kinase A (PKA) was first identified and described by Edwin G. Krebs in 1968 as catalyzing the transfer of phosphate from ATP to a target serine or threonine residue in substrate proteins (Walsh et al., 1968). Since the initial identification of this ubiquitous kinase, many studies have defined its regulation by regulatory subunits (R-subunits), of which there are four isoforms (RIα, RIβ, RIIα, RIIβ; Taylor et al., 2012). PKA regulatory subunits inhibit the activity of the PKA catalytic subunit (C-subunit) by occupying the substrate binding site of the C-subunit and preventing the phosphorylation of substrate proteins (Corbin et al., 1978). When cAMP binds to the R-subunits and inhibition is released, the C-subunit is able to assume its catalytic activity and phosphorylate nearby targets. In addition, each R-subunit isotype contains an N-terminal docking and dimerization domain (D/D domain) that is the basis for the formation of a heterotetramer composed of two R-subunits, each of which bind one C-subunit (2:2 stoichiometry;

Corbin et al., 1975; Newlon et al., 1999). In addition to the formation of R-subunit dimers, this D/D domain is responsible for docking to a genetically diverse but functionally related family of proteins called A-kinase anchoring proteins (AKAPs; Scott et al., 1990; Newlon et al., 2001).

The first AKAP to be identified was microtubule-associated protein 2 (MAP2) by analysis of associated cAMP-dependent kinase activity (Theurkauf and Vallee, 1982). The number of AKAPs identified since has vastly increased due to use of a far-western technique known as the RII overlay (Carr et al., 1991), as well as through more recent development of computational algorithms designed to predict R-subunit binding regions (Burgers et al., 2015). Some of the most characterized AKAPs include AKAP79/150, gravin, AKAP15/18, and mAKAP (Wong and Scott, 2004). In addition, some AKAPs have been shown to bind RI subunit isoforms, either with dual-specificity for RI and RII, or preference for the RI types (Huang et al., 1997a,b; Lacana et al., 2002; Kovanich et al., 2010; Means et al., 2011). However, the majority of AKAPs interact primarily with RII isoforms.

A-kinase anchoring proteins tether pools of readily stimulated PKA holoenzymes to subcellular compartments and organelles through a variety of mechanisms (Langeberg and Scott, 2015). Importantly, AKAPs also bind other signaling enzymes such as phosphodiesterases (PDEs), G-protein coupled receptors (GPCRs), ion channels, and protein phosphatases to form complexes that are able to integrate and modulate multiple second messenger signaling pathways and fine-tune cellular signaling responses. Many excellent reviews have described the range of binding partners these AKAPs associate with (Wong and Scott, 2004; Carnegie et al., 2009; Welch et al., 2010; Diviani et al., 2011; Sanderson and Dell'Acqua, 2011). In this review, we focus on the structural basis for anchoring of PKA as well as the protein phosphatases that oppose cAMP-mediated signaling.

Structural Basis for PKA Anchoring

Though AKAPs are not typically related to one another on a sequence level, a common unifying feature is their ability to bind the D/D domain of R-subunit dimers through a short (14–18 residues) amphipathic helix, which appears to have arisen relatively early in evolution (Peng et al., 2015). This helix is often one of the few ordered regions, as most AKAPs are intrinsically disordered (Gold et al., 2008). Therefore, this helix serves as a short linear motif (SLiM), which is an emerging concept in cellular signaling that has important implications for protein–protein interactions and drug development (Van Roey et al., 2014). For example, a recent study examining the scaffolding properties of the yeast deubiquitinating enzyme Ubp10 showed that the interplay of SLiMs and intrinsic disorder is essential for facilitating interactions with diverse substrates and binding partners (Reed et al., 2015). SLiMs are often isolated within intrinsically disordered proteins and can serve to facilitate transient interactions which allows a single anchoring protein to interact with a dynamic range of signaling partners (Ren et al., 2008). The first atomic model of an AKAP helix was solved using peptides derived from AKAP79 and Ht31 (AKAP-Lbc) (Newlon et al., 1999). It was obtained using NMR techniques and was solved in complex with the D/D domain (residues 1–44) of RIIα. Subsequently, other structures of the D/D domain in complex with various AKAP-derived helices have been solved using X-ray crystallography (**Figure 1A**; Gold et al., 2006; Kinderman et al., 2006; Sarma et al., 2010). The D/D domain has been shown to adopt an anti-parallel four-helix X-type bundle that forms a platform with a hydrophobic groove. This groove is the basis for a high affinity interaction with the hydrophobic face of amphipathic AKAP helices. The D/D domain is then connected via a flexible linker to two cAMP-binding cassettes per protomer that display cooperative binding of cAMP (Vigil et al., 2004; Zawadzki and Taylor, 2004). Upon binding of cAMP, a conformational change occurs that relieves inhibition of the PKA C-subunit and allows it to phosphorylate nearby substrates. Crystal structures have been solved for the cAMP-binding cassettes in complex with C-subunit or with cAMP (**Figure 1B**; Su et al., 1995; Diller et al., 2001; Wu et al., 2007; Zhang et al., 2012). Together with the known structure of the D/D in complex with AKAP helices, these structures have provided insights at the atomic level about the intricate topology and organization of the different functional elements of PKA holoenzyme.

Yet, there is currently no high-resolution structural information available for the 46 (in mammals) amino acid flexible linker that connects the D/D domain to the pseudosubstrate region that binds the C-subunit and to the tandem cAMP binding cassettes. Therefore, a recent study used single particle electron microscopy studies to examine the structure of an AKAP18γ-PKA holoenzyme complex (Smith et al., 2013). This study revealed that although many crystal structures of RII and C-subunits showed surface contact between each heterodimer of RII and C, the complexes likely occupy a much broader conformational space that is constrained by the length of the linker, yet facilitated by the intrinsic disorder of the linker (**Figure 1C**). This linker-guided conformation sampling may be a mechanism by which PKA preferentially phosphorylates substrates within the same macromolecular complexes upon elevation of cAMP levels. cAMP PDEs have been suggested to form a 'fence' around subcellular pools of elevated cAMP (Baillie et al., 2005). AKAP18γ has been shown to form a complex with PDE4D3 and regulate its activity via PKA phosphorylation (Stefan et al., 2007). In combination with local restraint of PKA conformations by the RII flexible linker, these local PDE fences represent an intriguing scheme by which spatiotemporal specificity may be regulated by macromolecular signaling complexes.

Targeting the PKA/AKAP Interaction for Therapeutics

Since PKA activity modulates a variety of physiological events, such as cardiac remodeling, disrupting the PKA/AKAP interface has been a long-standing area of interest for therapeutics (Troger

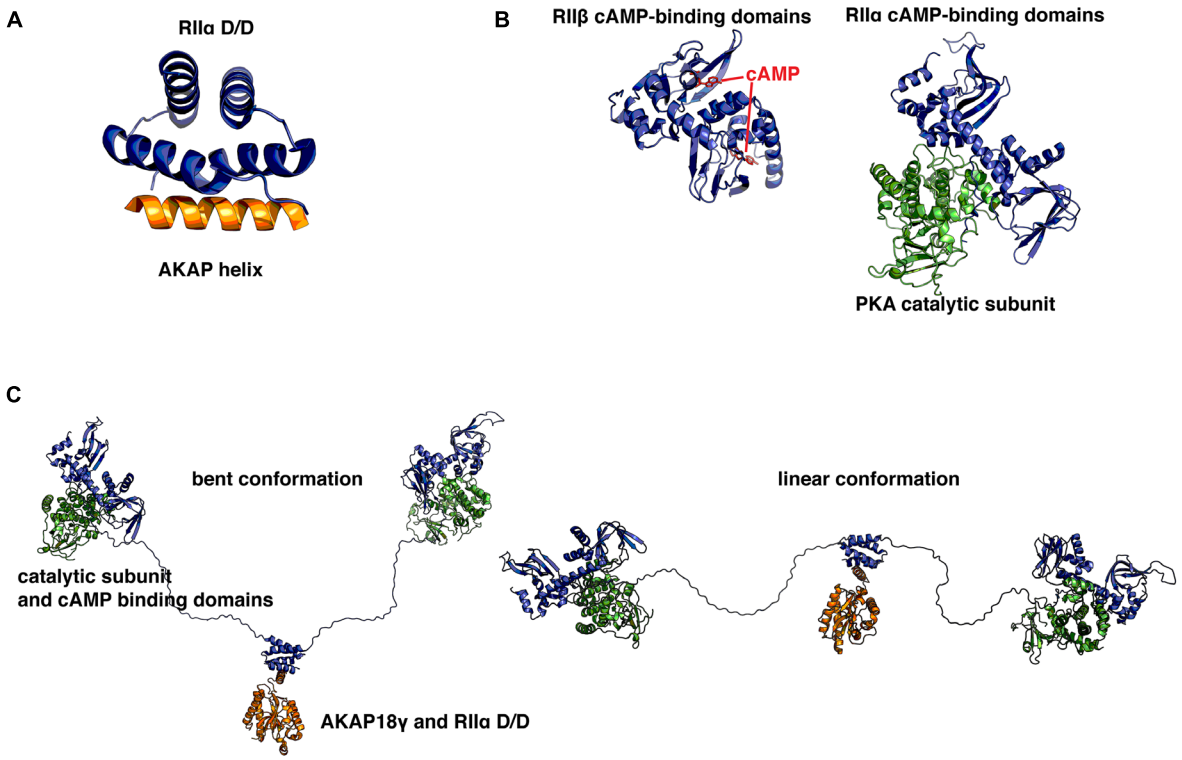

FIGURE 1 | Structural basis for protein kinase A (PKA) holoenzyme formation and anchoring. (A) Crystal structure of the synthetic A-kinase anchoring protein (AKAP) helix AKAP's (orange) in complex with the RIIα docking/dimerization (D/D) domain, residues 1–44 (blue). The AKAP amphipathic helix binds to a hydrophobic groove created by the antiparallel X-type helix bundle of the RII D/D domain. PDB ID: 2IZX. **(B)** Left: RIIβ cAMP-binding cassettes (blue) in complex with cAMP (red). Right: RIIα cAMP binding cassettes in complex with PKA catalytic subunit (green). With cAMP bound at each of two sites, RII releases inhibition of the catalytic subunit. When cAMP is not present, RII presents an inhibitory sequence to the active site, preventing phosphorylation of PKA substrates. PDB IDs: 1CX4, 2WVS. **(C)** A pseudo-atomic model of the PKA holoenzyme in complex with AKAP18γ derived from low-resolution EM data. This illustrates that the PKA holoenzyme has a constrained range of flexibility (~300 Å) provided by AKAPs, allowing the catalytic subunits to be poised near potential substrates. PDB IDs: 3J4Q, 3J4R. Models were prepared using PyMol (Schrödinger).

et al., 2012; **Table 1**). One of the first disruptors of the AKAP/RII interaction is the 24 amino acid peptide Ht31, named after human thyroid clone 31, which was later realized to represent a biologically active segment of the multifunctional scaffolding protein AKAP-Lbc (Carr et al., 1992). The Ht31 peptide has since been lipid modified with a stearol group to increase its membrane permeability for treatment of cell lines and elucidation of anchored PKA signaling events (Vijayaraghavan et al., 1997; Gold et al., 2012). *In silico* approaches have resulted in optimized peptides that mimic the AKAP amphipathic helix and bind to RII or RI with high affinity (Alto et al., 2003). Added to this, structure-based approaches have further increased the specificity of the peptide superAKAPis for RII to the low nanomolar affinity range with a 12,000-fold preference for RII over RI (Gold et al., 2006). Conversely, the RI-anchoring disruptor peptide (RIAD) has been engineered to specifically disrupt RI/AKAP interactions (Carlson et al., 2006). Peptidomimetics have been developed by several groups that mimic amphipathic helix structures and are able to disrupt RI/RII interactions with AKAPs (Schafer et al., 2013; Singh et al., 2014). Recent work has centered on developing stapled AKAP-mimetic peptides that are cell-permeable and have increased stability (Wang et al., 2014, 2015; Kennedy and Scott, 2015). This would increase the utility of these peptides for therapeutic purposes as well as for teasing apart the molecular mechanisms by which AKAPs influence local PKA signaling pathways.

TABLE 1 | Summary of molecules disrupting protein kinase A (PKA) anchoring.

Name	Type/mechanism	Reference
Ht31	Derived from A-kinase anchoring protein (AKAP) helix	Stokka et al. (2006)
SuperAKAPis	Optimized AKAP helix	Gold et al. (2006)
RI-anchoring disruptor peptide (RIAD)	Optimized AKAP helix	Carlson et al. (2006)
STAD peptides	Stapled AKAP helix	Wang et al. (2014, 2015)
RIAD-P3	Peptidomimetic of RIAD	Singh et al. (2014)
Terpyridine derivatives	Peptidomimetic of AKAP helix	Schafer et al. (2013)
Rselects	Engineered R-subunit D/D domain	Gold et al. (2013)
FMP-API-1	Allosteric interaction with R-subunits	Christian et al. (2011)

Since there are numerous AKAPs and only four R-subunit isoforms, any disruptor that relies on an interaction with an R-subunit is by definition non-selective. In order to disrupt a specific AKAP's ability to bind PKA, anchoring disruptors must bind to an AKAP helix with high affinity and recognize the unique structural features of one AKAP helix preferentially. Therefore, a phage-display screening approach, which used immobilized AKAP helices to enrich for phage variants that displayed mutant RII D/D domains, selected variants that exhibit preferential binding to specific AKAPs. These mutant D/D domains are termed Rselects, and have been shown in preliminary work to bind and label AKAPs in a cellular context as well as in to purified proteins (Gold et al., 2013). Further development of these Rselects could lead to high affinity binding variants that could disrupt individual pools of anchored PKA while allowing other anchored PKA signaling events to proceed unperturbed. The potential to isolate spatially constrained post-translational modifications is an important step forward for targeted therapeutics. However, utility of this approach as a cell based means of selectively interrupting particular PKA–AKAP interfaces has yet to be rigorously established.

Small molecule disruptors are another attractive means to pharmacologically target the PKA–AKAP interface. Although these studies are still in their formative stages there have been a few successful attempts at moderate-throughput screening for small molecule AKAP disruptors (Schachterle et al., 2015). Perhaps the most notable example is the development of 3,3′-diamino-4,4′-dihydroxydiphenylmethane (FMP-API-1), a small molecule antagonist that appears to allosterically inhibit the RII–AKAP interaction and activate anchored PKA C-subunit (Christian et al., 2011). Yet, despite extensive characterization of this compound the mechanism of action of FMP-API-1 has yet to be defined. Nonetheless, the future is bright for the discovery and development of cell soluble chemical entities that target PKA–AKAP interfaces.

Protein Phosphatase Anchoring

Classically, protein phosphatases are considered to be responsible for the opposing action to kinases, namely the removal of phosphate groups from serine, threonine, or tyrosine residues. In addition a burgeoning family of pseudokinases and pseudophosphatases are emerging a key players in cell signaling (Reiterer et al., 2014). Protein phosphatases fall into two main classes – serine–threonine phosphatases, and tyrosine phosphatases. While there are 428 serine/threonine kinases, there are only ∼40 serine/threonine phosphatases (Moorhead et al., 2007). This disparity in gene number infers that additional mechanisms come into play as a means to modulate and vary the substrate specificity of these critical regulatory enzymes. Philip and Tricia Cohen were the first to recognize that regulation of protein phosphatases by association with regulatory and targeting subunits is a crucial mechanism to allosterically modulate substrate specificity (Stralfors et al., 1985; Cohen and Cohen, 1989). Subsequently others have shown that most of the three classes of serine/threonine phosphatases are modulated by targeting subunits (Langeberg and Scott, 2015). In this article we focus exclusively on protein phosphatase 1 (PP1) and protein phosphatase 2B (PP2B, or calcineurin), since these ubiquitous phosphatases often oppose the action of PKA and are especially reliant on anchoring for their regulation.

Protein Phosphatase 1 Regulation by Auxiliary Proteins

Protein phosphatase 1 has an important role in a number of physiological processes, notably regulation of glycogen synthesis (Hubbard et al., 1990), nuclear events (Helps et al., 1998), and synaptic long term potentiation (LTP) and long term depression (LTD), (Morishita et al., 2001; Malinow and Malenka, 2002). The latter two events occur through phosphatase opposition of CaMKII and PKA phosphorylation of glutamate receptors at the post-synaptic density. The PP1 catalytic subunit (PP1c) associates with over 200 regulatory subunits, many of which bind via a conserved short linear peptide motif called the RVxF motif (Cohen, 2002; Roy and Cyert, 2009).

Some of these subunits serve primarily to inhibit the catalytic activity, such as the protein Inhibitor 1 (I-1) and dopamine and cAMP-regulated phosphoprotein 32 (DARPP32), (Williams et al., 1986). Notably some of these inhibitors are activated by PKA phosphorylation. Other regulatory subunits contain localization signatures that target PP1 to specific subcellular regions and may or may not also inhibit the enzymatic activity of PP1c. The most recognized examples of these targeting subunits are the myosin phosphatase targeting subunit MYPT1, the G_M regulatory subunit, p53-binding protein 2 (53BP2), and PP1 nuclear targeting subunit (PNUTS). Recent investigation of PNUTS has highlighted several properties shared by many PP1-binding proteins (Choy et al., 2014). First, the RVxF motif serves as a short linear interaction motif (SLiM) and is responsible for the primary interaction. Second, intrinsic disorder in PNUTS facilitates extended contact with PP1 on additional surfaces to fine-tune the phosphatase. Third, binding to these surfaces inhibits activity toward some substrates without physically blocking the active site of the phosphatase (**Figure 2A**).

A number of AKAPs have been shown to interact with PP1c, including AKAP220 (Schillace and Scott, 1999), D-AKAP1 (Steen et al., 2000), and yotiao (Westphal et al., 1999). Likewise, some isoforms of AKAP18 are thought to sequester PP1, although it would appear that this occurs via indirect mechanisms (Singh et al., 2011). All direct PP1–AKAP interfaces utilize some version of the degenerate RVxF motif. D-AKAP1 was suggested to be involved in anchoring PP1 for efficient nuclear envelope reassembly after mitosis (Collas et al., 1999; Steen et al., 2000). AKAP220 has been shown to anchor PP1 through a modified KVxF motif, and this has been proposed to play a role in regulating the activity of glycogen synthase kinase 3β (GSK3β), through modulation of the phosphorylation state of serine 9. Phosphorylation of this residue results in suppression of GSK3β activity (Schillace et al., 2001; Tanji et al., 2002; Whiting et al., 2015). Yotiao, a product of the AKAP9 gene, also contains an RVxF motif, and has been shown to

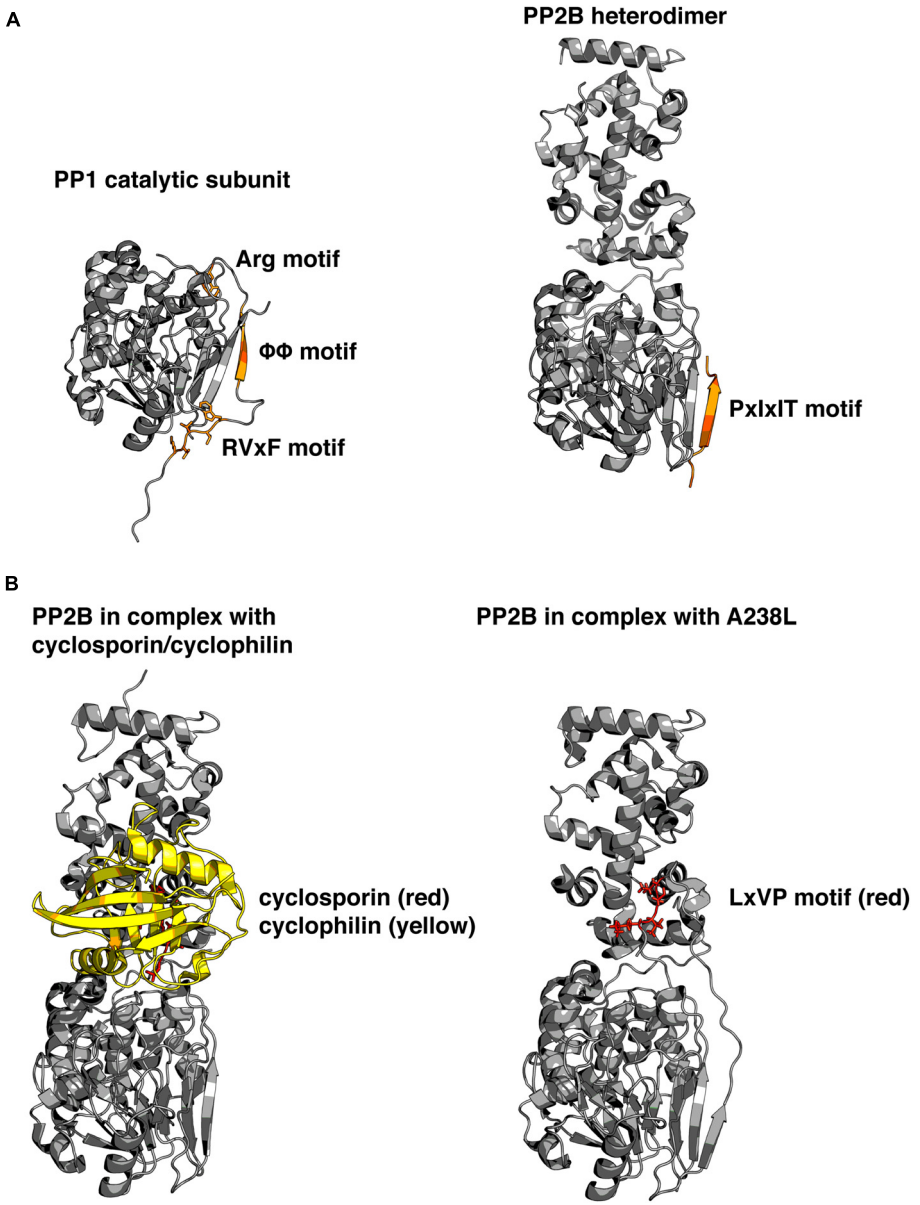

FIGURE 2 | Structural basis for phosphatase regulation and anchoring. (A) Left: PP1 catalytic subunit (gray) in complex with RVxF and auxiliary anchoring motifs from protein phosphatase 1 (PP1) nuclear targeting subunit (PNUTS; orange). Right: PP2B (gray) in complex with PIAIIIT sequence from AKAP79 (orange). Comparison reveals that similar surfaces are used for anchoring, and that multiple motifs can simultaneously interact with varied portions of the molecule. PDB IDs: 4MOY, 3LL8. **(B)** Left: PP2B in complex with cyclosporin (red)/cyclophilin (yellow) complex. Right: PP2B in complex with a viral peptide A238L, containing a PxIxIT motif, as well as an LxVP motif (red). Cyclosporin and LxVP peptides bind to overlapping surfaces on PP2B, formed by both the catalytic and regulatory subunits of PP2B. This surface does not occlude the active site of the phosphatase, yet immunosuppressants are able to allosterically inhibit PP2B activity toward substrates. PDB IDs: 1MF8, 4F0Z. Models were prepared using PyMol (Schrödinger).

regulate the phosphorylation state of NMDA receptors through localization of PP1 (Lin et al., 1998; Westphal et al., 1999). AKAP18 does not appear to interact directly with PP1, but some reports indicate that it binds Inhibitor-1 to promote its phosphorylation by PKA (Singh et al., 2011). The net effect of this later phosphorylation event is to promote local inhibition of PP1c.

PP2B Regulation by Auxiliary Proteins

PP2B, also known as calcineurin, is a broadly expressed obligate heterodimeric protein phosphatase that is activated by calcium and calmodulin (Stewart et al., 1982). Like PP1, PP2B is involved in diverse processes such as synaptic plasticity (Mulkey et al., 1994), glucose metabolism (Hinke et al., 2012), cardiac

signaling (Tandan et al., 2009), and immune responses (Clipstone and Crabtree, 1992). In addition, activation of PP2B can mobilize phosphatase cascade, through dephosphorylation of PP1 regulatory subunits (Mulkey et al., 1994). The catalytic A subunit of PP2B contains an autoinhibitory region that occludes the active site in the absence of calcium. Upon elevation of calcium levels, calcium ions bind directly to the regulatory B subunit, and to calmodulin, which in turn interacts with the autoinhibitory region and allows PP2B to resume catalytic activity toward phosphosubstrates (Li et al., 2011). Because calcium transients often envelop the whole cell rather than occurring locally, regulation of PP2B's activity toward substrates is accomplished primarily through protein–protein interactions. The best-known PP2B substrate is the nuclear factor of activated T-cells (NFAT) family. These transcription factors contain phosphoserine-rich regions, and when dephosphorylated, dimerize and translocate to the nucleus, where they are responsible for controlling a range of transcriptional responses such as inflammation in response to immune system signaling (Li et al., 2012). The common immunosuppressants FK506 and cyclosporine target PP2B and have their primary effect through inhibition of NFAT signaling (Liu et al., 1991).

Not only is NFAT a typical PP2B substrate, it also contains two SLiMs, which are typical of PP2B interacting proteins – the PxIxIT motif, and the LxVP motif (Roy et al., 2007; Rodriguez et al., 2009). The PxIxIT motif forms a beta strand that binds to a hydrophobic groove formed by a beta sheet on the PP2B A subunit (Li et al., 2007). This surface of the PP2B A subunit is analogous to the region of PP1 which interacts with the RVxF motif (**Figure 2A**). Proteins that contain PxIxIT motifs include NFAT, regulator of calcineurin 1 (RCAN1; Mehta et al., 2009), TWIK-related spinal cord potassium channel (TRESK; Roy and Cyert, 2009), and notably, AKAP79/150 (Dell'Acqua et al., 2002). The LxVP motif is a degenerate sequence that binds to the interface of the A and B subunits of PP2B, and only binds to activated calcineurin (Rodriguez et al., 2009). It has been challenging to describe a consensus LxVP sequence. Therefore, many LxVP motifs have been identified without originally being aware of their identity. The first LxVP motif to be described was that of the RII subunit by Blumenthal et al. (1986), although it was not recognized as a conserved binding mode until it was found in NFAT. Many substrates of PP2B contain an LxVP motif, and it has been suggested that all efficient substrates contain some type of sequence that interacts with the LxVP binding region on PP2B (Grigoriu et al., 2013). The characterization of multiple SLiMs that interact with various surfaces of PP2B parallels that of PP1, and suggests that other mechanisms may also be in common such as fine-tuning the location and activity of PP2B through a combination of disorder and SLiMs. Recently, a structure of PP2B in complex with a viral inhibitor peptide from African swine fever revealed the binding site for the LxVP motif in atomic level detail (Grigoriu et al., 2013). This crystal structure reveals that the leucine residue occupies a pocket formed by two aromatic residues, and when these are mutated to alanine residues they no longer interact with the LxVP motif. In addition, this binding site overlaps with the binding sites for cyclosporine and FK506 complexes (**Figure 2B**). However, no structure has been solved of the PP2B heterodimer bound to calmodulin in the fully active state, so the question of how LxVP motifs are able to impact PP2B activity remains unclear.

Some AKAPs have been shown to bind PP2B, such as the aforementioned AKAP79, and mAKAP (Li et al., 2010). In addition, the AKAP gravin has been suggested to be in the same complex as PP2B and beta-adrenergic receptors, however, evidence for a direct interaction is not immediately apparent (Shih et al., 1999). The mAKAP interaction has been mapped to the residues 1286–1345 in the mAKAPα splice variant. However, this region does not contain an easily identifiable PxIxIT or LxVP sequence. Loss of mAKAP-PP2B binding was shown to result in reduced cardiac myocyte hypertrophy in response to norepinephrine, as well decreased atrial natriuretic factor expression. Interestingly, the pool of PP2B bound to mAKAP appeared to be active, and required to dephosphorylate NFAT efficiently in response to phenylephrine treatment. Formation of the PP2B/mAKAP complex was enhanced *in vitro* by calcium/calmodulin, suggesting that the interaction may occur via a similar mechanism to the LxVP motif (Li et al., 2010).

A-kinase anchoring protein 79 is perhaps the best characterized AKAP, and its interaction with PP2B has been extensively investigated. Although original studies suggested an interaction site was restricted to the N-terminal third of AKAP79 (Coghlan et al., 1995), later work described the primary site of interaction as being a PxIxIT motif from residues 337-343 (Dell'Acqua et al., 2002; Oliveria et al., 2007). Use of a transgenic mouse model in which AKAP79 lacks this region, known as the AKAP79ΔPIX mouse, has revealed that AKAP79-anchored PP2B is required for NMDA-dependent hippocampal long-term depression and NFAT signaling in neurons (Oliveria et al., 2007; Sanderson et al., 2012). Intriguingly, the AKAP79ΔPIX mouse shows improved insulin sensitivity, indicating that this interaction may be a possible therapeutic target for Type II diabetes (Hinke et al., 2012).

Because of the importance of the AKAP79–PP2B interaction, much emphasis has been placed on understanding the structural basis of this interaction. Native mass spectrometry and biochemical approaches have suggested that there is an additional interaction site for PP2B between residues 1–153 of AKAP79 that is dependent on calcium/calmodulin (Gold et al., 2011). Although

TABLE 2 | Summary of molecules disrupting protein phosphatase-2B (PP2B) anchoring.

Name	Type/mechanism	Reference
Cyclosporine	Immunophilin complex, competes with LxVP	Liu et al. (1991)
FK506	Immunophilin complex, competes with LxVP	Liu et al. (1991)
VIVIT	Optimized PxIxIT peptide	Aramburu et al. (1999)
INCA-6	Allosteric disruptor of PxIxIT	Kang et al. (2005)
LxVP peptide	LxVP peptide derived from NFAT	Escolano et al. (2014)
PxIxIT disruptors from ZINC library	Organic compounds, direct competition for PxIxIT	Matsoukas et al. (2015)

a crystal structure of PP2B in complex with a synthetic PxIxIT motif was solved in 2007 (Li et al., 2007), the first structure of PP2B bound to a natural PxIxIT motif was that of AKAP79 (Li et al., 2012). This structure matched closely with the previously solved structure, in that crystal packing is such that each PIAIIIT sequence contacts two PP2B A subunits along the PxIxIT binding region. This, along with native mass spectrometry approaches, raises the question of whether AKAP79 is capable of binding two PP2B molecules simultaneously.

Because of PP2B's importance in a range of physiological contexts, there is great interest in developing disruptors that target specific PP2B anchoring proteins (**Table 2**). One of the first targeted approaches resulted in an optimized high-affinity PxIxIT motif called the VIVIT peptide (Aramburu et al., 1999), which is the aforementioned synthetic peptide that was co-crystallized with PP2B. In addition, fluorescence polarization screens for small molecules that disrupt binding to the PxIxIT motif have yielded a potential candidate known as INCA-6 that is able to inhibit PP2B-NFAT signaling with similar potency to cyclosporine and FK506, but through an alternate mechanism (Kang et al., 2005). Recently, an approach disrupting the LxVP interaction in macrophages through lentiviral expression of an LxVP peptide was shown to reduce inflammation and confer resistance to arthritis and contact hypersensitivity (Escolano et al., 2014). Understanding the molecular basis for PP2B anchoring has led to potential for therapeutics and the realization that primary and secondary binding sites may both be targeted for diverse physiological effect. Because the AKAP79–PP2B interaction is important in many processes, specifically targeting this interaction may be of great promise.

Conclusion

Protein kinase A phosphorylation events and the phosphatases that oppose them are tightly regulated by anchoring proteins. Recently, the use of new and sophisticated biochemical, biophysical, and structural techniques have forged two important concepts. First, the combination of SLiMs and intrinsic disorder allow anchoring proteins to allosterically and spatially control the range and specificity of phospho-signaling. Second, AKAPs are not just static anchors, but are conformationally and compositionally flexible. This allows them to adapt to a varied and continually changing cellular signaling environment. A recent paper characterizing binding partners of the AKAP ezrin by quantitative mass spectrometry revealed that conformational switches in ezrin are accompanied by changes in the complement of enzymes present in the complex (Viswanatha et al., 2013). This may well prove to be the case for many AKAPs allowing them to perform cell type specific roles. Moreover, the concept of AKAPs as conformational switches could account for how the same anchoring protein can simultaneously perform distinct functions at multiple locations within a single cell.

These new biological insights have been demonstrated by using hybrid structural techniques such as x-ray crystallography, NMR, hydrogen/deuterium exchange experiments and crosslinking/mass spectrometry (Burns-Hamuro et al., 2005; Gold et al., 2011; Choy et al., 2014). The advent of direct electron detectors for cryo-electron microscopy has increased attainable resolutions (Campbell et al., 2012), and will likely contribute to increased structural understanding of these flexible multi-protein complexes. In addition, computational advances in understanding heterogeneous cryo-EM samples will also advance our knowledge of multiple conformational states (Behrmann et al., 2015). Already, negative-stain approaches such as random conical tilt (RCT) experiments are allowing researchers to understand structural heterogeneity in protein complexes (Veesler et al., 2014). Combining these approaches with biosensors for enzymatic activity (Mehta and Zhang, 2014; Mehta et al., 2014) will provide a more comprehensive picture of how the structural properties of anchored kinase and phosphatase complexes are able to influence local signaling in a cellular context. Finally, as exemplified by a recent structure-guided pharmacophore screen for inhibitors of PP2B anchoring, atomic resolution structural insights will guide design of small molecules that target anchoring protein interactions in the context of SLiMs and intrinsic disorder (Matsoukas et al., 2015).

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PN, JS: Wrote, edited, and approved final version of the manuscript and figures.

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Impact of kinase activating and inactivating patient mutations on binary PKA interactions

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The second messenger molecule cAMP links extracellular signals to intracellular responses. The main cellular cAMP effector is the compartmentalized protein kinase A (PKA). Upon receptor initiated cAMP-mobilization, PKA regulatory subunits (R) bind cAMP thereby triggering dissociation and activation of bound PKA catalytic subunits (PKAc). Mutations in PKAc or RIa subunits manipulate PKA dynamics and activities which contribute to specific disease patterns. Mutations activating cAMP/PKA signaling contribute to carcinogenesis or hormone excess, while inactivating mutations cause hormone deficiency or resistance. Here we extended the application spectrum of a Protein-fragment Complementation Assay based on the *Renilla* Luciferase to determine binary protein:protein interactions (PPIs) of the PKA network. We compared time- and dose-dependent influences of cAMP-elevation on mutually exclusive PPIs of PKAc with the phosphotransferase inhibiting RIIb and RIa subunits and the protein kinase inhibitor peptide (PKI). We analyzed PKA dynamics following integration of patient mutations into PKAc and RIa. We observed that oncogenic modifications of PKAc(L206R) and RIa(Δ184-236) as well as rare disease mutations in RIa(R368X) affect complex formation of PKA and its responsiveness to cAMP elevation. With the cell-based PKA PPI reporter platform we precisely quantified the mechanistic details how inhibitory PKA interactions and defined patient mutations contribute to PKA functions.

Keywords: molecular interactions, patient mutations, cAMP-dependent protein kinase A, protein-fragment complementation assay, GPCR, Carney complex, Acrodysostosis

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Introduction

Small molecules represent the vast majority of cellular components. They are either substrates or products of numerous biochemical reactions. By acting as hormones or ligands, small molecules control protein functions, and molecular interactions which affect signal transmission. Molecular interactions of second messenger molecules with distinct proteins alter protein:protein interactions (PPIs) and signal propagation (Clapham, 2007; Scott and Pawson, 2009; Taylor et al., 2012; Li et al., 2013). The second messenger concept explains the dynamic wiring of membrane receptor pathways to a vast array of intracellular PPIs. One prominent example is the canonical second messenger molecule cyclic AMP (cAMP). It is an evolutionary conserved transmitter of membrane receptor originating input signals. A large number of G protein-coupled receptor (GPCR) cascade utilizes cAMP as intracellular second messenger (Neves et al., 2002; Pierce et al., 2002; Lefkowitz, 2007; O'Hayre et al., 2014). cAMP molecules dynamically bind to and regulate activities of its key effector

kinase, the cAMP-dependent protein kinase A (PKA). The mechanistic details of cAMP-PKA activation is a textbook paradigm for allostery and small molecule:protein interactions (Taylor et al., 2012; Zhang et al., 2012). In its inactive state the PKA holoenzyme consists of a PKA regulatory subunit homodimer (R; type Ia, Ib, IIa, IIb) which inhibits the phosphotransferase activity of two PKA catalytic subunits (PKAc) through binding. Upon selective GPCR activation cAMP production through adenylyl cyclase (AC) activities is initiated; on the contrary, phosphodiesterase (PDE) control cAMP degradation (Dessauer, 2009; Houslay, 2010). cAMP binding to spatially compartmentalized type I (RI_2:$PKAc_2$) or type II (RII_2:$PKAc_2$) PKA holoenzymes causes a conformational change, hence dissociation, and activation of phosphotransferase activities of PKAc. As a consequence PKAc phosphorylates compartmentalized substrates which affects enzyme activities, PPI, localization, or protein abundance (Scott and Pawson, 2009; Lignitto et al., 2011, 2013; Taylor et al., 2012; Bachmann et al., 2013; Scott et al., 2013; Filteau et al., 2015). To guarantee substrate specificity PKA R subunits bind A kinase-anchoring proteins (AKAPs) which organize the subcellular targeting of PKA activities (Wong and Scott, 2004; Skroblin et al., 2010; Scott et al., 2013). To desensitize PKA phosphotransferase activities nuclear PKAc subunits are specifically inhibited through binding to the protein kinase inhibitor peptide (PKI) (Walsh et al., 1971; Scott et al., 1985; Dalton and Dewey, 2006). Deregulation of cAMP/PKA functions contribute to different diseases. Inactivating *PRKAR1A* gene (RIa) and activating *PRKACA* gene (PKAc) mutations/fusions provoke deregulated PKAc activities. As examples, mutations in RIa and PKAc account for the generation of endocrine tumors, cortisol-producing adenomas, and chimeric fusions with PKAc have been linked to the manifestation of hepatocellular carcinoma (Groussin et al., 2002; Meoli et al., 2008; Stratakis, 2013; Beuschlein et al., 2014; Calebiro et al., 2014; Cao et al., 2014; Di Dalmazi et al., 2014; Espiard et al., 2014; Goh et al., 2014; Honeyman et al., 2014; Sato et al., 2014; Cheung et al., 2015; Zilbermint and Stratakis, 2015). However, specific *PRKAR1A* gene (RIa) mutations impair cAMP-dependent PKA activation and cause hormone resistance as observed in the rare disease Acrodysostosis (a form of skeletal dysplasia) (Linglart et al., 2011; Assié, 2012; Silve et al., 2012). Here we tested the impact of functionally different PKA mutations on mutually exclusive molecular protein interactions using an extended PPI reporter platform based on the *Renilla* luciferase protein-fragment complementation assay (*R*luc PCA) (Stefan et al., 2007; Röck et al., 2015). We show that the presented *R*luc PCA platform—which is based on binary PKA network interactions—can be applied to analyze the consequences of patient mutations and upstream receptor activities on defined PKA PPIs directly in living cells.

Materials and Methods

Cell Culture and Antibodies

HEK293 and U2OS cells were grown in DMEM supplemented with 10% FBS. Transient transfections were performed with Transfectin reagent (Biorad). Cells were treated with Forskolin (Biaffin) or Isoproterenol (Sigma) with indicated concentrations and for the indicated time frames. As primary antibody we used mouse anti-PKAc (BD Bioscience, #610981).

Constructs

The *R*luc PCA based hybrid proteins RIIb-F[1] and PKAc-F[2] have been designed as previously described (Stefan et al., 2007). *R*luc PCA fusions with PKI and RIa have been generated using an analogous cloning approach. Following PCR amplification of the of the human RIa (alpha) gene (protein accession number: NP_002725.1) and PKI alpha gene (protein accession number: AAA72716; addgene plasmid # 45066) (Day et al., 1989) we fused them C-terminally with either -F[1] or -F[2] of the *R*luc PCA. PKA subunits and PKI were subcloned into the 5′ end the 10aa linker $(GGGGS)_2$ and the *R*luc PCA fragments (pcDNA3.1 backbone vector). Site directed mutagenesis have been performed to generate the PKAc amino acid (aa) mutations G187V and L206R in the *PRKACA* gene and the RIa aa mutations R368X (X stands for the stop codon of the patient mutation; we generated the corresponding RIa truncation) in the *PRKAR1A* gene. In addition we deleted the corresponding nucleotide sequence from the aa 184–236 in the *PRKAR1A* gene.

Renilla Luciferase PCA of Detached Cells

HEK293 cells were grown in DMEM supplemented with 10% FBS. We transiently overexpressed indicated versions of the *R*luc PCA based reporter constructs in 24 or 12 well-plate formats. In case of the HEK293 cells we exchanged growth medium and resuspended cells in PBS 24 or 48 h post-transfection. Cell suspensions were transferred to 96-well plates and subjected to luminescence analysis using the LMax™-II-384 luminometer (Molecular Devices). *R*luc luminescence signals were integrated for 10 s.

Renilla Luciferase PCA of Attached Cells

We seeded U2OS cells into white-walled 96 well-plates with transparent bottom. We transiently overexpressed indicated versions of the *R*luc PCA based reporter constructs. 24 or 48 h post-transfection we exchanged growth medium with PBS. We performed luminescence analyses of attached U2OS cells at room temperature using the LMax™-II-384 luminometer (Molecular Devices). Time dependent changes of the *R*luc luminescence signals were integrated in 3-s intervals following addition of the *R*luc substrate benzyl-coelenterazine (5 μM; Nanolight) with or without Isoproterenol (final concentration 10 μM) (Stefan et al., 2007). Following determination of the *R*luc PCA signals we normalized the resulting signals on the control experiment (data points without Isoproterenol treatment).

Results

The purpose of our study was to extend the application spectrum of a PPI reporter platform (*R*luc PCA) to quantify mutually exclusive PKA interactions directly in living cells. It is the simplicity and sensitivity of the genetically encoded *R*luc PCA which enables different kinds of perturbation studies to unveil

mechanistic details of PKA dynamics. The *R*luc PCA facilitates the precise quantification of cellular PPI in an appropriate *in vivo* setting (Röck et al., 2015). In **Figure 1** we illustrate the *R*luc PCA design principle to analyze binary PPI of PKAc subunits. PPIs of R subunits and PKI with PKAc trigger folding and complementation of appended PCA-fragments. Following addition of the *R*luc substrate benzyl-coelenterazine *R*luc PCA emitted bioluminescence signals reflect quantifiable cellular PPI. Elevations of cAMP levels and defined patient mutations in RIa or PKAc affect PPI between the kinase inhibitory proteins (R/PKI) and PKAc which is indicated through alterations of bioluminescence signals. For the cloning of the PPI reporter constructs we fused the carboxy terminus of the PKA subunits (PKAc, RIIb, RIa) and PKI to complementary fragments of the *R*luc PCA (either -F[1] or -F[2]). In all cases the bait/prey proteins and the *R*luc PCA fragments are separated by an interjacent 10 amino acid (aa) linker with the sequence $(GGGGS)_2$. It is a standard linker we previously showed to function using the dynamic RIIb:PKAc *R*luc PCA reporter (Stefan et al., 2007). We started with analyzes of time and dose dependent effects of distinct cAMP elevating agents on binary PKA interactions before we initiated investigations of the correlation of cAMP elevation, PPI and patient mutations. In our first tests we transiently over-expressed indicated *R*luc PCA pairs in HEK293 cells. We detected significant *R*luc PCA originating bioluminescence signals of existing PPI between RIIb:RIIb, RIIb:PKAc, RIa:PKAc, and PKI:PKAc. No significant bioluminescence signals with R subunit:PKI combinations were detectable (**Figure 2A**). These data confirm the specificity of the assay and the suitability to analyze PKAc complexes with differentially localized phosphotransferase inhibitors. PKA R subunits sense and bind cAMP. We tested different modes of cAMP elevation. We used the general cAMP elevating agent Forskolin to maximally activate AC mediated cAMP production. In addition, we activated a prototypical GPCR which is coupled to cAMP production, the beta-2 adrenergic receptor (β_2AR), respectively. In this context it needs to be considered that distinct mechanism have been described how β_2AR switch their G protein coupling from stimulatory G proteins to inhibitory ones (Baillie et al., 2003). We used HEK293 cells stably expressing β_2AR. Activation of different βAR subtypes are related to proliferation, cardiac function, and memory and learning (Daaka et al., 1997; Wong and Scott, 2004; Zhang et al., 2005; Thaker et al., 2006; Lefkowitz, 2007). As predicted, we observed that both Forskolin exposure and the activation of β_2AR (with Isoproterenol) triggered R:PKAc complex dissociation. We observed no major changes of PPI between PKI:PKAc and RIIb homodimers following 15 min of cAMP elevation (**Figure 2B**). These results indicate that discrete GPCR/cAMP activation induces dissociation of heterodimeric RIa:PKAc and RIIb:PKAc complexes which underlines the suitability of *R*luc PCA to characterize transient and small molecule regulated PPI *in vivo*.

In order to evaluate dose dependent changes of type I and type II PKA dynamics we treated HEK293-β_2AR cells with increasing concentrations of the non-selective beta adrenergic agonist Isoproterenol. We observed that similar half maximal effective concentrations of Isoproterenol induce PKA type I and type II activation in the used stable HEK293 cell line (**Figure 3A**). As predicted, we observed no major impact of β_2AR-mediated cAMP elevation on RIIb homodimers and the PKI:PKAc complex. Next, we wanted to track time dependent changes of the studied PPIs. We switched to the well-attached human osteosarcoma cell line U2OS. We set out to monitor GPCR mediated changes of PPI in the first 4 min of agonist exposure of attached U2OS cells expressing endogenous β_2AR directly in the 96-multiwell plate (Stefan et al., 2007). Following transient overexpression of the four *R*luc PCA pairs (RIa:PKAc, RIIb:PKAc, RIIb:RIIb, PKI:PKAc) and full length *R*luc we observed that Isoproterenol immediately induced the dissociation of heterodimeric type I and type II PKA complexes with similar kinetics. Again, β_2AR activation for 4 min has no major effect on the RIIb dimer and on PKAc:PKI interactions (**Figure 3B**). The results from the real-time measurements of PKA kinetics using whole cell populations underline the suitability of the *R*luc PCA to compare subtype-receptor controlled PPI dynamics in distinct cell settings.

Next, we analyzed different kind of patient mutations of PKA subunits and their impact on binary PKA network interactions. We selected patient mutations of PKAc and RIa which contribute to disease etiology and progression (Carney et al., 1985; Horvath et al., 2010; Stratakis, 2013; Beuschlein et al., 2014; Calebiro et al., 2014; Cao et al., 2014; Di Dalmazi et al., 2014; Espiard et al., 2014; Goh et al., 2014; Honeyman et al., 2014; Salpea and Stratakis, 2014; Sato et al., 2014; Cheung et al., 2015; Zilbermint and Stratakis, 2015). We started with analyses of PKAc mutations and their effect on compartmentalized PPI of PKAc with R subunits (type I and II) and PKI (**Figure 4A**). In addition we investigated the impact of cAMP alterations on wild type and mutant PKA complexes. We introduced two patient mutations into the PKAc-F[2] *R*luc PCA constructs. It has been described that the PKAc mutation G187V diminished, whereas the hotspot mutation L206R of PKAc in

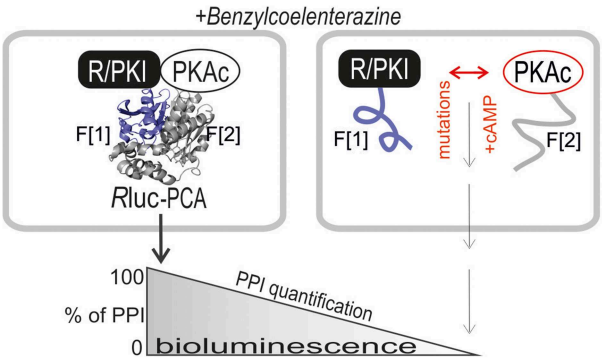

FIGURE 1 | *R*luc PCA design principle. The PKA subunits PKAc, RIa, and RIIb have been C-terminally tagged with *R*luc PCA fragments (-F[1], -F[2]). PPIs trigger folding and complementation of appended PCA-fragments. The emitted bioluminescence signal is a quantitative description of cellular PPIs. Elevations of cAMP levels and integration of distinct patient mutations in RIa and PKAc affect protein complex formation. Following addition of the *R*luc substrate benzyl-coelenterazine *R*luc PCA emitted bioluminescence signals reflect quantifiable PPI.

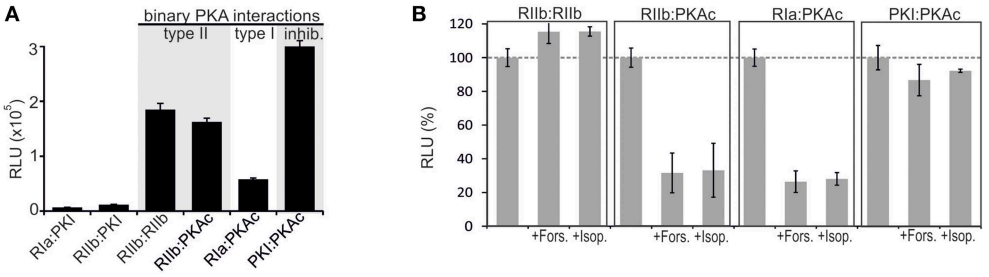

FIGURE 2 | Impact of receptor activities and kinase mutations on PPIs of PKA in human cell lines. (A) Co-transfection of HEK293 cells with indicated *R*luc PCA pairs followed by *R*luc PCA analyses have been performed. The amount of PKI hybrid constructs have been bisected for cell transfections (representative of $n = 5$; ±SD from triplicates). **(B)** Indicated combinations of *R*luc PCA tagged components of the binary PKA network have been subjected to *R*luc PCA measurements. The effect of Forskolin (50 μM; 15 min) and Isoproterenol (10 μM; 15 min) on complex formation has been determined (average from at least $n = 3$ independent experiments; ±SEM). Basal PPIs of RIIb:RIIb, RIIb:PKAc, RIa:PKAc, and PKI:PKAc have been set to 100%. The percentage change of PPI is shown.

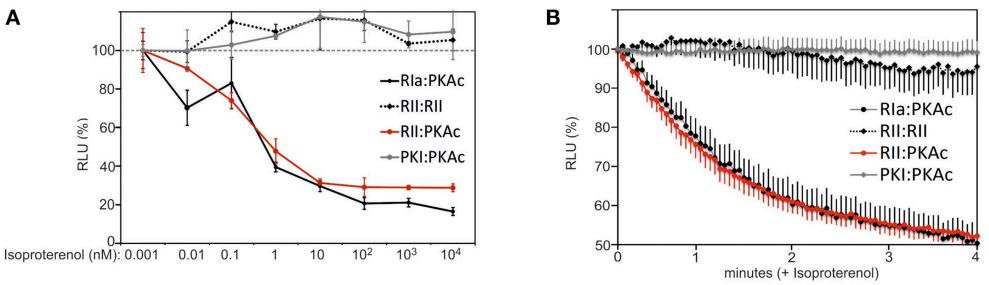

FIGURE 3 | Time and dose dependent impact of β_2AR activities on PPIs. (A) HEK293-β_2AR cells transiently expressing indicated *R*luc PCA pairs have been exposed to dose-dependent Isoproterenol treatments (15 min). PPIs have been determined using the *R*luc PCA as read out (representative experiment from $n = 3$; ±SD from triplicates). PPIs of RIIb:RIIb, RIIb:PKAc, RIa:PKAc, and PKI:PKAc upon 1 pM Isoproterenol exposure have been set to 100%. **(B)** U2OS cells transiently expressing indicated *R*luc PCA pairs have been exposed to time-dependent Isoproterenol treatments (10 μM). Time dependent changes of the *R*luc luminescence signals were integrated for 3 s intervals following addition of the *R*luc substrate benzyl-coelenterazine (5 μM) with or without Isoproterenol. Following determination of *R*luc PCA signals we normalized the data points on the experiments with the full length *R*luc (±Isoproterenol) and on the control experiment (without Isoproterenol). Shown is a representative experiment of $n = 3$; ±SEM.

adrenal Cushing's syndrome promoted PKAc activity (Soberg et al., 2012; Cao et al., 2014; Di Dalmazi et al., 2014; Sato et al., 2014). Following co-expression of PCA pairs in HEK293 cells we observed a significant reduction of complex formation of PKAc mutants with all three PKA-inhibitory proteins. To our surprise also the interaction with PKI was significantly reduced. cAMP binding to the heterodimeric wild type PKA reporter (RIa:PKAc, RIIb:PKAc) caused complex dissociation (Stefan et al., 2007, 2011; Bachmann et al., 2013). Only in the case of RIIb:PKAc-G187V the remaining PPI was still responsive to general cAMP elevation using Forskolin (**Figure 4B**). The expression level of mutated PKAc-F[2] was slightly reduced (**Figure 4C**). These data underline that the patient mutation PKAc-L206R and the mutant PKAc-G187V significantly reduce complex formation with RIa, RIIb, and PKI. However, we show that RIIb has still the potential to bind to the catalytic inactive PKAc-G187V mutant. No remaining PPIs of inhibitory R/PKI proteins with the critical PKAc-L206R mutant were detected.

Following analyses of PKAc patient mutations we tested the consequence of mutating RIa on complex formation with PKAc (**Figure 5A**). In the RIa R subunit a variety of mutations have been found which lead to distinct human diseases (**Figure 5A**) (Horvath et al., 2010). We decided to test a patient mutation which causes the expression of a RIa protein lacking the aa sequence encoded by exon 6 (R1a Δ184-236) which contribute to the multiple neoplasia and lentiginosis syndrome Carney complex (Groussin et al., 2002; Meoli et al., 2008). It is a disease described as "the complex of myxomas, spotty skin pigmentation and endocrine over activity" (Carney et al., 1985; Veugelers et al., 2004; Horvath et al., 2010; Salpea and Stratakis, 2014). In case of the Carney complex a collection of RIa mutations lead to PKA activation. As second mutation we introduced one stop codon at the position R368X in RIa which results in a truncation of the last 14 amino acids of the RIa protein. This heterozygous *de novo* mutation has been identified in patients with Acrodysostosis (a rare form of skeletal dysplasia) which is caused through a resistance to several hormones (Linglart et al., 2011). In case of Acrodysostosis RIa mutations lead to PKA inactivation.

To quantitatively evaluate the impact of these two patient mutations on PPI we transfected the same DNA amount of

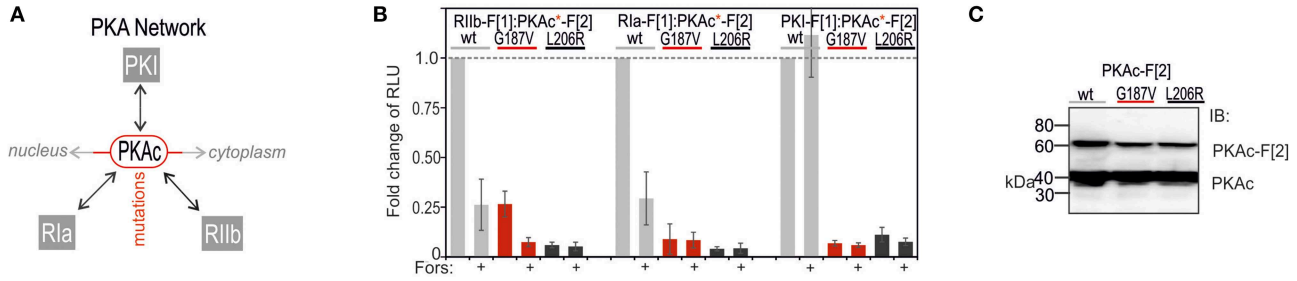

FIGURE 4 | Impact of PKAc mutations and cAMP elevation on PPIs emanating from PKAc. (A) cAMP elevation leads to PKA activation: cAMP binds to R subunits and triggers dissociation of active PKAc subunits which phosphorylate substrates in cytoplasm and nucleus. PKI inactivates nuclear PKI complexes. PKAc mutations deregulate the binary PKA network. **(B)** Following site directed mutagenesis of PKAc-F[2], combinations of wild type and mutant *R*luc PCA tagged PKA subunits have been subjected to *R*luc PCA measurements. The effect of forskolin (50 μM; 10 min) on PKA complex formation has been determined ($n = 4$ independent experiments, ±SEM). **(C)** Immunoblotting shows expression levels of endogenous PKAc and F[2]-tagged PKAc variants.

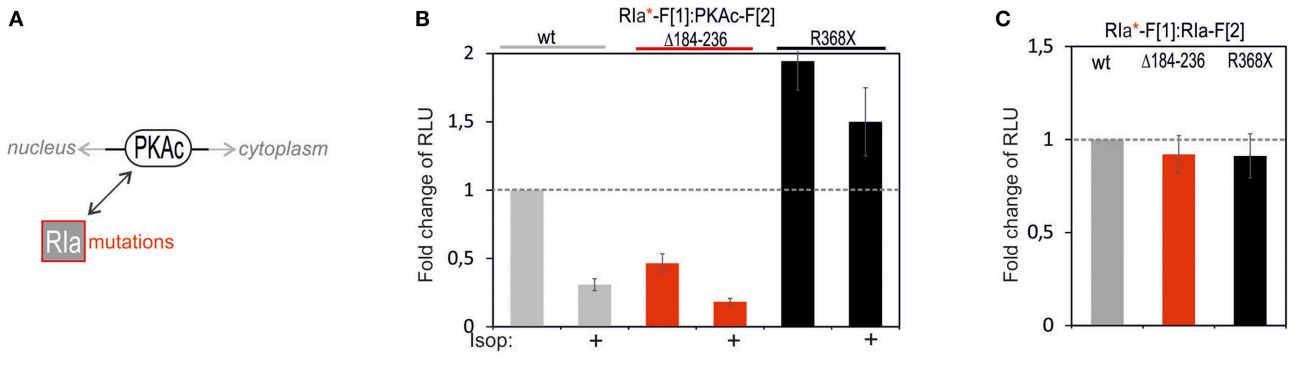

FIGURE 5 | Impact of RIa mutations and cAMP elevation on PKA type I complexes. (A) Ligand-mediated activation of GPCR pathways linked to cAMP production lead to activation of PKA holoenzymes. cAMP binds to RIa subunits and triggers dissociation of active PKAc subunits which phosphorylate substrates in cytoplasm and nucleus. RIa mutations deregulate RIa:PKAc dynamics. **(B)** Following indicated modifications of RIa-F[1] sequences, combinations of wild type and mutant *R*luc PCA tagged PKA subunits have been subjected to *R*luc PCA measurements. The effect of isoproterenol (1 μM, 10 min; 48 h transient PCA reporter expression) on PKA complex formation has been determined (at least $n = 3$ independent experiments, ±SEM). **(C)** Analyses of *R*luc PCA based homodimer formations of wild type and mutated RIa have been performed (at least $n = 3$ independent experiments, ±SEM).

indicated *R*luc PCA constructs into HEK293 cells. We observed that the R1a Δ184-236 deletion in RIa-F[1] reduced basal PPI with PKAc-F[2] to less than 50% (**Figure 5B**). No major changes of R subunit dimerization of R1a Δ184-236:RIa was detectable (**Figure 5C**). Upon cAMP elevation with Isoproterenol the remaining PKA complex can be activated to a similar extend as the wild type PKA type I complex. In parallel we analyzed the truncated RIa protein simulating the stop codon at position R368 of RIa (=14aa truncation). First, we observed elevated levels of inactive RIa:PKAc complexes. Second, upon cAMP elevation with Isoproterenol we recorded less PKA dynamics (**Figure 5B**). No major changes of R subunit dimerization of RIa-R368X:RIa was detectable (**Figure 5C**). Apparently the RIa-R368X mutated PKA type I complex is less responsive to cAMP elevations. The R368X mutation impairs the cAMP-dependent dissociation of RIa from PKAc. These data underline that specific patient mutations in RIa have different consequences on both the PKA holoenzyme complex formation and on cAMP-induced PKA activation.

Overall our data indicate that the presented *R*luc PCA PKA reporter platform is suitable to quantify dynamic and mutually exclusive protein complex assembly and disassembly of the binary PKA network, which is controlled through oscillations of cAMP levels. In addition, we clearly demonstrate with this proof of concept study that observed PPI dynamics of wild type and mutated PKA complexes support findings and hypotheses of disease relevant PKA associations.

Discussion

The ability to precisely record PPI dynamics of the binary PKA network is central for understanding compartmentalized cAMP signal transmission. We present a highly sensitive and easy applicable reporter platform to systematically quantify, map, and manipulate PKA protein complex formation in real time. We show that second messenger initiated changes of PKA reflect connections to upstream located receptor pathways. The implementation of the PPI reporter allows

for more accurate and quantitative specification of receptor-effector relationships directly in the preferred cell type. In light of disease relevant PKAc variations (mutations, fusions, decontrolled upstream pathways) application of the PKA *R*luc PCA platform offers the possibility to systematically test different means of kinase perturbations using the available biosensor toolbox. We quantified mutually exclusive PKAc interactions which account for different spatially controlled cell functions. We showed that the PPI reporter implementation simplified the determination of consequences of PPI relevant patient mutations. Such comparative *in vivo* analyses will facilitate the decision how to interfere with deregulated PKA functions. We showed that two PKAc mutations display differences in the binary PPI pattern. We revealed that cAMP-sensing R subunits and PKI form no PPIs with PKAc-L206R, a hotspot mutation of PKAc in adrenal Cushing's syndrome which promotes PKAc activity (Cao et al., 2014; Di Dalmazi et al., 2014). First, this implements that PKAc-L206R cannot be compartmentalized through R/PKI and indirect AKAP interactions. Second, PKAc-L206R operates independently of cAMP. Therefore, alternative means need to be prosecuted to reduce the deregulated phosphotransferase activity. Besides inhibitory small molecules we believe that targeting the stability of PKAc-L206R is one alternative.

We also tested two patient mutations of RIa which are related to two different diseases showing opposite effects on PKA activity. We demonstrated that the Carney complex relevant R1a-Δ184-236 mutation had a major impact on PKA complex formation when compared to the wild type PKA holoenzyme type I. Our data showed that R1a-Δ184-236 has less affinity for PKAc. We assume that the reduced affinity for PKAc contributes to disease relevant elevations of PKAc phosphotranferase activities. The second mutation RIa-R368X (=14aa truncation) caused a defect in PKA activation. The mutation introduces a stop codon and shortens the cAMP binding domain B. The resulting deletion of 14 aa at the C terminus reduced the cooperative binding of cAMP first to binding domain B and then to binding domain A, which is a prerequisite for PKAc dissociation and activation (Kim et al., 2007; Bertherat et al., 2009; Linglart et al., 2011; Taylor et al., 2012). We showed that this truncation is sufficient to elevate basal RIa-1-368:PKAc complexes. Primarily this complex was less responsive to GPCR/cAMP mediated PKA activation what correlates with the findings of Linglart and colleagues (Linglart et al., 2011; Silve et al., 2012). Interestingly, we detected marginal cAMP-dependent activation of the mutated PKA complex. This underscores the suitability of the PKA *R*luc PCA reporter to detect even modest changes of PPIs in response to second messenger elevations. Overall we underline with this study that the *R*luc PCA is a versatile reporter system to map transient and small molecule controlled PPI. Besides linking membrane receptor pathways to alterations of molecular interactions, the consequence of patient mutations on PPI can be tested with a simple protocol and in parallel fashion. The implementation of this PPI reporter platform will unveil consequences of distinct patient mutations on binary PKA network dynamics. We envision that the mutation approach will disclose hot spots in these critically regulated kinase complexes which become pharmaceutical targets to target both PPI and/or cAMP sensing. This will help to gain novel insights into mechanism of PKA activation which will be relevant for the diagnosis and for identifying treatments of kinase involved diseases.

Author Contributions

RR, JM, VB, and ES performed the experiments. RR, JM, VB, and ES analyzed the results. ES wrote the manuscript.

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GENERAL COMMENTARY
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Corrigendum: Impact of kinase activating and inactivating patient mutations on binary PKA interactions

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There was a mistake in the figure legend for **Figure 5** as published. The correct version of the figure legend **(Figure 5B)** appears below. The authors apologize for the mistake. This error does not change the scientific conclusions of the article in any way.

Figure 5. Impact of RIa mutations and cAMP elevation on PKA type I complexes. ... **(B)** Following indicated modifications of RIa-F[1] sequences, combinations of wild type and mutant *R*luc PCA tagged PKA subunits have been subjected to *R*luc PCA measurements. The effect of isoproterenol (1 μM, 10 min; 48 h transient PCA reporter expression) on PKA complex formation has been determined (at least $n = 3$ independent experiments, ±SEM).

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REVIEW
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The genetically encoded tool set for investigating cAMP: more than the sum of its parts

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Intracellular fluctuations of the second messenger cyclic AMP (cAMP) are regulated with spatial and temporal precision. This regulation is supported by the sophisticated arrangement of cyclases, phosphodiesterases, anchoring proteins, and receptors for cAMP. Discovery of these nuances to cAMP signaling has been facilitated by the development of genetically encodable tools for monitoring and manipulating cAMP and the proteins that support cAMP signaling. In this review, we discuss the state-of-the-art in development of different genetically encoded tools for sensing cAMP and the activity of its primary intracellular receptor protein kinase A (PKA). We introduce sequences for encoding adenylyl cyclases that enable cAMP levels to be artificially elevated within cells. We chart the evolution of sequences for selectively modifying protein–protein interactions that support cAMP signaling, and for driving cAMP sensors and manipulators to different subcellular locations. Importantly, these different genetically encoded tools can be applied synergistically, and we highlight notable instances that take advantage of this property. Finally, we consider prospects for extending the utility of the tool set to support further insights into the role of cAMP in health and disease.

Keywords: cAMP, PKA, AKAP, adenylyl cyclase, phosphodiesterase

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Introduction

The discovery that some hormones elevate the second messenger cyclic AMP (cAMP) without triggering canonical effects such as increased phosphorylase activity (Keely, 1979; Hayes et al., 1980) indicated that cAMP may be compartmentalized in cells (Edwards et al., 2012). Studies of cAMP compartmentalization rely upon methods to sense and manipulate signaling enzyme activities in space and time (Scott and Pawson, 2009). The first sensor of intracellular cAMP ('FlCRhR') was constructed by fluorescein-labeling PKA catalytic subunits and rhodamine-labeling the regulatory subunits of the tetrameric kinase (Adams et al., 1991). Release of the catalytic subunits upon binding of cAMP to the regulatory subunits can be detected as a reduction in Forster resonance energy transfer (FRET; Adams et al., 1991). The FlCRhR probe was revolutionary as it enabled the first visualizations of cAMP compartmentalization (Bacskai et al., 1993; Hempel et al., 1996). However, the sensor must be microinjected since its fluorescent labels are not genetically encoded. This technical hurdle has limited the influence of the FlCRhR probe.

The subsequent development of genetically encoded sensors of cAMP (Zaccolo et al., 2000) and PKA activity (Zhang et al., 2001), in tandem with methods for manipulating both the enzyme that synthesizes cAMP – adenylyl cyclase (AC) – and molecular interactions that support cAMP signaling, has initiated a new wave of discoveries concerning cAMP signaling

(Edwards et al., 2012). Studies with these novel tools have helped to establish the necessity for targeting PKA to its substrates by anchoring to A-kinase anchoring proteins (AKAPs; Rosenmund et al., 1994; Zhang et al., 2001; Di Benedetto et al., 2008). The tools have been applied to reveal a small nuclear population of anchored PKA (Sample et al., 2012). Furthermore, they have illuminated how PKA interacts spatiotemporally with phosphodiesterase (PDE) enzymes that degrade cAMP (Willoughby et al., 2006), with the exchange-protein activated by cyclic AMP (Epac; Dodge-Kafka et al., 2005), and with Ca^{2+} signals (Cooper and Tabbasum, 2014). This genetically encoded tool set has also been applied to establish a role for localized cAMP signaling in diseases (Gold et al., 2013b) such as heart failure (Nikolaev et al., 2010), muscular dystrophy (Roder et al., 2009), diabetes (Zhang et al., 2005), breast cancer (Hansen et al., 2009), and adrenal Cushing's syndrome (Beuschlein et al., 2014).

In this review, we introduce genetically encoded sensors of cAMP and PKA activity, before discussing tools based upon ACs. We describe sequences that are available for targeting such tool proteins to specific sub-cellular locations and for modifying protein interactions involving cAMP signaling proteins. Importantly, further functionality can arise when the different classes of tools are combined, and we highlight studies that exploit this kind of synergy. Finally, we consider how the tool set might be extended and combined in novel ways to enable further advances in the understanding of cAMP signaling.

Genetically Encoded Sensors for Monitoring cAMP Dynamics

There are three types of cAMP receptors in higher organisms: PKAs, Epacs, and cyclic-nucleotide-gated channels (CNGCs). Genetically encoded sensors are available that derive from each class of endogenous cAMP receptor (Willoughby and Cooper, 2008). Sensors derived from PKAs and Epacs rely upon fusions to variants of green fluorescent protein (GFP) that undergo decreased FRET upon elevation of cAMP. Sensors derived from CNGCs can also employ measurements using patch clamp electrophysiology to detect changes in conductance upon binding of cAMP to the channel. The first genetically encoded FRET-based cAMP sensor was constructed by fusing an improved GFP to the C-terminus of the PKA catalytic subunit and a blue GFP variant (EBFP) to the C-terminus of type-II regulatory (RII) PKA subunits (Zaccolo et al., 2000). Respective substitution of EBFP and GFP with cyan and yellow fluorescent proteins yielded an improved sensor that is less prone to photobleaching (Zaccolo and Pozzan, 2002). This allowed real time imaging of cAMP fluctuations in cardiomyocytes in response to the β-adrenergic agonist norepinephrine or the broad spectrum PDE inhibitor isobutyl-methyl-xanthine (IBMX), and helped to establish that type IV PDEs are the critical PDE class for degrading cAMP following activation with norepinephrine in cardiomyocytes (Mongillo et al., 2004).

The discovery of Epac1 and Epac2 (de Rooij et al., 1998; Kawasaki et al., 1998) opened the door for development of unimolecular cAMP sensors based upon these cAMP-dependent GTPase activators (DiPilato et al., 2004; Nikolaev et al., 2004; Ponsioen et al., 2004). Unimolecular sensors based on Epacs exhibit higher FRET efficiency (increase in FRET signal from maximal to minimal cAMP) of ∼20–30% compared to ∼8% for multimolecular PKA-based sensors. Epac probes also show better temporal resolution (Nikolaev et al., 2004), and there are no concerns regarding balancing expression of the donor and acceptor fluorophores in unimolecular probes. For these reasons, Epac probes are now the most popular option for sensing cAMP fluctuations. The latest optimized Epac-based probes include pH-insensitive Ci/Ce Epac2-camps (Everett and Cooper, 2013) (**Figure 1A**) and the Epac1-based probes ICUE3 (DiPilato and Zhang, 2009) and $^{T}Epac^{VV}$ (Klarenbeek et al., 2011; Li et al., 2015) although the original probes exhibit good FRET efficiency and are still popular. CNGC-based cAMP sensors excel in temporal resolution of cAMP fluctuations (Rich et al., 2000; Fagan et al., 2001). The first example of this approach exploited rat CNG2 expression in human embryonic kidney-293 (HEK-293) cells (Rich et al., 2000). Measurement of Ca^{2+} flux through the channels as a proxy for cAMP elevation using electrophysiology supported the existence of cellular cAMP microdomains. Mutations can be incorporated into the CNG2 to tailor it for sensing cAMP: C460W improves cAMP sensitivity, E583M improves cAMP specificity over cGMP, and removal of residues 61-90 abrogates channel regulation by Ca^{2+}/calmodulin (Rich et al., 2001) (**Figure 1B**). A C460W/E583M double CNG2 mutant was used as a sensor to reveal that both G protein coupled receptor kinases (GRKs) and PKA stimulate PDE degradation of cAMP following β2-AR simulation of HEK-293 cells (Xin et al., 2008). A common way to apply CNG2 is to combine expression of the channel with the Ca^{2+} dye Fura-2, allowing measurement of Ca^{2+} influx by imaging rather than electrophysiology (Fagan et al., 2001; Rich et al., 2001, 2007; Rochais et al., 2004). For example, the E583M CNG2 variant was applied in this way to establish the necessity of PKA anchoring for negative feedback through PKA activation of type IV PDEs (Willoughby et al., 2006). CNGCs can also be adapted as FRET sensors (Nikolaev et al., 2006). The hyperpolarization-activated CNG2 (HCN2) has a higher sensitivity than CNG2, and exhibits as wide a dynamic range as a cAMP FRET sensor when YFP and CFP are fused either side of a single HCN2 cAMP binding domain (Nikolaev et al., 2006).

Genetically encoded cAMP probes have been applied to investigate different facets of cAMP signaling. For example, they have helped build upon initial observations of cAMP oscillations in Brooker (1973) and Gorbunova and Spitzer (2002). Epac-based probes demonstrate that cAMP oscillations can be evoked in cell lines including MIN6 cells (Landa et al., 2005) and HEK293 cells (Willoughby and Cooper, 2006). cAMP oscillations have also been monitored in β-cells using evanescent-wave-microscopy in combination with fluorescently labeled RII-CAAX and C subunits (Dyachok et al., 2008). This approach shows that Ca^{2+} amplifies but is not essential for glucose-induced cAMP oscillations in β-cells (Dyachok et al., 2008). Ca^{2+} typically oscillates in tandem with cAMP, and a related area of focus has been the basis of interplay between cAMP and

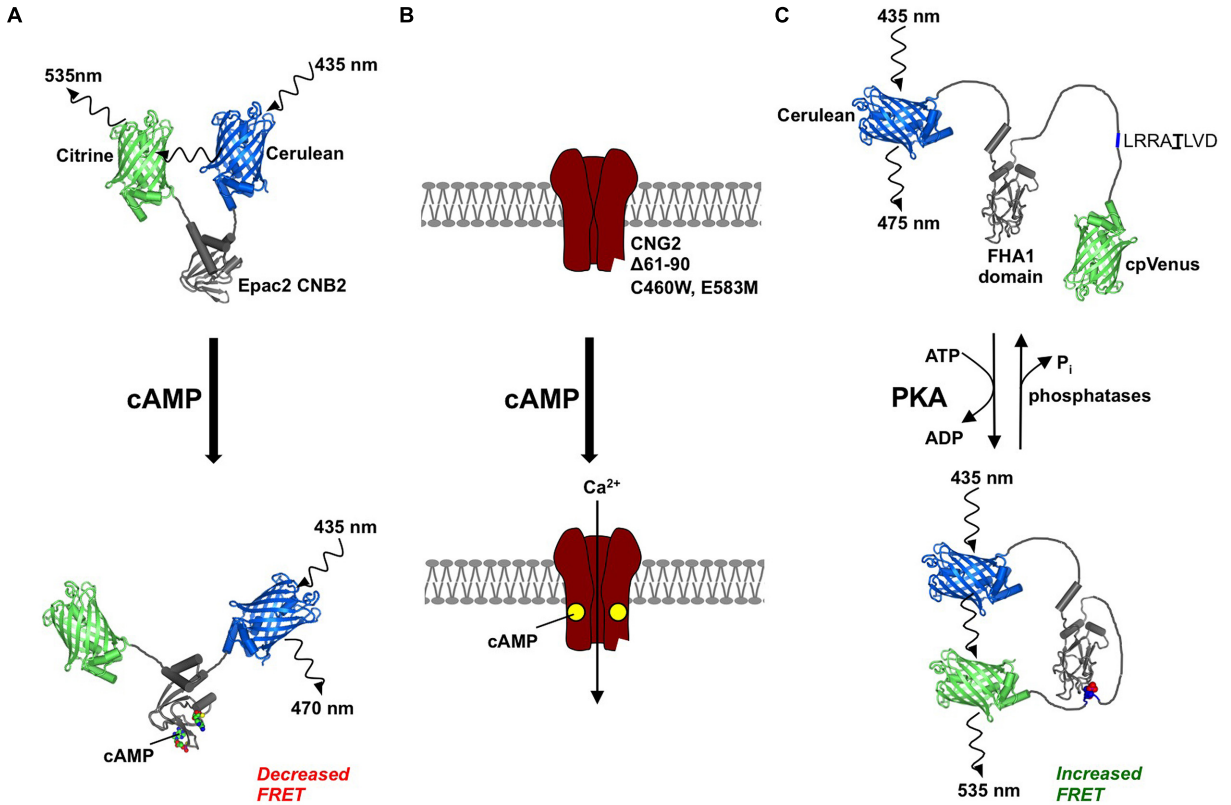

FIGURE 1 | Tools for monitoring cyclic AMP (cAMP) signaling. Two cAMP sensors and an A-kinase activity reporter are illustrated. **(A)** Cartoon showing mechanism for cAMP sensing by 'Ci/C-Epac2-camps,' which is based on the second cAMP binding domain of Epac2. Elevation of cAMP leads to separation of citrine and cerulean fluorescent proteins that can be detected as a decrease in FRET **(B)** cAMP sensor based on cyclic nucleotide-gated channel 2 (CNG2), which is modified with removal of residues 61–90 to prevent Ca^{2+}/calmodulin regulation, and the substitutions C460W/E583M for high affinity and specificity for cAMP. Elevation of cAMP leads to increased flux of Ca^{2+} which can be detected using Ca^{2+} dyes or by measuring current across the membrane. **(C)** AKAR4 contains a central FHA1 domain. PKA phosphorylation of threonine within the motif 'LRRATLVD' leads to association of phosphothreonine with FHA1, which can be detected as an increase in FRET between the terminal fluorescent proteins.

Ca^{2+} signals at the level of signaling proteins. Epac probes reveal that distinct pools of cAMP center on specific isoforms of AC (Wachten et al., 2010), with the Ca^{2+}-activated cyclase AC8 occupying a prominent role in linking Ca^{2+} signals to localized cAMP elevation (Willoughby et al., 2010; Ayling et al., 2012).

A key advantage of genetically encoding sensors is that transgenic animals expressing the sensors can be generated. Imaging of cAMP fluctuations has been achieved in pancreatic islets (Kim et al., 2008) using genetically engineered mice that selectively express a PKA-based cAMP sensor in pancreatic islets. This approach was exploited to show that that glucose triggers cAMP elevation independent of Ca^{2+} (Kim et al., 2008). Another study monitored cAMP changes upon activation of either β1 or β2 adrenergic receptors within small sarcolemmal areas by employing adrenergic receptor knockout mice transgenically expressing Epac2-camps (Nikolaev et al., 2010). This study revealed that β2 adrenergic receptors are restricted to deep transverse tubules (Nikolaev et al., 2010). Development of transgenic fruit flies expressing cAMP sensors (Lissandron et al., 2007) has also proved to be useful. In particular, *Drosophila* expressing Epac1-camps with an upstream activating sequence for GAL4 (Shafer et al., 2008) have enabled detailed investigation of how neuropeptides including pigment dispersing factor modulate cAMP in neurons during circadian rhythms (Duvall and Taghert, 2012; Pirez et al., 2013; Vecsey et al., 2014; Yao and Shafer, 2014). In sum, an impressive array of sensors and delivery options are now available for monitoring intracellular cAMP fluctuations.

Fluorescence-Based Sensors for Monitoring PKA Activity

One potential limitation of cAMP sensors is that they may not reflect cAMP receptor activation if the receptors and active cyclase are not co-localized. For PKA, genetically encoded A-kinase activity reporters (AKARs) may be utilized to monitor kinase activity more directly (Mehta and Zhang, 2011). The first AKAR was constructed by placing YFP and CFP either side of a PKA consensus phosphorylation sequence derived from Kemptide and the phospho-serine/threonine-binding protein

14-3-3τ (Zhang et al., 2001). Phosphorylation at the PKA consensus sequence causes association of the central elements that may be detected as a concomitant increase in FRET (Zhang et al., 2001). A second-generation sensor, AKAR2, incorporates a Forkhead-associated (FHA) domain in tandem with a lower affinity PKA recognition site. This modification improves the reversibility of the reporter (Zhang et al., 2005). This reporter was first applied to investigate interaction of insulin and isoproterenol stimulation of adipocytes. Using AKAR2, the authors found that chronic insulin treatment delayed PKA activation following addition of low concentrations of the β-AR agonist isoproterenol (Zhang et al., 2005). Conversely, PKA response to stimulation with either forskolin or caged cAMP was not affected by prior chronic insulin treatment. This suggested that insulin reduces PKA localization to a pool of cAMP associated with β-ARs. This notion was corroborated by anti-β-AR immunoprecipitation experiments that revealed decreased interaction of PKA RII subunits with β-ARs following dual treatment with insulin and isoproterenol (Zhang et al., 2005). This detail might have been missed if a cAMP sensor had been employed rather than AKAR2. Systematic improvement of AKAR reporters is ongoing (Liu et al., 2011; Oldach and Zhang, 2014). The latest reporter, AKAR4, features the fluorescent protein variants Cerulean and cpVenus (Depry et al., 2011) (**Figure 1C**).

A-kinase activity reporters have also been widely applied. For example, they have been used to validate stapled PKA anchoring disruptor peptides (Wang et al., 2014), and to confirm that Leu206Arg substitution in Cα subunits (associated with Adrenal Cushing's Syndrome) leads to constitutive kinase activation (Beuschlein et al., 2014). They are often utilized in parallel with cAMP sensors, for example, in imaging fluctuations of cAMP and PKA activity induced by neural activity in retinal cells (Dunn et al., 2006); to investigate cAMP/PKA dynamics at the centrosome (Terrin et al., 2012); and in the study of neurite outgrowth including a forskolin-coated glass bead contact procedure for cultured hippocampal neurons (Shelly et al., 2010). As with genetically encoded cAMP sensors, it is possible to express AKARs in transgenic animals. For example, PKA activity dynamics have been imaged in *Drosophila* expressing AKAR2 (Gervasi et al., 2010). In this study, Kamioka et al. (2012) performed crosses with learning and memory deficient mutant fly lines to establish that the AC Rutabaga acts as a coincidence detector during aversive and appetitive learning (Gervasi et al., 2010). AKAR-expressing mice have also been developed, and exploited to image real-time PKA activity in mouse epidermis and small intestine (Kamioka et al., 2012). These whole-organism studies underline the benefits of genetically encoding reporters in comparison to techniques that rely upon microinjection (Adams et al., 1991).

Tools for Manipulating Adenylyl Cyclase Activity

The ability to control cAMP elevations with spatiotemporal precision can help to reveal how cAMP signaling is organized in time and space (Scott and Pawson, 2009). In analogous fashion to the discovery of light-activated channelrhodopsins for artificially depolarizing cells (Nagel et al., 2003), photo-active adenylyl cyclases (PACs) have been identified in photo-sensitive microbes (Iseki et al., 2002; Ryu et al., 2010; Stierl et al., 2011). Advantages of genetically encoded PACs over cAMP uncaging approaches (Ponsioen et al., 2004; Saucerman et al., 2006) include the ability to deliver into whole animals, and the option to localize the PAC within cells by fusion to subcellular targeting sequences. The first PAC to be characterized and utilized was discovered in *Euglena*. This unicellular flagellate relies on a PAC in photophobic behavior (Iseki et al., 2002). *Euglena* PAC comprises two subunits, PACα and PACβ. Each subunit consists of two blue light receptor using flavin adenine nucleotide (BLUF) domains paired with two AC domains. Activation of the BLUF domains with blue light leads to a conformational change that activates the AC domains. Both PAC subunits respond to blue light with a maximal increase in AC activity of ~80-fold. The PACα subunit is more active than PACβ in both light and dark conditions (Iseki et al., 2002), so applications of *Euglena* PAC have utilized the α subunit (Bucher and Buchner, 2009; Bellmann et al., 2010; Weissenberger et al., 2011).

Smaller PACs have subsequently been discovered in species other than *Euglena*, including *Beggiatoa* PAC (bPAC; Ryu et al., 2010; Stierl et al., 2011) (**Figure 2A**). bPAC comprises 350 residues, which facilitates transgenic delivery in comparison to PACα (1019 residues). bPAC also exhibits better responsiveness to blue light than PACα, and cyclase activity decreases faster for bPAC upon return to the dark (Ryu et al., 2010; Stierl et al., 2011). A different class of PAC has been identified in *Microcoleus* (mPAC) that relies on a blue light-responsive light oxygen voltage domain coupled to an AC domain (Raffelberg et al., 2013). mPAC compares favorably to bPAC when expressed

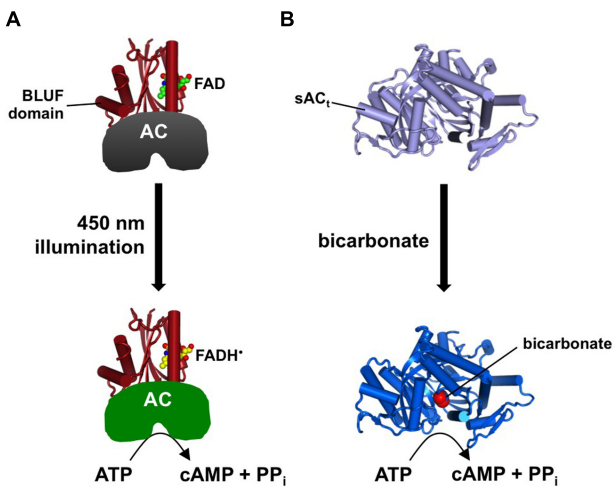

FIGURE 2 | Tools for triggering cAMP elevation. (A) Illustration of *Beggiatoa* PAC (bPAC) demonstrating how light illumination of the BLUF domain is coupled to activation of AC activity. **(B)** A truncated version of soluble AC (sAC_t) responds to elevated bicarbonate by catalyzing cAMP synthesis.

in vivo, although its responsiveness to blue light is worse *in vitro*, suggesting that a cellular co-factor may support its proper function (Raffelberg et al., 2013). Both bPAC and mPAC require lower light intensity for cyclase activation than PACα (Ryu et al., 2010; Stierl et al., 2011; Raffelberg et al., 2013), so are better suited for application in tissue samples where light penetration is a challenge. A noteworthy alternative to PACs is to employ a C-terminally truncated version of soluble AC (sAC_t) that can be activated by addition of bicarbonate (Sample et al., 2012) (**Figure 2B**). This approach is compatible with simultaneous monitoring of cAMP concentration and PKA activity with FRET-based probes (Sample et al., 2012; see Combinatorial Applications).

The utility of PACs is demonstrated by studies up to the level of whole animals. PACα has been expressed in cholinergic neurons (Weissenberger et al., 2011), motor neurons (Bucher and Buchner, 2009), and olfactory receptor neurons (ORNs; Bellmann et al., 2010) in *Drosophila*. This has enabled conceptual progress including the establishment of a specific class of ORNs that mediate olfactory avoidance behavior (Bellmann et al., 2010). PACα has been applied to demonstrate a key role for cAMP in growth cone turning in *Xenopus* (Nicol et al., 2011). Nicol et al. (2011) found that local pulses of blue light on distal parts of growth cones expressing PACα were sufficient to maintain orientation of axon outgrowth toward the midline despite blockade of a key Netrin-1 receptor. The potential utility of bPAC has been demonstrated in *Aplysia* (Nagahama et al., 2007), *Toxoplasma* (Hartmann et al., 2013), and in *Xenopus*, HEK293 cells and *Drosophila* (Schroder-Lang et al., 2007). Jansen et al. (2015) have shown how bPAC can be used to control sperm motility and maturation. Furthermore, specific expression of bPAC in *Drosophila* renal tubule in combination with pharmacological manipulations has revealed the distinct roles fulfilled by PKA and Epac in controlling secretion from principal and stellate cells (Efetova et al., 2013). The field is poised for further insights as application of PACs becomes more widespread.

Sequences for Subcellular Targeting and Modifying Protein–Protein Interactions

An important characteristic of genetically encoding sensors and manipulators of cAMP signaling is that they can be combined with targeting sequences to direct them to specific subcellular compartments. This approach facilitates investigation of spatial aspects of cAMP signaling. In addition, a number of sequences are available for manipulating interaction between PKA and its anchoring sites. A straightforward way to control the location of a given genetically encoded tool is to fuse it with a full-length signaling protein of interest. This option has been taken with proteins including AC8 (Willoughby et al., 2012, 2014; Everett and Cooper, 2013), full-length PKA RII (Zhang et al., 2001), different PDEs (Herget et al., 2008), SOD2 (Zhang et al., 2015), phospholamban (Sprenger et al., 2015), and Hsp20 (Sin et al., 2011). In cases where a good structural understanding is available, fusions are possible to shorter protein domains, such as with the dimerization and docking (D/D) domain of PKA regulatory subunits for tethering at anchoring sites (Di Benedetto et al., 2008), or with the PDE4D-association region from mAKAP (Dodge-Kafka et al., 2005).

The structural interface between PKA and AKAPs is well understood: all AKAPs present a ∼20 amino acid amphipathic helix that binds within a shallow hydrophobic groove on the D/D domain of PKA regulatory subunits (Gold et al., 2006; Kinderman et al., 2006; Sarma et al., 2010). Native and synthetic AKAP anchoring helix sequences are available for modifying PKA anchoring or targeting genetically encoded tools including cAMP sensors (summarized in **Table 1**). A popular sequence is Ht31 (Rosenmund et al., 1994), which is the native anchoring helix of AKAP-Lbc. Using a combination of structural information and peptide array screening, synthetic sequences have been developed with altered binding preferences for RI and RII PKA regulatory subunits. These include the RII-selective sequences AKAP-*is* (Alto et al., 2003), Super-AKAP-*is* (Gold et al., 2006), and AKB-II (Burns-Hamuro et al., 2003); and the RI-selective sequences RIAD (Carlson et al., 2006; Torheim et al., 2009) and AKB-RI (Burns-Hamuro et al., 2003). Studies that utilize sequences for modifying protein–protein interactions typically apply them exogenously as peptides, such as stearated Ht31 (Gold et al., 2012), and delivery approaches in this vein are still improving (Wang et al., 2014, 2015). Some studies have taken advantage of their genetic encodability. For example, sequences including AKAP-*is* (Dodge-Kafka et al., 2005) and AKB-RI/RII (Burns-Hamuro et al., 2003) have been expressed using transfection, and Ht31 has been incorporated in a transgenic mouse (Park et al., 2014).

An additional recent innovation is the development of variants of PKA regulatory subunits with selective anchoring properties (Gold et al., 2013a). A structure-based bacteriophage screening procedure (Walker-Gray and Gold, 2015) enabled identification of variants of the RII D/D domain with substitutions that enable selective anchoring to AKAP18 and AKAP2 (Gold et al., 2013a). RSelect subunits can potentially be applied to drive PKA either specifically to or away from individual AKAPs (Gold et al., 2013a). In most tissues, with rare exceptions (Gold et al., 2012), multiple AKAPs are present and an important challenge is to identify the PKA substrates associated with different AKAP–PKA signaling complexes (Gold et al., 2013b). It is possible to genetically abrogate specific PKA anchoring sites, for example, in the case of AKAP150 ΔPKA knock-in mice in which the PKA anchoring helix in AKAP150 is absent (Murphy et al., 2014). Therefore, a potentially more novel application of RSelect subunits is to combine them with anchoring disruptors such as Ht31. This approach enables PKA catalytic subunits to be driven to a single AKAP within the cell (Gold et al., 2013a).

A variety of sequences may be utilized for targeting to organelles (**Table 1**). Inclusion of C-terminal polybasic-CAAX sequences enables targeting to the plasma membrane (DiPilato et al., 2004; Dyachok et al., 2006; Saucerman et al., 2006; Depry et al., 2011), whereas N-terminal addition of a sequence derived from Lyn kinase targets specifically to cholesterol-rich regions of plasma membrane (Terrin et al., 2006; Depry et al.,

TABLE 1 | Sequences for modifying interactions between cyclic AMP (cAMP) signaling proteins and subcellular targeting.

Purpose	Name and specifications	Reference
Sequences for modifying protein–protein interactions		
Binding to the D/D domain of PKA RII subunits	Ht31 (AADLIEEAASRIVDAVIEQVKA). K_D = 2.2 nM for RIIα; 1.3 μM for RIα	Rosenmund et al. (1994), Alto et al. (2003)
	AKAP-*is* (QIEYLAKQIVDNAIQQA). K_D = 0.4 nM for RIIα, 277 nM for RIα	Alto et al. (2003), Dodge-Kafka et al. (2005)
	Super-AKAP-*is* (QIEYVAKQIVDYAIHQA). On filter assay binds RIIα 4× more and RIα 12.5× less efficiently than AKAP-*is*	Gold et al. (2006)
	AKB-RII (VQGNTDEAQEELLWKIAKMIVSDVMQQ). K_D = 2.7 nM for RIIα, 2.5 μM for RIα	Burns-Hamuro et al. (2003)
Binding to the D/D domain of PKA RI subunits	RIAD (LEQYANQLADQIIKEATE). K_D = 1 nM for RIα, 1800 nM for RIIα	Carlson et al. (2006), Torheim et al. (2009)
	AKB-RI (FEELAWKIAKMIWSDVFQQ). K_D = 5.2 nM for RIα; 450 nM for RIIα	Burns-Hamuro et al. (2003)
Localizing with type I PKA	RIα (1–64)	Di Benedetto et al. (2008)
Binding to AKAP anchoring helices	RIIβ (amino acids 1–49)	Di Benedetto et al. (2008)
Specifically binding to the AKAP18 anchoring helix	RSelectAKAP18 (PKA RIIα 1–45 with I3V, I5L, T10D, Q14G substitutions)	Gold et al. (2013a)
Binding to PDE4D	mAKAP (1286–1831)	Dodge-Kafka et al. (2005)
Sequences for subcellular targeting		
Plasma membrane	C-terminal addition of polybasic-CAAX sequence, e.g., GKKKKKKSKTKCVIM. CAAX box undergoes farnesylation	DiPilato et al. (2004), Dyachok et al. (2006), Saucerman et al. (2006), Depry et al. (2011)
Plasma membrane (cholesterol-rich)	N-terminal addition of Lyn kinase sequence, e.g., MGCIKSKRKDNLNDD, that undergoes myristoylation and palmitoylation	Terrin et al. (2006), Depry et al. (2011), Sample et al. (2012)
Nuclear localization	C-terminal addition of nuclear localization signal PKKKRKVEDA	DiPilato et al. (2004), Terrin et al. (2006), Sample et al. (2012)
Nuclear export	C-terminal addition of nuclear export sequence LPPLERLTL	Sample et al. (2012)
Sarcoplasmic reticulum	C-terminal addition of the helical transmembrane region (PQQARQKLQNLFINFCLILICLLLICIIVMLL) of phospholamban	Liu et al. (2011)
Outer mitochondrial membrane	N-terminal addition of the targeting peptide yTom70	Lefkimmiatis et al. (2013)
	N-terminal addition of MitoDAKAP1 (MAIQLRSLFPLALPGMLALLGWWWFFSRKK), a mitochondrial signal sequence derived from D-AKAP1	Burns-Hamuro et al. (2003), DiPilato et al. (2004), Lim et al. (2007), Depry et al. (2011)
Mitochondrial matrix	N-terminal addition of the mitochondrial matrix targeting signal encoded in the first 12 amino acids of subunit IV of human cytochrome oxidase *c*	DiPilato et al. (2004), Lefkimmiatis et al. (2013)

2011; Sample et al., 2012). C-terminal addition of the sequence 'PKKKRKVEDA' enables nuclear localization (DiPilato et al., 2004; Terrin et al., 2006; Sample et al., 2012), whereas addition of 'LPPLERLTL' at the same terminus drives nuclear export (Sample et al., 2012). Targeting to the sarcoplasmic reticulum is possible by fusing with the helical transmembrane domain of phospholamban (Liu et al., 2011). Furthermore, sequences are available for targeting to both the outer mitochondrial membrane (OMM; Burns-Hamuro et al., 2003; DiPilato et al., 2004; Lim et al., 2007; Depry et al., 2011; Lefkimmiatis et al., 2013), and to the mitochondrial matrix (DiPilato et al., 2004; Lefkimmiatis et al., 2013). At the whole animal level, systems are also available for driving cell-type specific expression of genetically encoded tools. These include the UAS/GAL4 system in *Drosophila* (Shafer et al., 2008), and the reverse tetracycline transactivator (rtTA)/doxycycline system in transgenic mice (Kim et al., 2008). Overall, there is an impressive arsenal of sequences at the experimenter's disposal for manipulating the location and binding properties of cAMP signaling proteins and genetically encoded tools.

Combinatorial Applications

The tools described in this review have been combined in different ways to make conceptual breakthroughs that would not have been possible if the tools were applied in isolation. The majority of combinations consist of fusions to either sequences derived from cAMP signaling proteins or to subcellular targeting sequences. We will also consider two exceptional studies that have utilized highly innovative combinations of the tool set (Ni et al., 2011; Sample et al., 2012).

The first study involving AKAR1 set a precedent for combining a cAMP sensor with sequences derived from cAMP signaling proteins. Fusion of AKAR1 to the PKA RII subunit revealed that PKA phosphorylation occurs more quickly if PKA is tethered in proximity to its substrate (Zhang et al., 2001). This finding underlined the importance of anchoring PKA to its substrates. A similar approach has been to fuse an Epac-based cAMP sensor to AC8 (Willoughby et al., 2012, 2014), for example, to demonstrate that this Ca^{2+}-sensitive AC responds to Ca^{2+} entering through L-type Ca^{2+} channels (Everett and

Cooper, 2013). Fusions to isolated domains of cAMP signaling proteins have also been insightful. Fusing Epac-based sensors to the D/D domains of different PKA regulatory subunit classes (Di Benedetto et al., 2008; Roder et al., 2009) has helped to establish that PDE4 associates with RII subunits, whereas PDE2 acts in the vicinity of RI subunits (Di Benedetto et al., 2008). By applying this approach in cardiomyocytes, Di Benedetto et al. (2008) also revealed that the β-AR agonist isoproterenol triggers relatively higher cAMP accumulation with RII subunits, whereas hormones including glucagon induce raised cAMP in the vicinity of the RI subunit. This mechanism allows G protein-coupled receptor specific patterns of cAMP signaling to occur within the same cell. A fusion of the PDE-binding region of mAKAP (residues 1286–1831) with the AKAR2 reporter has also been applied to demonstrate that dominant active MEK5 prolongs PKA activity after cAMP elevation with forskolin (Dodge-Kafka et al., 2005).

Targeting FRET-based sensors using subcellular targeting sequences has also proved to be valuable. Fusions of AKAR reporters with membrane-targeting sequences (Saucerman et al., 2006; Depry et al., 2011) have revealed, for example, that basal PKA activity is higher in cholesterol-rich 'raft' regions of membrane (Depry et al., 2011). Similarly, plasma membrane-targeting of an Epac-based cAMP sensor has helped to establish how a pre-assembled protein complex including RXFP1, AKAP79, AC2, β-arrestin 2, and PDE4D3 enables responses to sub-picomolar circulating concentrations of relaxin peptide (Halls and Cooper, 2010). Comparison of PKA activity using AKAR4 targeted to either the mitochondrial matrix or OMM shows that PKA phosphorylation is more enduring at the OMM than in the cytosol due to diminished phosphatase activity at the OMM (Lefkimmiatis et al., 2013). A related approach has been to fuse a sequence that targets to the outer mitochondrial membrane to the PKA regulatory subunit-specific anchoring disruptors AKB-R1 and AKB-R2 (Lim et al., 2007). Lim et al. (2007) expressed these fused sequences to prevent access of either type I or type II PKA to the plasma membrane. This approach revealed that leading-edge phosphorylation of PKA substrates requires type I PKA, with re-localization of type I PKA to the mitochondria inhibiting both the directional persistence and speed of cell migration (Lim et al., 2007).

Two highly innovative combinatorial approaches have been exploited to examine cAMP dynamics in recent years (Terrin et al., 2006; Sample et al., 2012). Sample et al. (2012) developed a novel technique called spatiotemporal manipulation of cAMP using sAC_t (Sample et al., 2012). They targeted sAC_t by fusing the bicarbonate-activated AC to sequences that target to either the plasma membrane, cytosol, or the nucleus (Sample et al., 2012). By monitoring cAMP accumulation or PKA activity with cytosolic or membrane-tethered sensors, the authors revealed that cAMP and PKA activity can be localized at either the plasma membrane or in the nucleus. Modeling the responses of cAMP and PKA to forskolin and nuclear sAC indicated that a pool of PKA resides in the nucleus. This prediction was subsequently confirmed by immunohistochemistry and immunoblotting (Sample et al., 2012). Another elegant innovation has been to red-shift FRET-based sensors of cAMP and PKA activity to enable simultaneous application with other fluorescent sensors. Red-shifted sensors typically include red fluorescent protein (RFP) variants such that FRET emission occurs at longer wavelengths than with typical fluorescent probes (Ni et al., 2011). This approach was taken to study a cAMP-Ca^{2+}-PKA oscillatory circuit in MIN6 cells (Ni et al., 2011). Ni et al. (2011) performed imaging of Fura-2 with either red-shifted AKAR or a red-shifted Epac-based cAMP sensor. Remarkably, the authors also utilized a novel dual detector that enables simultaneous PKA activity and cAMP detection (ICUEPID). Application of these tools showed that oscillations in MIN6 cells can be triggered by cAMP alone, and the authors speculate that such oscillations may provide a way for local PKA activity to be maintained for long periods of time (Ni et al., 2011). These two studies from the Zhang laboratory exemplify the potential benefits of combining different categories of tools in novel ways.

Considerations for Experimental Design

When deciding whether to employ one of the tools outlined in this review, one considers how the strengths, limitations, and challenges associated with the tool match up with the aims of an experiment. If spatial aspects of cAMP signaling are the emphasis of investigation, then the Epac-based cAMP probes (Klarenbeek et al., 2011; Li et al., 2015) are a good option as they consist of relatively short sequences that can be directed to subcellular locations with targeting sequences (**Table 1**). On the other hand, if a maxim is placed on temporal resolution, the CNG2 system may be the better option (Rich et al., 2000; Fagan et al., 2001). Similarly, PACs such as bPAC (Ryu et al., 2010; Stierl et al., 2011) allow faster activation and de-activation than the bicarbonate-activated sAC_t (Sample et al., 2012). For tandem applications, the bicarbonate-activated sAC_t cyclase has the advantage that there is no concern about unwanted photoactivation when combined with FRET-based cAMP/PKA sensors (Sample et al., 2012). Similarly, red-shifted sensors can allow cAMP and PKA activity sensors to be applied simultaneously (Ni et al., 2011). The choice of tools will also be dictated by the availability of specialist equipment. For example, application of FRET-based sensors typically relies on a confocal microscope with appropriate lasers. If such a microscope is unavailable then recordings may be performed using a plate reader (Robinson et al., 2014) although studies will be limited to the population level following this approach.

It is important to consider the potential off-target effects and distortions that may be caused by the tools. For example, if PKA is the focus of investigation, it is wise to avoid PKA-based cAMP sensors that may distort signaling by interacting with native PKA subunits. Any unwanted cellular changes resulting from long-term expression of genetically encoded tools can be limited by using inducible expression systems such as the tetracycline system (Meyer-Ficca et al., 2004). Another consideration is that cAMP sensors act as buffers for cAMP, potentially altering the amplitude and duration of cAMP transients in a similar way to alteration of free Ca^{2+} transients by dyes such as Fura-2 (Neher, 2008). Buffering effects can be at least ameliorated by taking care

to express cAMP sensors at levels no higher than is necessary for reliable detection. Finally, one should also bear in mind that targeting and disruptor sequences may not always behave as desired. The specificity of disruptor sequences can be tested by performing negative control experiments with sequences such as Ht31-P in which the disruptor helix is destabilized by incorporation of a central proline (Willoughby et al., 2006). It is also good practice to image cells to check that localization sequences have partitioned within the cell as anticipated (Lim et al., 2007). Overall, the wide range of available tools means that a good technical solution is at hand for most experimental aims.

Conclusions and Prospects

The genetically encoded tool set for investigating cAMP signaling has expanded rapidly over the last 15 years. There are now a multitude of options for monitoring intracellular fluctuations in cAMP, with Epac-based probes emerging as the most popular sensor class (DiPilato and Zhang, 2009; Klarenbeek et al., 2011). AKAR reporters enable PKA activity to be monitored more directly, while experimenters can artificially elevate cAMP levels by photoactivating PACs or stimulating sAC with bicarbonate. The functionality of these tools can be enhanced by combining with an impressive array of sequences for modifying protein–protein interactions and subcellular targeting (**Table 1**).

There is scope for improving the tools described in this review, developing novel tools, and combining the tools in new ways. Biosensors typically improve over time by incorporating modifications based on user feedback (Oldach and Zhang, 2014). This process may have reached the point of diminishing returns for FRET-based sensors and peptides derived from AKAP anchoring helices, whereas there is more potential for improvement of the more recently developed PACs and RSelect subunits. For example, an AC with a synthetic domain architecture has been shown to be activated by near-infrared light (Ryu et al., 2014). This PAC is likely to be particularly advantageous for studies that require deep tissue penetration (Ryu et al., 2014). It is worth noting that methods for calibrating FRET-based cAMP reporters are still improving (Koschinski and Zaccolo, 2015). Genetically encoded sensors that incorporate Renilla luciferase for Bioluminescence resonance energy transfer (BRET) imaging (Prinz et al., 2006; Jiang et al., 2007) exhibit higher maximal signal-to-noise ratios than FRET-based sensors. Application of BRET sensors in live cell imaging could become more popular if light detectors can improve to overcome the relatively low light output of BRET compared to FRET. Although sequences for disrupting or selectively binding to either side of the AKAP–PKA interface are advanced (**Table 1**), no equivalent sequences exist for interactions mediated by ACs, PDEs, or Epacs. If the structural and molecular basis of interactions involving these protein classes can be determined more precisely, it should be relatively straightforward to develop improved sequences using established peptide array screening (Alto et al., 2003; Burns-Hamuro et al., 2003) and directed evolution (Walker-Gray and Gold, 2015) approaches.

There are currently no existing technologies for activating PKA or Epac activity with temporal precision. One future avenue is to genetically encode unnatural amino acids such as caged lysine using the amber codon TAG (Gautier et al., 2011; Kim et al., 2013) as a basis for enzyme activation upon illumination in a similar way to PACs. Future studies may also exploit an approach for light-gating protein-protein interactions that can be genetically encoded using sequences from a phytochrome signaling network (Levskaya et al., 2009). Further combinatorial possibilities that have been underexploited include fusions of PACs and sAC_t with the sequences listed in **Table 1**. Many targeting studies have focused on the relation of cAMP signaling with different sub-structural features of cardiomyocytes (Di Benedetto et al., 2008; Nikolaev et al., 2010). It will be valuable to perform analogous experiments to probe cAMP signaling in different neuronal compartments. Finally, the ICUEPID sensor (Ni et al., 2011) sets a precedent for how red-shifting FRET-based sensors can enable their combination with other technologies that rely upon illumination.

It is important to question the value of determining how cAMP signaling processes proceed with high spatial and temporal detail. The conceptual breakthrough that supported the development of the first β-blocker propranolol (Stapleton and Black, 1997) indicates how current research targeted at spatiotemporal cAMP signaling might be useful in the long term. James Black was inspired to target β-ARs by experiments conducted by Raymond Ahlquist. Ahlquist established that there are two major classes of adrenergic receptor, with β-ARs underpinning responses to norepinephrine in the heart (Stapleton and Black, 1997). An analogous classification of cAMP signaling processes at the sub-cellular level can provide a framework for selectively intervening in cAMP signaling with more precision than drugs that act at the level of the receptor. Combinatorial application of the genetically encoded tools described in this review is central to achieving the level of spatiotemporal detail necessary to open a path to a next generation of drugs that manipulate cAMP signaling.

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Genetically-encoded tools for cAMP probing and modulation in living systems

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Intracellular 3′-5′-cyclic adenosine monophosphate (cAMP) is one of the principal second messengers downstream of a manifold of signal transduction pathways, including the ones triggered by G protein-coupled receptors. Not surprisingly, biochemical assays for cAMP have been instrumental for basic research and drug discovery for decades, providing insights into cellular physiology and guiding pharmaceutical industry. However, despite impressive track record, the majority of conventional biochemical tools for cAMP probing share the same fundamental shortcoming—all the measurements require sample disruption for cAMP liberation. This common bottleneck, together with inherently low spatial resolution of measurements (as cAMP is typically analyzed in lysates of thousands of cells), underpin the ensuing limitations of the conventional cAMP assays: (1) genuine kinetic measurements of cAMP levels over time in a single given sample are unfeasible; (2) inability to obtain precise information on cAMP spatial distribution and transfer at subcellular levels, let alone the attempts to pinpoint dynamic interactions of cAMP and its effectors. At the same time, tremendous progress in synthetic biology over the recent years culminated in drastic refinement of our toolbox, allowing us not only to bypass the limitations of conventional assays, but to put intracellular cAMP life-span under tight control—something, that seemed scarcely attainable before. In this review article we discuss the main classes of modern genetically-encoded tools tailored for cAMP probing and modulation in living systems. We examine the capabilities and weaknesses of these different tools in the context of their operational characteristics and applicability to various experimental set-ups involving living cells, providing the guidance for rational selection of the best tools for particular needs.

Keywords: cAMP signaling, cyclic nucleotide, biosensor, genetically encoded probe, optogenetics, cell-based assays

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Introduction

The small molecule 3′-5′-cyclic adenosine monophosphate (cAMP) is an established second messenger, involved in signal transduction in most living organisms. Being instrumental in regulation of a plethora of processes both in health and pathological states, principles of cAMP signaling have remained in the research spotlight for the best part of the last century and the interest

to this cyclic nucleotide does not seem to fade away (Beavo and Brunton, 2002; Lefkimmiatis and Zaccolo, 2014). To a certain degree this is attributed to the immense pharmacological significance of G protein-coupled receptors (GPCRs), which have cAMP for an immediate intracellular effector and continue to serve as drug targets for as much as one third of the pharmaceutical compounds marketed worldwide (Filmore, 2004; Overington et al., 2006).

As it typically happens with branches of knowledge that are built up on empirical observations, the perceptions of cAMP-mediated signal transmission in living systems have been (and continue to be) shaped by the input from the experimental and analytical tools available to research community. The truism "*we can only go as far as our instruments will allow us to*" is nicely exemplified by the changes in the paradigm of cyclic nucleotide signaling, occurring in the last decades.

Conventional biochemical methods, such as chromatography with tritium—prelabeled adenine pool and antibody-dependent competition assays, that made the platform for cAMP studies till 1990-x, though robust and applicable to essentially any bodily tissue, can measure only the unresolved *bulk* of cAMP levels in pooled cellular populations, thus leaving us to guess what is going on with cAMP molecules in any given single cell. Apart from the limited spatial resolution, biochemical assays typically require cAMP liberation from specimens under study, which is usually accomplished by cell lysis (Williams, 2004; Hill et al., 2010). This way, biochemical assays in essence provide a single time point measurement, reflecting the total cAMP levels present in a specimen at the time of cell disruption. Though it is possible to deduce the overall kinetic trend of total cAMP over a time period by preparing a set of biological replicates and lysing them at certain intervals, the resulting kinetic curve is usually only a faint reflection of the actual cAMP oscillations in a given biological sample.

Experimental data on cAMP, obtained with biochemical methods with limited temporal and spatial resolution, formed the basis for a widely accepted model of cAMP signaling. This model implies cAMP generation by membrane-bound adenylyl cyclases (ACs) in response to GPCRs activation and its subsequent free diffusion into the cytoplasm. The ensuing activation of immediate cytoplasmic effectors of cAMP, such as protein kinase A (PKA), convey the signal further to the level of cell nucleus, eventually translating extracellular stimuli into transcriptional response (Beavo and Brunton, 2002).

However, cAMP network and governing principles of its functional and structural organization happen to be far more complex. Indeed, the conceptualization of cAMP signaling as of a highly compartmentalized process, occurring in separated subcellular domains, shaped by anchoring proteins and phosphodiesterases (PDEs), with organization of the key players of cAMP-mediated signal relay machinery into supramolecular complexes or "signalosomes," has just started to evolve (Willoughby and Cooper, 2007; Lefkimmiatis and Zaccolo, 2014). Apart from the intricate laws of spatial organization of cAMP generation, trafficking and degradation, this burgeoning model recognizes the multifaceted nature of signal encoding by cAMP (strength vs. duration vs. frequency) and pays due regards to the crosstalk between cAMP and other intracellular regulators (Rich et al., 2014).

It wound not be an overstatement to say, that the major insights into the complexity of cAMP signaling, served to fuel the above conceptual framework, were gained by studies exploiting next generation of tools for cAMP probing and modulation. Most of these tools are genetically encoded proteins, tailored for sensing and modulation of cAMP in living systems. These engineered proteins provide excellent spatial resolution down to desired subcellular domains, can respond to genuine oscillations of cAMP levels in real time and are designed to uncover cAMP signaling partners, and as such have enabled a paradigm-shift in cyclic nucleotide research.

Evidently, in order to scrutinize a complex phenomenon, a set of diverse probing tools is required. Align with this and thanks to the intricate nature of cAMP signaling relay and never-ceasing attempts to gain insights into the *laws of the game*, several *families* of biosensors for cAMP have been developed (reviwed in Willoughby and Cooper, 2008; Hill et al., 2010; Sprenger and Nikolaev, 2013). However, besides being genetically-encoded proteins and hence applicable to studies in living cells, the modern biosensors do not have much in common, as they strive to probe different aspects of cAMP signaling, are governed by distinct biological phenomena and rely on diverse biophysical techniques.

Considering this heterogeneity and in order to make this review more sound and cohesive, we decided to categorize the biosensors into two major groups: **tools for direct measurement of cAMP** and **tools for indirect cAMP probing**. As the name implies, direct probes provide *first hand* cAMP measurements, with a readout typically being generated immediately after binding between cAMP and the sensor molecules. Additionally, the readout intensity from direct biosensors is usually proportional to the intensity of stimulation, which allows the direct probes to convey valid data on actual oscillations of intracellular cAMP levels. In contrast with direct probes, indirect biosensors typically measure the effects of cAMP on its downstream effectors. In case of indirect cAMP sensing, there is always at least one *intermediary* between actual concentration of cAMP and the readout from the probe. Obviously, in order to deduce genuine cAMP levels from such an indirect readout, one has to be aware of the relations between cAMP and its effector being probed and to account for

Abbreviations: AC, adenylyl cyclase; AKAR, A-kinase activity reporter; BLUF, blue light receptor using FAD; BRET, bioluminescence resonance energy transfer; CaM, calmodulin; cAMP, 3′-5′-cyclic adenosine monophosphate; CCD, charge-coupled device; CFP, cyan fluorescent protein; cGMP, 3′, 5′-cyclic guanosine monophosphate; CNBD, cyclic nucleotide-binding domain; CNGC, cyclic nucleotide-gated channel; CRE, cAMP-response element; CREB, cAMP-response element-binding protein; Epac, exchange protein, directly activated by cAMP; FlCRhR, FRET sensor based on PKA (Fluorescein + Catalytic unit/Rhodamine + Regulatory unit); FRET, Förster resonance energy transfer; FSK, forskolin; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; HCN, hyperpolarization-activated cyclic nucleotide-gated channels; HTS, high-throughput screening; IBMX, 3-isobutyl-1-methylxanthine; LAPD, light-activatable phosphodiesterase; LOV, light-oxygen-voltage-sensing domain; NO, nitric oxide; PAC, photoactivatable adenylyl cyclase; PDE, phosphodiesterase; PKA, protein kinase A; RIIβ, regulatory subunit IIβ of protein kinase A; Rluc, *Renilla reniformis* luciferase; sAC, soluble adenylyl cyclase; YFP, yellow fluorescent protein.

possible interference that may happen on the way of signal relay. Though the above inherent factors limit the use of indirect sensors for measurement of actual levels of cAMP, the very nature of these sensors makes them excellent tools for studies of cAMP downstream effects.

In the following sections, we consider the modern cAMP biosensors with respect to the above categorization and discuss the principal groups of probes in more detail, highlighting their strengths and limitations. After that, we provide some guiding landmarks that would be of some assistance to the reader facing an uneasy task of selection of the most appropriate tool for cAMP probing for a particular experiment involving living cells. We finish the article with a short prospect on selected genetically-encoded tools for cAMP modulation in living systems. Combinatorial use of these cutting-edge instruments with biosensors for cAMP creates the most powerful research platform, which opens new avenues for fruitful studies of the life cycle and signaling properties of this cyclic nucleotide.

Tools for Direct cAMP Measurement in Living Cells

Irrespectively of the nature of readout produced upon cAMP binding (e.g., fluorescence or luminescence), direct sensors detect cAMP molecules by means of native or modified cyclic nucleotide-binding domains (CNBDs) invariably present in its structure. All the CNBD exploited in modern sensors have been adopted from cAMP-binding domains of the three principal downstream cAMP effectors: PKA, exchange proteins activated by cAMP (Epac) or cyclic nucleotide-gated channel (CNGC; Kamenetsky et al., 2006; Willoughby and Cooper, 2007).

A prototypical direct biosensor for cAMP is a conformationally flexible multidomain protein, which undergoes a structural change once cAMP molecule lands in the pocket of the CNBD, followed by a change in the signaling intensity of the sensor, which is used to deduce the concentrations of cAMP the sensor is exposed to. Below we review the principal classes of direct cAMP sensors based on the type of signal they generate in response to cAMP (**Figure 1**).

Förster Resonance Energy Transfer (FRET)-Based Sensors

Introduced in the early 1990s, biosensors based on FRET phenomenon have been instrumental in shaping of our understanding of the actual principles governing cAMP generation, flow, crosstalk and decay in defined compartments of living cells (Willoughby and Cooper, 2007; Lefkimmiatis and Zaccolo, 2014). Not surprisingly, FRET sensors remain the most popular and widely used tool for cAMP studies in living cells, with several dozens of distinct probes generated over the last three decades. For this reason, we will not discuss all the available cAMP FRET-based sensors in very detail—the reader is addressed to several comprehensive review papers instead (Willoughby and Cooper, 2008; Sprenger and Nikolaev, 2013; Gorshkov and Zhang, 2014). Instead, we shall try to highlight the basic principles of the method, advantages it offers and limitations one should keep in mind once planning an experiment with FRET-derived readout.

Förster resonance energy transfer refers to a mechanism of non-radiative energy transfer between a pair of light-sensitive molecules (fluorophores), a donor and an acceptor, that have partially overlapping spectral characteristics. For the transfer of energy, fluorophores making a FRET pair should be in close proximity (typically, within several nm from each other) and have a favorable relative orientation. As the efficiency of donor—to—acceptor energy *flow* is inversely proportional to the six power of the distance between fluorophores of any given FRET pair, the method is exceptionally sensitive to tiny alterations in FRET pair collocation (Forster, 1946; Correa and Schultz, 2009). This way, FRET intensity and its alterations provide an excellent reflection of a distance change between two fluorophores.

Förster resonance energy transfer intensity is typically measured by calculating a ratio of emission intensities of donor and acceptor molecules registered upon donor fluorophore excitation with light of the appropriate wavelength. Another way to estimate the degree of FRET is to photobleach an acceptor fluorophore and to track the rise in donor emission intensity. FRET efficiency estimates obtained with any of the above approaches are ratiometric in essence and are usually corrected for possible bleed-through of donor emission into the acceptor channel (Correa and Schultz, 2009).

In general, all FRET biosensors are designed to operate in the similar fashion. A prototypical FRET sensor represents a complex molecular structure with three principle domains: a sensor domain, that is in charge of binding to a molecule or compound of interest to be measured, and two fluorophore domains making a FRET pair. A binding of a target molecule to a sensor domain triggers a chain of conformational changes in a biosensor tertiary structure, which should lead to a change of distance between donor and acceptor fluorophores and result in alteration of FRET efficiency. Though multitude of variations in sensor design are possible, the above operational principle remains valid for all FRET biosensors, with the ones developed for cAMP not being an exception.

Since the number of molecules forming a sensor is an important factor that has several significant implications (reviewed below), we are considering all FRET cAMP indicators in the context of two big "*families*": a family of **multimolecular sensors**, members of which are build up of at least two different molecular units carrying separate domains, and a family of **unimolecular sensors** having a sensor and a FRET pair within a single molecule. Units of a multimolecular sensor can either dimerise or dissociate, and any of these events should be accompanied by a measurable change in FRET intensity.

Multimolecular FRET Sensors for cAMP

In essence, all of the multimolecular FRET sensors for cAMP generated so far represent different genetically engineered modifications of PKA, one of the principal downstream effectors of cAMP. PKA holoenzyme is a heterotetrameric complex conformed by two subunits: the catalytic (C) and the regulatory (R) subunit, arranged in a C2:R2 ratio. Binding of cAMP to the R subunits results in conformational change and the dissociation of the two, now active, C subunits from a tandem of R units, that remain attached to each other.

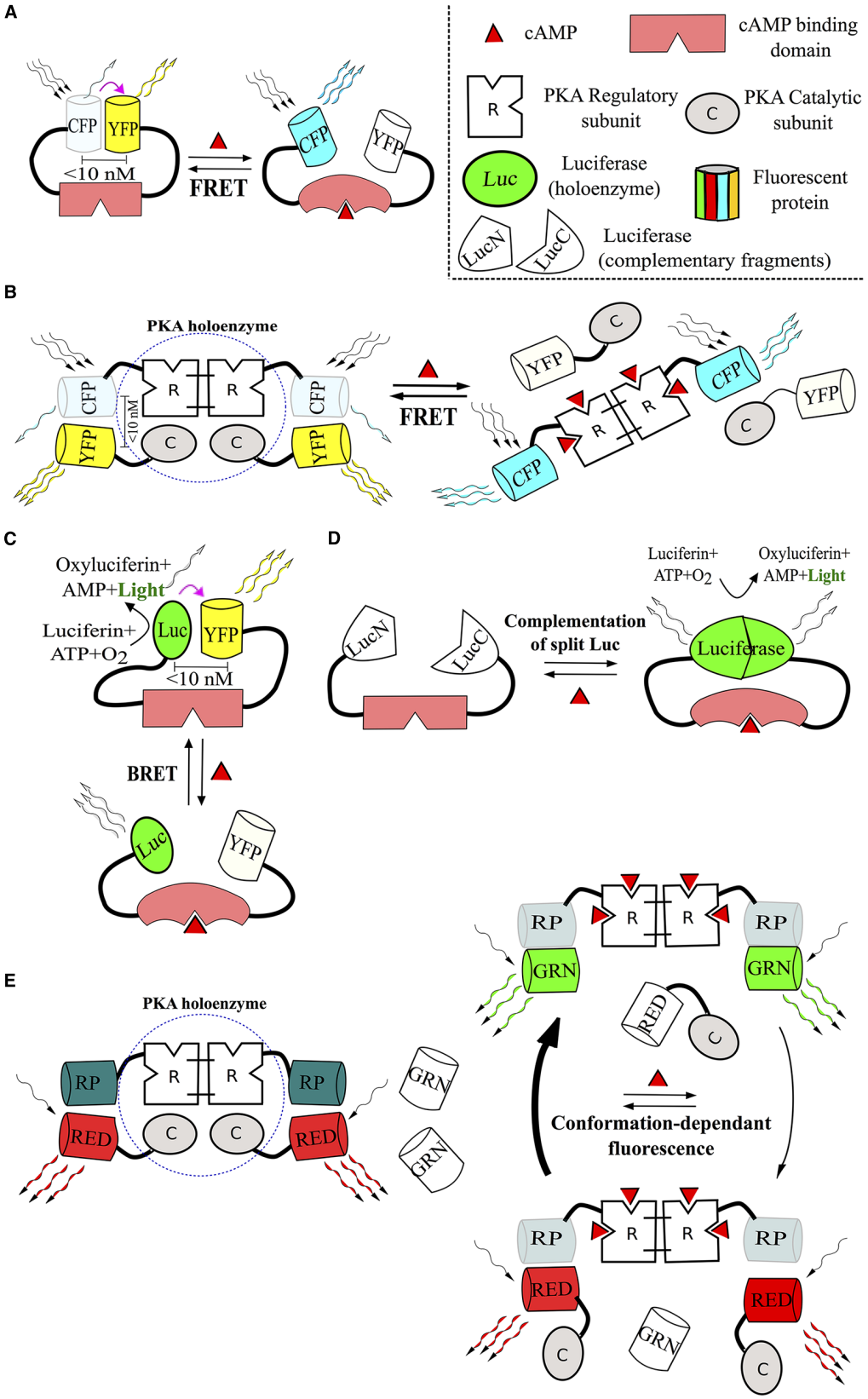

FIGURE 1 | (Continued)

FIGURE 1 | Main classes of direct biosensors for cAMP. A prototypical structure and mechanism of action of unimolecular and multimolecular FRET sensors (**A** and **B** respectively), BRET sensors **(C)**, sensors, based on luminescent enzymes **(D)**, and probes operating as conformation-sensitive fluorophores **(E)**, are depicted. All the listed sensors directly bind cAMP molecules and react to binding events with conformational changes that affect their signaling properties—FRET or BRET efficiency (purple arrow), intensity of light production or fluorescence (winding arrows). For more details, please, refer to corresponding sections of the text. Abbreviations: C, PKA catalytic subunit; CFP, cyan fluorescent protein; GRN, green fluorophore; Luc, luciferase; LucN/C, fragments, forming luciferase holoenzyme; R, PKA regulatory subunit; RED, red fluorophore; RP, regulatory protein; YFP, yellow fluorescent protein.

More than 25 years ago, the group of Tsien reported on the first successful use of FRET sensor for cAMP measurement in living cells (Adams et al., 1991). This sensor, named FlCRhR, represented the chemically modified PKA, which had its C subunits labeled with fluorescein fluorophore and R subunits tagged with rhodamine (hence the name: Fluorescein + Catalytic unit/Rhodamine + Regulatory unit) in FRET distance in the inactive holoenzyme. Binding to cAMP evoked PKA holoenzyme dissociation with measurable drop in FRET efficiency, as fluorescein and rhodamine were not in the immediate vicinity any longer for successful FRET to occur.

FlCRhR turned out to be a groundbreaking tool, as it allowed for real-time measurement of intracellular cAMP in physiological concentrations and provided a wealth of information on spatial cAMP dynamics. However, this sensor was produced *ex vivo* by means of chemical labeling and hence had to be introduced into cytoplasm of single cells via microinjection that was obviously technically demanding and significantly limited the processivity of the method.

Such limitation was overcome a few years later with development of a set of genetically-encoded FlCRhR analogs, that could be expressed in living cells by means of transfection or viral transduction (Zaccolo et al., 2000). Additionally, PKA-based FRET sensors evolved improved characteristics in successive generations, including broader dynamic range and higher resistance to photobleaching (Lissandron et al., 2005).

Nonetheless, despite all the modifications, PKA-based sensors still bear several common shortcomings, and most of these are actually related to the very nature of PKA and its mode of operation. Firstly, despite being labeled with a pair of FRET fluorophores, PKA holoenzyme—based sensors preserve catalytic activity. This way, a rise in cAMP triggers the complex chain of signaling events, involving effectors downstream of PKA and altering transcriptional activity of cAMP-dependent genes (Beavo and Brunton, 2002; Cooper, 2003; see also the section of this review on Reporter Gene-Based Systems). These effects form a potential basis for short and long-term interference, which in some cases might not be immediately obvious and quite difficult to correct for, e.g., in prolonged studies with either chronic or multiple exposure to certain agents. Secondly, as PKA holoenzyme naturally dissociates upon cAMP binding, one should expect the reassembled holoenzyme to have incorporated endogenous non-fluorescent C subunits inevitably present in cell cytoplasm. Obviously, this might lead to loss of overall FRET efficiency with each dissociation–reassociation cycle, as the fraction of reassembled PKA holoenzymes will not carry FRET pairs any more. Besides, it should be recognized, that more complex structural dynamics of PKA-based probes, involving integration of a tetrad of subunits, might negatively affect temporal resolution of these sensors, potentially making them inferior to unimolecular probes in this regard (Rich et al., 2014).

In order to alleviate unwanted interference from endogenous PKA subunits, PKA-based sensors are to be heavily expressed. However, high levels of sensor expression combined with its naturally high affinity to cAMP might lead to significant cAMP buffering, thus distorting genuine patterns of cAMP spatio-temporal dynamics (Rich and Karpen, 2002). Another related issue is the necessity of equal expression levels of both PKA subunits, which are usually encoded by separate vectors and hence require co-transfection (Zaccolo et al., 2000). Lastly, some experimental evidence suggest that PKA might be activated by 3′, 5′-cyclic guanosine monophosphate (cGMP), which opens new avenues for interference and might lead to decrease in assay specificity (Forte et al., 1992).

The multimolecular structure and cooperative nature of action of PKA-based cAMP probes underpin significant part of the above limitations, which in essence are inherent to most multimolecular FRET sensors. Conversely, unimolecular FRET sensors, developed in the ensuing years, allowed to bypass some of the *old* problems and made a valuable addition to the cAMP toolbox (discussed below).

Unimolecular FRET Sensors for cAMP

The majority of unimolecular FRET sensors for cAMP currently available are based on genetically modified Epac proteins—exchange factors, directly activated by cAMP. Epac1 and Epac2 are closely related multidomain proteins that basically act as guanine nucleotide-exchange factors for the small GTPases Rap1 and Rap2. Binding of a single cAMP molecule to the CNBD of Epac1/2 triggers a conformational change with ensuing exposure of initially *shielded* catalytic domain, allowing it to bind and activate Rap1/2. In such fashion, Epac proteins, apart from PKA and CNGCs, are recognized as another immediate downstream effectors of cAMP (Bos, 2006).

The first reports on generation of Epac-based FRET sensors and their successful use for cAMP measurements in living cells trace back to 2004, when three independent research teams published their data (DiPilato et al., 2004; Nikolaev et al., 2004; Ponsioen et al., 2004). Since then the engineering activity in the field of unimolecular biosensors does not seem to cease and a host of improved sensor modifications have been developed, showing better signal-to-noise ration, enhanced temporal resolution and decreased sensitivity to light (Van der Krogt et al., 2008; Gorshkov and Zhang, 2014; Klarenbeek et al., 2015). All Epac-based sensors have a unimolecular structure and follow the similar design: a native or modified CNBD from Epac protein is typically decorated by two fluorophores involved in FRET. Binding of cAMP to the CNBD evokes a change in the sensor's tertiary conformation, which is accompanied by alteration of FRET intensity.

Unimolecular FRET sensors provide several advantages over multimolecular cAMP probes. As all the functional domains are localized within a single molecule, the kinetics of conformational changes is more favorable—hence, unimolecular sensors have a faster response to cAMP (= higher temporal resolution) in comparison to more *bulky* multimolecular PKA-based sensors that require binding of four cAMP molecules (Nikolaev et al., 2004). Again, with unimolecular sensors only a single vector is needed for transfection and the unbalanced expression of different subunits that might be observed with multimolecular PKA sensors is no longer an issue. A handful of catalytically *dead* Epac sensors have been developed as well, thus eliminating the possible interference from Epac downstream targets (Nikolaev et al., 2004; Ponsioen et al., 2004; Klarenbeek et al., 2011). Endogenous Epac molecules have a lower sensitivity to cAMP than PKA holoenzyme (Bos, 2006). In line with this, PKA-based sensors are readily activated by nanomolar levels of cAMP, but affinities of Epac probes typically lie within low micromolar range (Bacskai et al., 1993; Ponsioen et al., 2004). Yet, higher sensitivity of PKA sensors comes with the price of early saturation and a narrowed dynamic range. Considering this, Epac-based probes appear advantageous, as they allow for measurements of cAMP at higher, but more physiologically relevant levels.

Apart from Epac probes, a few other unimolecular FRET sensors for cAMP have been engineered. One example is PKA-camps sensor, that represents a truncated regulatory IIβ-subunit (RIIβ) of PKA flanked by yellow and cyan fluorescent proteins (YFP and CFP respectively) making a FRET pair. PKA-camps has no catalytic activity and operates in the same fashion as Epac sensors: binding of one molecule of cAMP to RIIβ triggers a conformational rearrangement, manifested by FRET efficiency changes (Nikolaev et al., 2004). HCN2-camps is another unimolecular FRET probe for cAMP, which is based on a single cAMP binding domain of murine hyperpolarization-activated CNGC sandwiched between YFP and CFP. HCN2-camps has a lower sensitivity to cAMP and extended dynamic range (1–100 μM). Therefore, HCN2-camps is not prone to early saturation and thus *tailored* to cell types with elevated basal levels and amplitude oscillations of cAMP, e.g., cardiomyocytes (Nikolaev et al., 2006; see also the section of this review on CNGCs).

Though most of the FRET biosensors are designed for direct binding (and hence sensing) of cAMP, there are several related genetically-encoded FRET probes, that can be used for indirect measurement of cAMP levels, using PKA activity as readout. The so-called AKAR sensors (A-Kinase Activity Reporters) operate in the very similar fashion to unimolecular FRET sensors for cAMP and hence are briefly discussed herein. AKARs have a prototypical tetra-domain structure comprised of a PKA-specific substrate and a phosphoamino acid-binding unit sandwiched between two fluorophores making a FRET pair. Upon cAMP binding to PKA holoenzyme the released and activated C subunits phosphorylate the substrate sequence of AKARs, thus making it an appealing *bait* for the neighboring phosphoamino binding domain. This results in a change of sensor tertiary conformation, leading to alteration in FRET efficiency (Zhang et al., 2001; Allen and Zhang, 2006; Erard et al., 2013). It should be noted that AKARs have all the functional domains residing in a single protein, and as such enjoy all of the benefits of modern unimolecular sensors, including possibility of targeting to defined intracellular compartments (Allen and Zhang, 2006; Depry et al., 2011). On one hand, the indirect nature of the readout provided by AKARs and the propensity of PKA to amplify the incoming signal from cAMP, alongside with difficulties of calibration, render AKARs suboptimal tools for cAMP measurement, while on the other hand, when used for *direct indication*, i.e., for PKA activity studies, AKARs can show excellent performance.

Both multi- and unimolecular FRET sensors share a principle advantage—targetability to discrete subcellular compartments. By introducing mutations to native domains (e.g., alteration or removal of Disheveled/Egl/10-Pleckstrin domain of Epac, responsible for membrane anchoring) or fusing Epac proteins or PKA subunits with appropriate targeting signals, e.g., nuclear or mitochondrial localization signals, or membrane anchoring motifs (farnesylation, palmitoylation, or polybasic sequences), several sensors were successfully routed to desired compartments, including plasma membrane, cytoplasm, mitochondria, and nucleus (DiPilato et al., 2004; Ponsioen et al., 2004; Dyachok et al., 2006; Terrin et al., 2006; Sample et al., 2012). This very ability to provide outstanding spatial resolution, unattainable with other biosensors, combined with quick response and reversible nature of conformational changes induced upon cAMP binding, underpins the true power of FRET sensors as tools for cAMP measurement in living cells. Currently FRET sensors remain the tools of choice once cAMP real-time dynamics in defined subcellular regions comes to question.

All modern FRET sensors for cAMP are genetically-encoded proteins and hence can be used not only in cell cultures, but in laboratory animals as well, providing insights into cAMP life-cycle in non-perturbed microenvironment (Nikolaev et al., 2006; Calebiro et al., 2009; Jacobs et al., 2010; Sprenger et al., 2015). The ratiometric nature of FRET brings additional benefits, allowing to correct for cell-to-cell variation in sensor expression levels and to minimize the effects of auto-fluorescence and light scattering. Besides, FRET biosensors do not require additional expensive substrates for signal generation, as it is the case for all bioluminescence-based methods (see below).

Nevertheless, just like as all the other methods in life sciences, FRET sensors for cAMP have certain limitations. Firstly, FRET methodology is heavily dependent on microscopy and image analysis. Though a basic microscopy set-up suitable for FRET should be affordable for most labs (Börner et al., 2011), conversion of images into figures (i.e., from pixel intensity to cAMP levels) requires manual image processing, which is not only laborious, but might inadvertently lead to introduction of bias, e.g., while defining regions of interest to be analyzed (Rich et al., 2014). However, computer-assisted tools for automated image processing and emerging methods of signal acquisition and interpretation such as hyperspectral imaging already offering new solutions to old problems and hold promise for the future (Francis et al., 2012; Leavesley et al., 2013).

Another facet of complicated image processing is a relatively poor suitability of FRET-based biosensors for high-throughput applications. Though it is possible to run FRET assays in multiwell

plate format on a fluorescence platereader and some attempts to increase throughput have been made (Allen and Zhang, 2006), currently other tools (see the section of this review on luciferase-based biosensors) appear to be preferential when high processivity or compound screen are desired.

When dealing with fluorescent proteins, it is prudent to account for possible effects of pH, temperature and other environmental factors, as these variables are known to influence performance of fluorophores and hence FRET efficiency (Correa and Schultz, 2009; Betolngar et al., 2015). This issue might become more pronounced, when targeted FRET biosensors are used, as sensor exposure to certain environmental factors might be different in distinct subcellular domains (e.g., pH and ROS levels in mitochondria; Putnam, 2012; Rich et al., 2014). Resistance to photobleaching is another variable affecting ultimate performance of a FRET pair to keep in mind once choosing the right sensor for experiment. At the same time, there has been a significant progress in the field, with recent generations of FRET sensors for cAMP appear to be more resistant to pH and photobleaching (Klarenbeek et al., 2011, 2015; Salonikidis et al., 2011).

It should be mentioned, that the conversion of FRET signal into absolute values of cAMP is not a trivial task. The most frequently used approach relies on calibration of a purified sensor against different concentrations of cAMP *in vitro*—however, this set-up is obviously unable to mimic physiologic conditions a sensor is exposed to inside living cells (Börner et al., 2011). An alternative and more physiological calibration method that has been recently introduced involves perfusion of sensor-carrying cells with different concentrations of cAMP via patch-clamp pipette, so the sensor remains and responds to cAMP in the native milieu (Koschinski and Zaccolo, 2015). Still, this protocol is obviously quite technically demanding and requires ample expertise with patch-clamp.

Last, but not least, FRET biosensors, like any other genetically encoded proteins, tend to have relatively high, non-physiological levels of expression, which may lead to excessive binding and thus *buffering* of cAMP. *Buffering* ability of a sensor becomes of more concern once targeted proteins are used, resulting in high local concentrations of cAMP-avid sensor molecules within certain subcellular compartments. The *buffering* phenomenon might distort natural patterns of cAMP synthesis, transport and breakdown, limiting a sensors ability to convey actual information on cAMP changes in living cells (Willoughby and Cooper, 2008). Noteworthy, cAMP *buffering* is not a unique limitation of FRET probes—this problem is inherent to all genetically encoded cAMP sensors that function as cAMP binders.

Bioluminescence Resonance Energy Transfer (BRET)-Based Sensors

In essence, BRET and FRET are closely related phenomena, that are governed by the same physical laws and rely on the same principle of non-radiative energy transfer between a couple of closely opposed molecules (Forster, 1946). The only difference between FRET and BRET is in the type of a donor molecule: in case of FRET a fluorophore acts as a donor of energy, but in BRET a bioluminescent protein is used instead.

Enzymatically active variants of *Renilla reniformis* luciferase (Rluc) are typically used for donor molecules in the majority of BRET pairs. Oxidation of the substrate (coelenterazine) by Rluc results in the release of energy, which is either emitted as photons of light or transferred to an acceptor fluorophore, provided the latter is in the close proximity. In this fashion, no external source of light to excite donor molecule is required, which underpins most of the advantages of the BRET approach, including absence of photobleaching, phototoxicity and autofluorescence (Bacart et al., 2008). Besides, BRET is applicable to studies of light-dependent and light-sensitive processes, e.g., visual perception and photosynthesis (Saito et al., 2012). Lastly, such experimental tools as optogenetics (discussed below) and caged compounds liberated upon exposure to light can be combined with BRET with minimal chances of interference, which is not the case with FRET, requiring strong external illumination (Ellis-Davies, 2007; Fenno et al., 2011).

Only a handful of genetically encoded sensors suitable for live cell measurements of cAMP based on BRET phenomenon have been developed so far. All of these essentially exploit the same design principle and operate in similar fashion as the multi- and unimolecular FRET cAMP sensors reviewed earlier. The only available multimolecular BRET sensor for cAMP probing to our knowledge is based on PKA holoenzyme, which has its regulatory and catalytically subunits decorated with Rluc and a green fluorescent protein (GFP) variant, respectively. Hence, PKA holoenzyme dissociation upon cAMP binding is paralleled by pronounced drop in BRET, captured as a ratio of the emission values (Prinz et al., 2006). Unimolecular BRET cAMP indicators typically feature a cAMP binding motif of either Epac or PKA regulatory subunit sandwiched between a luciferase and a fluorescent protein, making a BRET pair (Jiang et al., 2007; Barak et al., 2008; Saito et al., 2012).

A slightly different design principle was exploited by Saito et al. (2012), who inserted Epac1-derived cAMP binding motif into the non-structural loop of a chimera YFP (Venus)-Rluc. The resulting sensor, named Nano-lantern (cAMP1.6), has the Rluc separated by the Epac1 motive into two complementary parts, rendering enzyme inactive as far as the cAMP-binding domain remains vacant. cAMP binding to the sensor evokes a conformational change and the restoration of Rluc structure and enzymatic activity, leading to BRET with the chimeric Venus protein. In such fashion, Nano-lantern (cAMP1.6) combines BRET and complementation of split luciferase principles, providing lower background signal and increased sensitivity for the assay.

Similar to FRET probes, BRET sensors are applicable to transgenic animals and can be targeted to desired subcellular compartments by means of introduction of appropriate localization signals (Barak et al., 2008; Saito et al., 2012). However, the intensity of BRET signal is still much weaker than emission from FRET indicators, which presently hinders efficient use of BRET sensors for single cell measurements or for tracing cAMP fluctuations in subcellular domains (Willoughby and Cooper, 2008; Saito et al., 2012).

In general, BRET sensors have a favorable dynamics of spatial rearrangements (several seconds) in response to changes in cAMP levels. The fast and reversible nature of BRET response makes

the indicators of this class well-suited for real-time measurement of cAMP in pools of living cells (Jiang et al., 2007). Another principal advantage of BRET sensors is their applicability for high-throughput screening (HTS). As generation of BRET signal is not dependent on external source of light, the signal capture becomes much easier. BRET sensors do not need dedicated microscopy set-up, required for FRET sensors, and the assays can be easily carried out in multiwell plates on a platereader equipped with the appropriate filter set. Simplified readout expressed as a ratio of two emissions obviates the tedious image processing typical of FRET and adds to the processivity of the assay (Boute et al., 2002; Jiang et al., 2007; Bacart et al., 2008).

At the same time, apart from the highlighted specialties, BRET and FRET-based cAMP indicators are largely similar. Hence, most of the strong points and shortcomings inherent to unimolecular and multimolecular FRET sensors reviewed in the previous section remain valid for BRET sensors of similar design as well.

Direct Sensors for cAMP, Based on Luminescent Enzymes

Though luminescent enzymes have an impressive record as reporters in functional studies of gene expression, their use as biosensors for direct probing of various intracellular molecules has started less than a decade ago (Hill et al., 2001; Jiang et al., 2008; Binkowski et al., 2009).

At large, all sensors of this class are genetically encoded proteins of similar operational structure, which are based on a variant of luciferase fused with functional domains responsible for sensing of desired analytes. Interaction of a sensing domain with its ligand typically triggers conformational rearrangement of the luciferase, altering its enzymatic activity and changing the output of light.

Bioluminescent sensors only require appropriate substrate (e.g., luciferin or coelenterazine) and molecular oxygen for generation of photons and hence are not dependent on exogenous light source. As most of biological samples do not produce any light *per se*, this feature of luminescent probes not only endows them with excellent sensitivity and signal-to-noise ratio, but also helps to avoid a multitude of problems inherent to fluorescent proteins and fluorophore-based sensors (discussed in the previous section).

A few dedicated biosensors for cAMP based on mutated variants of luminescent enzymes have been engineered so far. An elegant approach is the dual-wavelength cAMP indicator engineered by Takeuchi et al. (2010). This unimolecular genetically-encoded sensor is based on *N*-terminal fragments of two different click beetle luciferases (ElucN and CBRN) and one *C*-terminal fragment (McLuc1) which could dimerize with any the above *N*-terminal fragments, forming two distinct luminescent enzymes with well-separated emission peaks (613 nm for ElucN and 538 nm for CBRN).

The cAMP binding domain of PKA RIIβ, that is linked to the luciferase fragments by peptide bridges, serves for cAMP sensing unit. In the absence of cAMP, McLuc1 and ElucN form a functional enzyme that generates red light. Upon cAMP binding to PKA RIIβ the sensor undergoes conformational rearrangement, leading to separation of the red light-producing luciferase and *migration* of McLuc1 toward CBRN, culminating in restoration of CBRN holoenzyme and green light production. This way, the sensor was originally designed to provide ratiometric readout of cAMP levels expressed as a shift of red to green light emission intensities.

Sadly, the resulting sensor refused to *behave* strictly according to the initial design assumptions: although cAMP binding did result in generation of green light, this was not paralleled by a drop in red light emission. This irresponsiveness of the red light luciferase to probe occupancy with cAMP narrows the signal-to-noise ratio of the sensor and deprives it of all the benefits that genuine ratiometric readout provides, e.g., insensitivity to differences in sensor expression levels or variations in substrate availability. Nevertheless, apart from this issue, the indicator has a good cAMP sensitivity range (1.0×10^{-7} to 1.0×10^{-4} M) and is easy to use for semi-quantitative cAMP probing in cell populations, including high-throughput setting. Though the relatively weak light intensity precludes studies of cAMP in subcellular compartments, the sensor can produce fair kinetic data on total cAMP oscillations in cellular pools over extended periods of time.

Another example of cAMP luminescent indicators is a dyad of similar sensors (pGloSensor-20F and its successor with improved characteristics pGloSensor-22F), based on a on circularly permutated variant of a firefly luciferase fused with cAMP-binding domain B of PKA RIIβB, which were developed by Wood and colleagues (Fan et al., 2008; Binkowski et al., 2011a). In these probes, RIIβB domain acts as an allosteric regulator of the adjoined luciferase—"*landing*" of a single cAMP molecule in the RIIβB pocket allows the silent luciferase to regain the enzymatically active conformation, which in the presence of the substrate (luciferin) immediately leads to light production. With a detection limit in low nanomolar range and outstanding dynamic window (0.003—100 μM for the improved 22F version of the sensor), GloSensor proteins are some of the most sensitive cAMP sensors presently available. These features, combined with the benefits of using the light emission as a readout (very low levels of non-specific signal) and broad linearity of response to cAMP, endow the probes with unsurpassed signal-to-noise ratio of up to 800-fold (Binkowski et al., 2011a). On top of it, fast dynamics and reversibility of sensor conformational changes in response to alterations in cAMP levels (within several seconds) make GloSensor probes excellent tools for direct tracing of real-time cAMP dynamics in living systems.

Encoded by a single plasmid, GloSensor probes avoid difficulties related to non-equality of expression of different domains making a functional sensor, that are inherent to, e.g., CNGCs and multimolecular FRET probes, and can be used in a variety of cell types and in principle should be applicable to laboratory animals. Importantly, due to simplicity of signal capture, attainable by a plain luminometer or luminescent platereader, and ease of data analysis, the probes are quite user-friendly. Most of the above features render GloSensor proteins readily suitable for high-throughput tasks, e.g., massive library screens of putative GPCRs interacting compounds (Pantel et al., 2011).

At the same time, the luminescent signal from GloSensor probes is not easily convertible to absolute cAMP values. In principle, the sensors can be calibrated against any end-point

assay measuring cAMP levels in cell lysates. Calibration of this kind, however, is not quite straightforward and reliable, as the intensity of luminescence in living cells transfected with GloSensor probes appears to be affected by several factors, including temperature, availability of the substrate and sensor protein expression levels, which might not be easy to account for (Binkowski et al., 2011b). Due to limited spatial resolution GloSensor probes are mainly suited for measurements of cAMP in cell populations rather than in single cells. Though GloSensor proteins technically may be targetable to certain subcellular compartments by fusion with localization signals, no reports on generation of that kind of modified sensors have been published so far. Altogether, when studies of intracellular cAMP spatial organization are in question, biosensors with higher spatial resolution, e.g., FRET-based probes, offer a better solution.

Of note, GloSensor proteins have been harnessed for indirect measurements of cAMP in non-transfected primary cell cultures by means of co-incubation of primary cells (donor cells) with a cell line stably transfected with GloSensor probe (*sensor* cell line), in an assay called CANDLES (cyclic AMP iNdirect Detection by Light Emission from Sensor cells; Trehan et al., 2014). This coculture set-up allowed for bi-directional transport of cAMP molecules via gap junction channels formed by neighboring cells. In such fashion, a rise in cAMP concentration in donor cells, triggered upon activation of GPCRs expressed exclusively in these cells and not in the *sensor* cells, could be registered in a kinetic fashion by a luminescent signal from the *sensor* cells.

Although the authors showed the interspecies detection of cAMP by this method, this assay might not be applicable to all cell types, even from the same organism, as some cells might be *reluctant* to form gap junctions with *sensor* cells, thus rendering intercellular flow of cAMP impossible. Another drawback of this method lies in its dependence on PDE inhibitors [such as 3-isobutyl-1-methylxanthine (IBMX)] to inhibit cAMP degradation (Trehan et al., 2014). Inability to register significant flow of cAMP molecules between the cells once cAMP breakdown machinery was fully active and not silenced with IBMX limits the *resolution* of the method, as the readout captured in the absence of PDE activity reflects severely distorted patterns of cAMP kinetics and intracellular distribution.

Apart from this, it should be recognized, that the above coculture set-up allowing for cAMP studies in primary cultures in principle might be implemented with any other modern indicator for cAMP expressed in *sensor cells,* e.g., with Epac and PKA-based FRET probes, as was successfully demonstrated by Ponsioen et al. (2007).

Lastly, when planning experiments with luminescent-based enzymes, do not forget to account for one additional universal drawback of this methodology—its dependence on a substrate for the luciferase. This not only increases the expenditure on the assay, but may also affect the resulting readout, as the concentration and availability of the substrate are important factors for signal generation. Moreover, some substrates are short living, affected by pH and temperature, and hence limiting the assay's capabilities and usability.

cAMP Sensors, Based on Conformation-Sensitive Fluorophores

The story of conformation-sensitive fluorophores starts from the pioneering work of Tsien and colleagues, that culminated in development of a novel class if Ca^{2+}-biosensors, coined "*camgaroos*" (Baird et al., 1999). Subsequently, the main principle exploited in *camgaroos* was tailored to other applications, providing the research community with a handful of robust genetically-encoded indicators for various intracellular targets in living cells, including Ca^{2+}, cGMP, and some kinases (Whitaker, 2012; Gorshkov and Zhang, 2014).

In essence, the indicators from this family rely on different circularly permutated variants of enhanced GFP, which fluorescent properties are conformation-dependent. A prototypical conformation-sensitive indicator consists of two principal domains, a sensor and a fluorescent reporter, that are fused together. A sensor domain is designed to bind a desired target [e.g., calmodulin (CaM) for Ca^{2+} cations], and the binding event is ultimately relayed into a conformational change of a reporter protein, leading to a change in its fluorescence. Though the readout is typically expressed as a ratio of two emissions intensities (i.e., registered in basal and stimulated states of a sensor), its fundamentally different from FRET, as all the fluorescence is coming from a single fluorophore.

Despite the appealing design principle, no conformation-sensitive non-FRET sensors for direct probing of cAMP have been developed so far. Bonnot et al. (2014) reported the generation of single fluorophore sensor for PKA activity in living cells, that represents a modification of the original AKAR2 FRET biosensor (see also the section of this review on FRET-based probes). Conformation-sensitive mutant of GFP in this probe responds to phosphorylation of the attached PKA substrate by a change in fluorescence intensity and lifetime. Though this genetically encoded indicator may be used for indirect probing of cAMP levels, it is obviously inferior to direct sensors for cAMP and should be reserved for the original application—studies of PKA activity.

Another related methodology based on conformation sensitive fluorophores that is applicable for cAMP measurements in living systems have been recently published (Ding et al., 2015). The assay general principle is based on heterologous co-expression of a trio of interacting proteins in a single cell. While two of the proteins carry fluorophores with different excitation/emission spectra, the third member of the trio lacks any fluorescent properties, but is able to dimerize with either fluorophores, acting as their allosteric regulator. In monomeric (or unbound) state, the fluorescent proteins are quenched and unresponsive to light stimulation, but dimerization with the allosteric regulator triggers conformational rearrangement of the pair, leading to dramatic rise in fluorescence. Provided that the rate and preferential direction of dimerization events between the regulator and the fluorophore-carrying proteins are governed by defined stimuli (e.g., intracellular calcium levels or certain enzymatic activity), that can be achieved by fusion monomeric proteins with various functional domains or *bridging* them with recognition sequences for desired enzymes, this trio of proteins make an extremely versatile platform, that have already been successfully used for

probing of cAMP, calcium–calmodulin interactions, caspase-3 and extracellular signal-regulated kinase activity (Ding et al., 2015).

For intracellular cAMP measurements in living cells, one of the fluorophores (red) and the regulatory protein were fused with the C subunit and R subunit of PKA, respectively, while the second fluorescent protein (green) was designed to remain in the cytoplasm in unbound state. This way, upon basal cAMP levels the red fluorophore and the regulatory subunit remain in close proximity, leading to strong emission of red light against the faint emission of green light from the quenched unbound second fluorophore. A rise in intracellular cAMP leads to PKA holoenzyme dissociation and liberation of the regulatory protein-R subunit chimera into cytoplasm, allowing it to bind the second fluorophore with resulting increase in the green-to-red emission ratio. As both fluorophores respond to cAMP in antipodal directions, it improves sensitivity of the assay, allowing for *recognition* of low-amplitude changes in cAMP concentration. However, due to the necessity of cotransfection with a set of vectors and inevitable differences in expression levels of the regulator and the fluorescent proteins, the assay provides qualitative rather than quantitative readouts. Measurements of absolute values of cAMP are further hindered by different binding affinities of the regulatory protein for its fluorescent counterparts.

To summarize, genetically encoded sensors for cAMP based on conformation-sensitive fluorophores make a new class of tools, which holds good promise for the future. These sensors are principally targetable to discrete subcellular compartments by fusion with relevant localization signals and thus might be used to gain insightful data on spatial organization of cAMP signaling. Simplified readout and data analysis (in comparison to FRET) makes conformation-sensitive probes more *user-friendly* and brings them closer to HTS. However, issues with calibration, absence of comprehensive data on kinetics of conformational alterations, narrow signal-to-noise ratio and vulnerability to pH changes leave some space for improvement and we are to await till this sensor class has found its niche in cAMP research field.

Tools for Indirect cAMP Measurement in Living Systems

As was previously mentioned, indirect probes are originally designed to measure cAMP downstream effects rather than its absolute concentrations. Therefore, though one can get a good idea of cAMP levels with selected indirect probes, these tools are generally inferior to direct sensors in this regard. But if used wisely, indirect probes can provide a wealth of information on cAMP signaling outcomes and hence remain actively involved in cyclic nucleotide studies (Hill et al., 2010).

Probes, Based on CNGCs

Cyclic nucleotide-gated channels comprise a big family of structurally related membranous ion channels, which are activated by cGMP and cAMP. Upon binding with the above cognate ligands, CNGCs undergo conformational change and become permeable for Na^+, K^+, and Ca^{2+}, allowing these cations to enter cytoplasm, which ultimately leads to either a depolarization or a hyperpolarization event. In such fashion, CNGCs basically function as switches that sense changes in intracellular cyclic nucleotide levels and transform them into a change of membrane potential and Ca^{2+} concentration. Originally discovered in retinal photoreceptor and olfactory neurons, CNGCs have a pivotal role in signal transduction of vision and olfaction. Besides this, CNGCs have been shown to be of importance in functional regulation of several other cell types and organs, including kidney, sperm cells, endocrine tissues and pacemaker cells of the heart (Kaupp and Seifert, 2002; Craven and Zagotta, 2006; Biel and Michalakis, 2009).

Though CNGCs and related hyperpolarization-activated cyclic nucleotide-gated channels (HCNs) share the common CNBD and bear general structure similarity, they operate in somewhat different mode: CNGCs are solely cyclic nucleotide-dependent and become permeable for Na^+, K^+, and Ca^{2+} upon activation, while HCNs are mainly governed by voltage with cGMP and cAMP acting only as *fine regulators* that adjust activation threshold (activation voltage and activation time). In active state, HCNs preferentially allow entry of Na^+ and K^+ but provide very limited *access* to calcium ions (Kaupp and Seifert, 2002; Biel and Michalakis, 2009; Akimoto et al., 2014). Of importance, prolonged exposure to cyclic nucleotides does not lead to CNGCs desensitization (Dhallan et al., 1990).

In such a way, a change in transmembrane electric current and/or intracellular Ca^{2+} levels in CNGCs harboring cells in principle may be used as an indirect measure of cAMP oscillations. However, naturally occurring CNGCs are poor sensors for cAMP due to several reasons: The major *limitation* of wild-type CNGCs lies in their low responsiveness to cAMP and preferential avidity for cGMP (Rich et al., 2001; Kaupp and Seifert, 2002). Secondly, CNGCs activation threshold and sensitivity to cyclic nucleotides are under negative feedback control of calcium–calmodulin complex (Ca^{2+}-CaM), which not only narrows the dynamic range of wild-type CNGCs-based assays, but brings possibility of interference with other signaling events, that affect intracellular Ca^{2+} levels (Liu et al., 1994; Trudeau and Zagotta, 2003). Another possible limitation to keep in mind is that wild-type CNGCs may be directly activated by nitric oxide (NO; Broillet, 2000).

In order to make CNGCs usable as cAMP sensors, a set of genetically modified CNGC subunits has been generated and tested in heterologous expression systems. Several combinations of different mutant subunits were shown to form functional CNGCs with desired properties, including higher affinity to cAMP and decreased cGMP responsiveness, loss of negative regulation by Ca^{2+}-CaM due to alteration of CaM binding site and inability to respond to NO. These genetically engineered variants of CNGCs made the platform for cAMP measurements in a majority of successive studies (Altenhofen et al., 1991; Liu et al., 1994; Varnum et al., 1995; Rich et al., 2001; Reinscheid et al., 2003; Wunder et al., 2008).

Intracellular cyclic nucleotide levels in living cells were measured with CNGCs for the very first time by Trivedi and Kramer (1998), who used a change in membrane potential

registered by a keen technique named *patch-cramming* for real-time probing of cytoplasmic cGMP levels in single living cells. Patch cramming involves heterologous expression of appropriate sensor channels in donor cells (chimeric CNGCs consisting of α—subunits from bovine rod and rat olfactory receptors were expressed in *Xenopus* oocytes in the above study), removal of a piece of cell membrane carrying CNGC and its calibration with different concentrations of cGMP via inside-out excised patch, with subsequent insertion (*cramming*) of the calibrated membrane into a recipient cell so the inside part of the sensor membrane is in direct contact with the cytoplasm of a recipient cell. This way, the sensor membrane on a tip of a pipette responds to changes in cytoplasmic cGMP levels of recipient cell by activation of CNGCs, leading to alterations in electric current that can be readily registered with patch clamp. The "patch cramming" approach allowed Trivedi and Kramer (1998) to follow cGMP levels in living neuroblastoma cells and rat neurons in real-time fashion, providing kinetic readout of cGMP oscillations scarcely attainable before. However, this methodology was obviously quite labor-intensive, involved multiple technically demanding steps and was applicable only to relatively large cells with a diameter of about 40 μm and higher.

A refinement followed a few years later—by means of viral transduction heterologous CNGCs and HCNs were directly expressed in the cells of interest and cytoplasmic cAMP fluctuations upon exposure to different stimuli were registered in single living cells in real time by means of whole-cell, inside-out, and perforated patch clamp (Rich et al., 2000, 2001; Heine et al., 2002). Thus, the limitations of the cell size and the need of harvesting a piece of CNGCs-carrying sensor membrane from donor cells were eliminated, facilitating more rapid and flexible assay flow.

Despite the above advances, CNGC-mediated measurements of cAMP were still invariably based on registration of electric currents by patch-clamp, which restricted the processivity of the method (one measurement—one cell) and required sophisticated probing tools and a skillful operator. The above limitations have been partially circumvented in the recent decade via development of automated patch-clamp instruments, though these tools are not readily available to majority of laboratories and remain to be tested for most of the cell types (Farre and Fertig, 2012).

Another general shortcoming of patch-clamp technique (excluding whole-cell setup) is that it can probe electric currents only in the immediate vicinity of a patch pipette tip, which restricts the measurements of cAMP to cell compartments that can be patched. For CNGCs, which expression is confined to plasma membrane, it means that patch-clamp allows to probe cAMP changes occurring exclusively in submembranous regions, providing little information on the events in other cellular compartments (Rich et al., 2000, 2014).

The aforementioned drawbacks can be bypassed by harnessing Ca^{2+} cytoplasmic oscillations as a measure of cAMP-driven CNGCs activation. Various methods for Ca^{2+} probing have been successfully applied to this aim, but most frequently Ca^{2+}-sensitive cell permeant dyes such as fura-2/AM, indo-1/AM and fluo-3 AM/fluo-4 AM have been used (Grynkiewicz et al., 1985; Fagan et al., 2001; Rich et al., 2001; Visegrády et al., 2007). These established Ca^{2+} indicators provide either direct (increase in fluorescence quantum yield upon Ca^{2+} binding—fluo-3 and -4 dyes) or ratiometric (fura-2 and indo-1 dyes) measure of intracellular Ca^{2+} levels. Though both types of dyes are quite sensitive and easy to use, ratiometric indicators appear to be advantageous, as readout expressed as an emission/excitation wavelength shift allows to avoid unwanted effects of uneven loading, photobleaching and noise from non-specific autofluorescence, thus yielding higher signal-to-noise ratio and less variability.

Besides imaging of CNGCs-mediated Ca^{2+} surges in cytoplasm of a single cell with standard fluorescent microscopy, fluorescent Ca^{2+} dyes are readily applicable for measurements in cell populations, i.e., with the help of a multiwell platereader/spectrofluorometer equipped with appropriate filter sets, thus rendering this assay principle readily suitable for HTS (Reinscheid et al., 2003; Visegrády et al., 2007).

Apart from Ca^{2+}-sensitive dyes, another approach initially developed for Ca^{2+} probing in living cells have been subsequently harnessed for cAMP measurements. Oscillations of intracellular Ca^{2+} triggered by cAMP-dependent activation of CNGCs can be registered with aequorin-based sensors (Sheu et al., 1993; Wunder et al., 2008; Richter et al., 2015). Consisting of two subunits, the apoprotein apoaequorin and its cofactor coelenterazine, aequorin is a well-studied bioluminescent protein from the jellyfish *Aequorea victoria*. Binding of free Ca^{2+} Ca^{2+} to aequorin triggers oxidation of coelenteramide, leading to generation of light and the holoprotein complex dissociation. The amount of photons emitted is proportional to Ca^{2+} ions concentration and can be easily registered with either a charge-coupled device (CCD) camera or a luminometer (Shimomura, 1985). In such fashion, successful co-expression of CNGCs and apoaequorin allows to measure cAMP in virtually any cell type. Besides, this methodology is not technically demanding and HTS-friendly (Wunder et al., 2008).

However, the generation of cell lines stably transfected with CNGCs and apoaequorin is quite time-consuming and might happen to be problematic, especially if one does not have all the components making the functional system in a single vector. Another limitation of this type of assay is the need of spiking the cells with Ca^{2+} in order to trigger aequorin break-up and light emission. This, together with short-lasting nature of Ca^{2++} responses ($\lesssim$30 s), limits the processivity of the method. At the same time, the latter issue might be bypassed by running the assay in a multiwell format (e.g., 96- or 384-well plates) on a platereader that is equipped with liquid dispensing system.

Apart from the Ca^{2+}-sensitive fluorescent dyes and apoaequorin-based assays, past decades witnessed the generation of another type of Ca^{2+} sensors, that have literally changed the field of calcium signaling research—this entails the class of genetically-engineered fluorescent proteins, capable of measuring Ca^{2+} oscillations by means of change in FRET intensity (Miyawaki et al., 1999; Whitaker, 2012). Though FRET sensors can provide excellent spatial resolution down to subcellular domains (discussed earlier) and obviously have a great potential for studies of crosstalk between cAMP and Ca^{2+} signaling, they have not been widely used in conjunction with CNGC.

Yet another quite similar approach for indirect intracellular cAMP measurement in living cells is based on membrane potential sensitive dyes such as $DiSBAC_2(3)$ and HLB 021-152. These molecules are impermeable to cells until plasma membrane depolarization occurs; once in the cytoplasm they exhibit a dramatic rise in fluorescence after binding with intracellular solutes and proteins, which can be registered with a conventional spectrofluorimeter or fluorescent microscopy. This assay format has been optimized for HTS as well (Tang et al., 2006; Visegrády et al., 2007).

To conclude, genetically-engineered CNGCs with increased sensitivity to cAMP represent a versatile analytical tool that can be tailored to wide array of research needs (**Figure 2**). Measurements of inward currents and membrane potential with patch-clamp allow one to have all the advantages that the very nature of CNGCs provides, including high spatial resolution, *snap-shot* responses to change in cyclic nucleotide levels within dozens of milliseconds, lack of saturation, excellent sensitivity and fair dynamic range, possibility to obtain absolute values of cAMP levels by means of excised patch calibration. However, these *benefits* are only available to dedicated and well-equipped labs with a strong record in electrophysiology and typically come at the expense of low processivity.

Ca^{2+}-sensitive dyes and apoaequorin-based sensors are easier to use and suitable for HTS, although absolute levels of intracellular cAMP usually remain elusive due to infeasibility of calibration. Another shortcoming of using Ca^{2+} levels as a measure of cAMP is the possible interference from other pathways involving Ca^{2+} signaling.

Necessity of CNGCs expression in heterologous systems by means of transfection/transduction makes an inevitable common limitation of CNGCs-based assays. This issue becomes especially important once the different subunits comprising a functional CNGC are delivered by separate vectors or another exogenous genetically encoded sensor is used in conjunction (e.g., apoaequorin). Another thing to keep in mind is that high spatial resolution of cAMP measurements in submembranous regions achievable with CNGCs-based methods turns into a disadvantage once cAMP dynamics in another cellular domains comes into question, as membrane-anchored CNGCs can respond to cAMP only in the immediate vicinity of thereof. Last, but not least, despite rapid activation and kinetic-like pattern of signal, with CNGCs one should not expect to obtain genuine kinetic data on cAMP fluctuations over time, as the measurable response to cyclic nucleotides is indirect in essence and mediated by either inward ionic currents or Ca^{2+} levels. In inherently complex living systems there is virtually no place for strictly linear relations between any two given cross-talking pathways—and each of these has its own mode of signal transduction and tends to be intertangled with a number of *third parties*. Ca^{2+} signaling and transmembrane electric currents do not make an exception from the above.

Reporter Gene-Based Systems

Reporter gene-based systems perhaps make the most illustrative example of indirect assay for cAMP, as these tools score on one of the most remote effects of cAMP, i.e., its effect on transcriptional activity. A rise in intracellular cAMP triggered by a variety of stimuli (most frequently, due to activation of ACs via $G\alpha_s$—coupled GPCRs) leads to dissociation of PKA with successive entry of liberated C subunits into cell nucleus and the phosphorylation of cAMP-response element (CRE)-binding proteins (CREBs). Phosphorylated CREBs then bind to CRE elements within promoter regions of a multitude of genes, thus altering their transcription (Neves et al., 2002; Wettschureck and Offermanns, 2005; Musnier et al., 2010). In such way, a change in protein levels may be used as indirect measure of cAMP oscillations.

CRE consensus sequence (TGACGTCA) and its variations might be found in promoter regions of thousands of genes across human genome (Lalli and Sassone-Corsi, 1994; Zhang et al., 2005). However endogenous promoters have not been frequently used for cAMP studies, as they tend to encompass binding sites for other transcriptional factors as well, thus rendering reporter protein synthesis not solely cAMP-dependent (Hill et al., 2001). Genetically-engineered promoters harboring only CRE elements do not have this limitation and thus provide a viable alternative. Along with this, synthetic promoters are not totally problem-free and require thorough validation in order to yield the best transcriptional response, including testing of different CRE elements and various numbers of repetitive CRE sequences, as well as the reporter gene (Dinger and Beck-Sickinger, 2002; Shan and Storm, 2010; Hald et al., 2015).

The choice of reporter proteins is broad and the selected reporter generally should be tailored to particular needs. The possible options include different enzymes allowing for absorbance (e.g., alkaline phosphatase and β-galactosidase) or luminescence-based readout (different types of luciferases), fluorescent proteins and combinations of thereof (Hill et al., 2001; Jiang et al., 2008). If the assay in question is well-optimized and sensitive enough, all of the above reporters are able to provide a reasonable estimate of cAMP effects in living cells.

Despite relative ease of use, all reporter gene-based assays share a number of limitations, which should be taken into account already at the stage of experimental design. As all the reporter-based assays are dwelling on the readout coming from a protein product of a synthetic construct, this construct has to be delivered to cell nucleus for successful expression by means of transfection or viral-mediated transduction. Obviously, this brings along all the possible issues with transfection/transduction, including low success rates in certain cells types, significant inter-run variation in terms of transfection efficacy, excessive toxicity due to gene delivery procedure *per se* and poor applicability of the method to *in vivo* settings (Kim and Eberwine, 2010). However, as already discussed earlier, similar problems are in essence inherent to all of the genetically-encoded proteins and hence to the bulk of cAMP biosensors presently available.

Some of the transfection-related difficulties like run-to-run variation in reporter expression levels, leading to high heterogeneity of response and/or poor signal-to-noise ratios, may be circumvented by generation of stable cell lines with relatively uniform and equal expression of reporter. Of note, this strategy, although offering a good solution, is not flawless, as even with stable clones, the number of transgene copies, the location of the

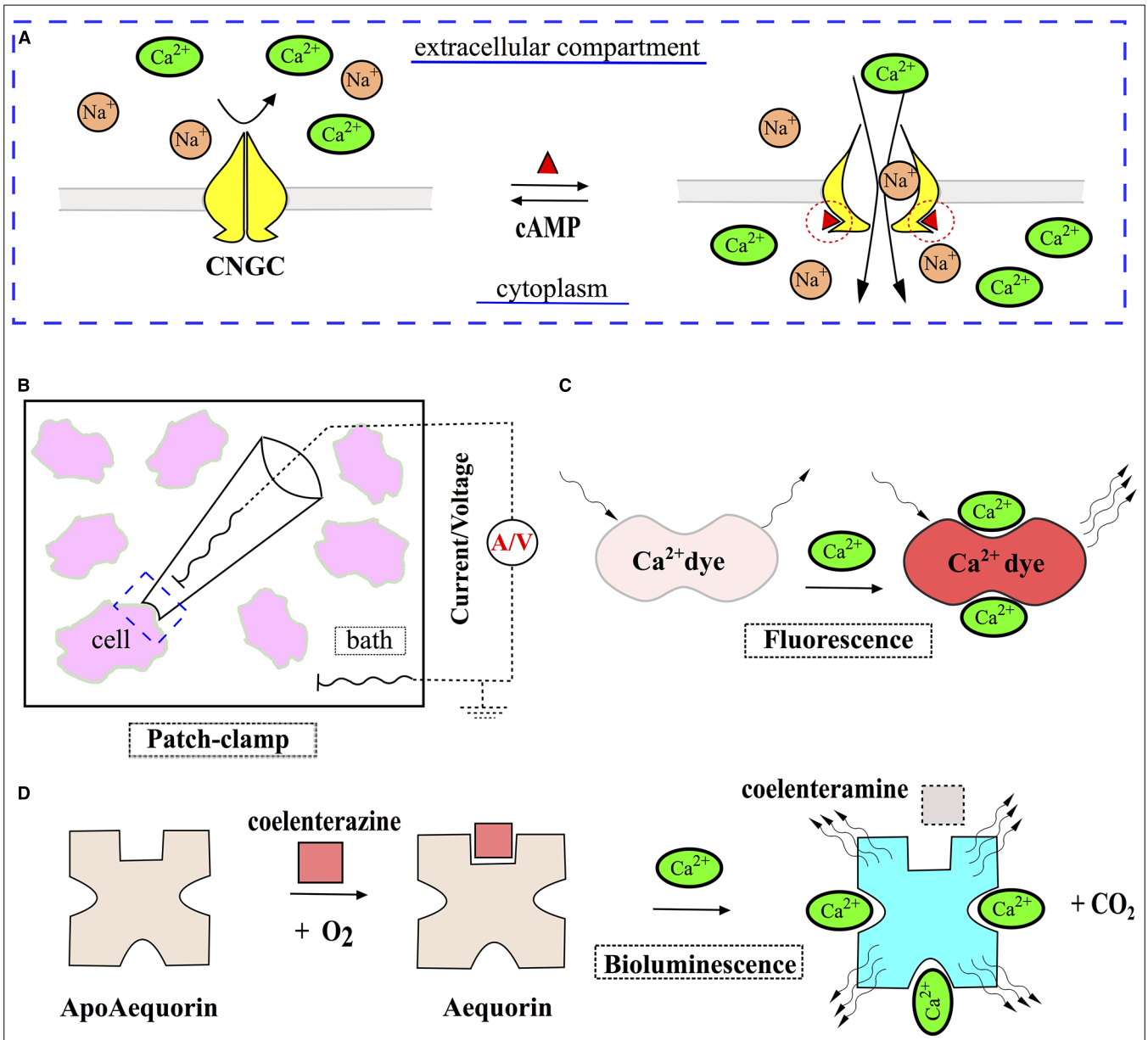

FIGURE 2 | Harnessing CNGCc for indirect measurement of cAMP. (A) Basic operational principle of CNGCs: binding of cAMP (red triangle) to CNGCs results in channel opening for cations, allowing Na^+ and Ca^{2+} to enter the cytoplasm. **(B)** Changes of the membrane potential and transmembrane electric currents, mediated by cAMP-driven CNGCs activation, can be registered with patch-clamp technique and serve as an indirect measure of cAMP levels. **(C,D)** Instruments, initially designed to measure concentration of intracellular Ca^{2+}, can be applied in combination with CNGCs for indirect sensing of cAMP. Inorganic dyes (e.g., fluo-3), that demonstrate a pronounced rise in fluorescence upon binding of Ca^{2+} **(C)**, and bioluminescent protein apoaequorin, responding to Ca^{2+} by generation of light **(D)**, have been successfully used to this end.

genomic loci of insertion and the state of the cells, e.g., passage number, might introduce massive variability (Jonas et al., 2013). The establishment of a stable line is a laborious process and does imply extra requirements to reporter construct, as it should confer antibiotic resistance for efficient selection of stable clones. Besides, the reporter construct should ideally encode a fluorescent tag allowing for negative selection of a fraction of stable clones with highest basal levels of reporter expression, providing the resulting cellular system with optimal sensitivity to cAMP (Hald et al., 2015). On top of it, the above approach is essentially non-applicable to primary cell cultures with a finite life span.

Another potential problem with reporter gene-based systems is related to basal promoter activity in the absence of its cognate transcription factor (CREB). This might happen when the strong promoter is being used (e.g., cytomegalovirus or phosphoglycerate kinase), thus leading to reporter protein synthesis in response to basal cAMP levels, which undermines assay performance and might lead to false-positives (Scarff et al.,

2003; Hald et al., 2015). At the same time, this problem might be minimized via promoter *rejigging* or via prior negative selection of the cells exhibiting significant reporter signal in unstimulated state, thus yielding a homogeneous basally *silent* population. The latter strategy is feasible in cases when the reporter gene encodes for either a fluorescent protein (Kotarsky et al., 2001; Hald et al., 2015) or certain enzymes coupled to fluorescent readout (e.g., cleavage of FRET substrate CCF2/AM by reporter β-lactamase, leading to drop in FRET efficiency, as reported by Zlokarnik et al., 1998), thus allowing for facile removal of basally *active* cells via fluorescent-activated cell sorting.

Excessive reporter protein stability presents yet another possible limitation of reporter gene-based assays. Obviously, if a reporter protein is quite stable and has a natural decay period of more than several hours [which is the case with many conventional fluorescent or enzymatic reporters (Van Diggelen et al., 1980; Corish and Tyler-Smith, 1999)], this will have a huge impact on an assay sensitivity and dynamic range, as first reporter molecules that have been synthesized upon initial cAMP stimulation will still be present and actively *signaling* after several hours, thus adding to any resulting readout from successive surges of cAMP (Hill et al., 2001).

The above limitation might be partially circumvented by using genetically-engineered reporters destined for fast proteolytic degradation, as was successfully achieved by fusing PEST domain of mouse ornithine decarboxylase or mouse cyclin B1 destruction box to *C*- or *N*-termini of GFP, respectively, shortening GFP half-life to 2–6 h (Li et al., 1998; Corish and Tyler-Smith, 1999). Leclerc et al. (2000) applied similar strategy to a firefly luciferase, engineering the mutant enzyme with a functional half-life of only 50 min (in contrast to 4 h of wild-type enzyme).

Another way to obtain data on cAMP-driven kinetics of gene expression is to use a reporter that is released into cell medium, e.g., secreted isoforms of placental alkaline-phosphatase or β-lactamase (Cullen, 2000; Qureshi, 2007). By repeatedly removing cell medium and analyzing multiple sequential samples, one can minimize the effect of reporter protein accumulation and increase the dynamic range of the assay. Although this multiple sampling approach can convey some idea of intracellular cAMP fluctuations and expression of cAMP-dependent genes over time, it is still a way from deducing the genuine kinetics of cAMP-driven transcription, which is attributed to inherent features of signal transduction from the level of cAMP to CRE regions. As was previously shown by Hagiwara et al. (1993) slow entry pace of PKA catalytic units into cell nucleus, their low affinity for CREB and low concentrations of the latter basically promote a slow and relatively long-lasting transcriptional response with early saturation of the downstream signal, which is in striking contrast with seconds-lasting amplitude surges of cAMP within cytoplasm.

The amplification of the downstream signals on the way from the plasma membrane to the nucleus is a well-known phenomenon (Wettschureck and Offermanns, 2005). Considering this, the initial signal, encoded by a rise in cAMP upon GPCRs activation, will be augmented manifoldly by the time it reaches CRE loci of reporter genes in the nucleus. This general nature of signal transduction might be advantageous if one is studying the effects of low-amplitude oscillations of cAMP. However, in the presence of pronounced changes of cAMP the above benefit turns into a limitation, as the signal relay quickly gets saturated of on the levels of nucleus entry and transcriptional regulation (discussed above), narrowing the dynamic range of the assay and *blunting* its ability to reflect actual magnitude of cAMP oscillations. Besides, the phenomenon of signal amplification should be duly accounted for when interpreting the data from reporter gene studies—the importance of this in nicely exemplified by partial GPCR agonists, which might appear to act as full agonists (Baker et al., 2003).

As reporter assays rely on the readout from the remote point of cAMP signaling cascade, there is always a possibility of interference with other signal transduction pathways. The presence of such confounding input might significantly affect the overall results of an assay, obscuring the actual changes of cAMP in specimens under study (Hill et al., 2010). Besides, as the way from cAMP to reporter protein encompasses multiple steps, any compound or investigational molecule to be used in the assay must not interfere with all the components of normal cell machinery involved in protein synthesis (i.e., transcription, translation, protein trafficking, and post-translational modifications). In such a way, results of essentially any reporter gene-based assay might warrant further validation in at least one extra assay of different format, which is not immediately dependent on protein synthesis.

Finally, when used separately, reporter gene assays are poorly applicable for studies of cAMP spatial dynamics in various subcellular compartments, as the readout from the assays indirectly reflects only the net change in total pool of free cAMP. Hence, a signal from a reporter protein usually confers little information on the initial *hot spots* of cAMP generation and its successive intracellular flow.

To conclude, reporter gene-based assays make a reasonable choice once the transcriptional effects of cAMP are in the focus of research interest. However, if one wants to have a closer look at cAMP dynamics above the level of gene expression, e.g., patterns of cAMP synthesis, diffusion and decay, biosensors for direct cAMP probing clearly should be used instead.

Selection of the Right cAMP Sensor for Experiment

Given the wide array of different biosensors for cAMP currently available, selection of the right one for a given experiment sometimes might present an uneasy task. Naturally, the choice of a sensor is dependent on particular research objectives. Indeed, if aiming at the levels of cAMP *per se*, direct sensors apparently make the best choice, but once the *effects* of cAMP signaling, such as transcriptional changes of cAMP-responsive genes, are in question, appropriate indirect sensors should come into action. Though the indirect sensors at large allow for the reverse scenario, i.e., reporter gene-based systems or CNGCs may be applied for measurements of cAMP oscillations, the limitations of such approach and its general inferiority to direct biosensors used for the same task should be duly recognized. Once the principle research goals have been identified, additional requirements to the readout (resolution to subcellular domains? HTS? Real-time

cAMP kinetics?) should ultimately spotlight the most appropriate biosensor for the particular experimental project.

Another issue worth considering is the expertise with desired experimental techniques and availability of equipment, as some biosensors require a skillful operator and a dedicated set of tools for optimal performance (e.g., CNGCs-based sensors coupled with patch-clamp or FRET probes applied to gain subcellular resolution). Lastly, one should not disregard conventional biochemical assays for cAMP, such as ELISA, RIA, HTRF (homogeneous time resolved fluorescence) or AlphaScreen. If no high spatial or temporal resolution is required and the total cAMP levels in a sample at a single time point is all that is needed, biochemical assays for cAMP obviously are the easiest and highly reliable choice. These established analytical tools offer ready-to-use solutions with minimal requirements for optimization and are applicable to vast array of cell types and tissues, providing robust data on total cAMP levels in cellular populations (Williams, 2004; Hill et al., 2010).

A decision diagram, which we hope will aid the reader in cAMP biosensor selection, is presented in **Figure 3**. Universal limitations, that are inherent to the bulk of the genetically encoded probes irrespectively of the nature of signal they produce, are summarized in **Figure 4**.

Cutting-Edge Genetically-Encoded Tools for Modulation of cAMP in Living Systems

Apart from the biosensors reviewed earlier, recent progress in synthetic biology endowed the research community with principally novel and extremely powerful tools for modulation of cAMP in living systems. Photoactivatable adenylyl cyclases (PACs) and PDEs perhaps make the most remarkable example of such achievements. These ingenious optogenetic enzymes allow researchers to enjoy all the benefits of light as the *steering wheel*, including exquisite spatio-temporal dosing of exposure, minimal toxicity and negligible interference with physiological intercellular processes. Naturally occurring photoactivatable proteins carry light sensitive domains that translate the energy of absorbed photons into conformational rearrangements of the whole molecule. The change in protein structure triggered by light typically affects protein functional state, e.g., leading to alteration in its enzymatic activity (Karunarathne et al., 2014).

The first PAC was isolated from the unicellular flagellate *Euglena gracilis* (Schröder-Lang et al., 2007). Each subunit of this tetrameric EuPAC protein harbors a couple of photosensitive BLUF domains (Blue Light receptor Using FAD) and has own AC activity that is stimulated by blue light.

EuPAC showed excellent performance in heterologous expression systems (HEK293 cells, *Xenopus laevis* oocytes and central neural system of *Drosophila melanogaster*), with up to 10-fold enhancement of cAMP production upon exposure to blue light. AC activity of EuPAC was clearly dependent on magnitude of stimulation (both light intensity and duration of exposure) and exhibited an ultra fast on-off response within dozens of milliseconds. However, despite the remarkable functional features, EuPAC has not been widely used in successive studies, mainly due to significant dark activity and big molecular weight precluding efficient and problem-free expression.

Another PAC, discovered a few years thereafter in soil bacteria *Beggiatoa sp*. (hence the name—bPAC), did not suffer from the above limitations of EuPAC and made an extremely valuable addition to the cAMP researchers toolbox (Ryu et al., 2010; Stierl et al., 2011). This small BLUF domain-containing enzyme (350 amino acids, that is roughly one-third of the size of EuPAC) has negligible activity in the dark, which is inducible up to 300-fold upon exposure to blue light. Besides, in comparison to EuPAC this native bacterial photoenzyme requires much less irradiation to generate comparable concentrations of cAMP and preserves light-induced active conformation for longer time, thus providing more durable response. Blue light stimulates enzymatic activity of yet another PAC, identified in cyanobacterium *Microcoleus chthonoplastes* (Raffelberg et al., 2013). In contrast to the BLUF PACs discussed above, this enzyme, coined mPAC, has a LOV (Light, Oxygen, Voltage) domain for light-sensitive unit, but otherwise operates in the similar fashion.

Despite successful use of blue-light sensitive PACs *in vitro* and in selected small animal models (i.e., *Drosophila*), the general application of these tools to *in vivo* setting is hindered by low tissue penetration depth of blue light (Wan et al., 1981). PACs, activatable by near-infrared light, which can pass in bodily tissues as far as several centimeters, provide an excellent alternative. The first PAC of this kind was engineered by Ryu et al. (2014), who fused together a light-sensitive domain of phytochrome from *Rhodobacter sphaeroides* and AC from bacterium *Nostoc sp*. The resulting chimeric enzyme was efficiently regulated by red light, including *in vivo*, as was demonstrated by an alteration of locomotory activity in *Caenorhabditis elegans*, expressing this PAC in cholinergic neurons.

On the opposite end of cAMP regulation, a light-activatable phosphodiesterase (LAPD), recently introduced by Gasser et al. (2014), represents a fine-tunable instrument for cAMP degradation. A chimera protein of photosensitive phytochrome from *Deinococcus radiodurans* and human PDE2A, LAPD is an extremely versatile optogenetic enzyme, as it responds to both blue and red light with an increase of PDE activity reaching sixfold, and is down-regulated by far-red light. LAPD was already successfully tested in cell cultures and zebrafish embryos, and will probably soon be applied in larger animal models (Gasser et al., 2014).

To conclude, ACs and PDEs engineered as optogenetic tools open breath-taking avenues for cyclic nucleotide research. As genetically-encoded single chain proteins, these photoactivatable enzymes are proven to be applicable to majority of modern model systems, including *in vivo* setting. Secondly and luckily enough, the indispensable cofactors for all of the discussed optogenetic proteins, that are required for efficient sensing of photon energy and its relay to conformational changes, are naturally present in sufficient quantitates in most types of living cells and tissues (i.e., biliverdin chromophore for LAPD and red light-dependent PACs, flavins for BLUF and LOV-harboring PACs), thus obviating the need for their exogenous supply. In such a way, all that is needed for triggering the desired activity (AC or PDE) in a biological sample under study—is to illuminate it with the light of appropriate wavelength and intensity. With spatial resolution

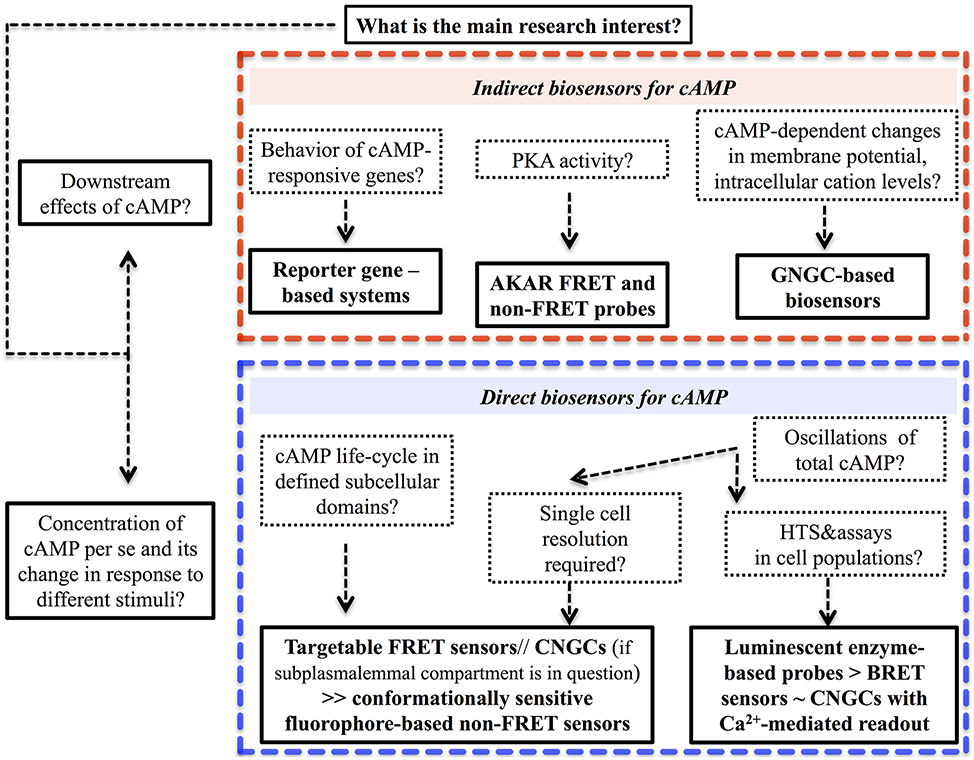

FIGURE 3 | Selection of the task-specific biosensor for cAMP probing in living cells—a decision tree.

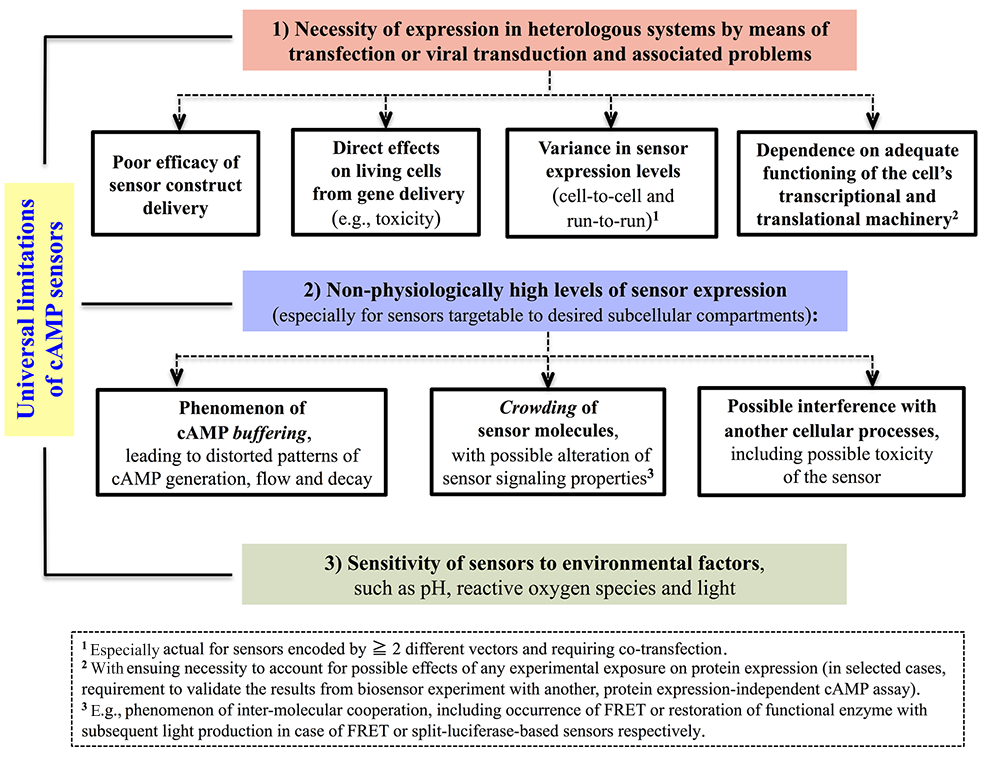

FIGURE 4 | Universal limitations inherent to most genetically encoded biosensors for cAMP.

defined by the width of the light beam and ultra-flexible timing of stimulation, optogenetics enzymes are a way ahead of the pharmacological agents traditionally used in cAMP research, such as non-specific AC stimulator forskolin (FSK) and PDE inhibitor IBMX. The engineering of PACs and PDE activated by red and near-infrared light paves the way for non-invasive modulation of cAMP in living animals, allowing to study and fine-tune cyclic nucleotide signaling in otherwise non-disturbed tissue microenvironment. Moreover, one can readily envision the combined used of photoactivatable ACs and PDEs in a single cellular system or animal model for a *full cycle* cAMP control, provided that both enzymes have well-separated spectral characteristics.

Apart from the photoactivatable proteins, we would like to highlight another pair of genetically-encoded tools that may be fruitfully employed in living systems for cAMP modulation. The first instrument is based on a modified soluble AC (sAC), a special isoform of AC that is directly regulated by bicarbonate ions (HCO_3^-) and involved in pH sensing in a variety of tissues (Rahman et al., 2013). By decorating truncated catalytically active rat sAC with the appropriate localization signals, Sample et al. (2012) were able to express sAC constructs in desired domains of living cells. Stimulation of the cells with bicarbonate readily triggered fast and sustained activation of sAC leading to rise in cAMP in the subcellular compartments sAC was targeted to. Moreover, sAC activity was proportional to the concentration of bicarbonate anions and quickly returned to basal state after bicarbonate removal. Therefore, the engineered sACs provide quite precise and versatile means of chemical control of cAMP production, making a nice addition to PACs.

The second instrument, named *cAMP sponge*, represents a truncated variant of the RIb regulatory subunit of PKA, which preserves high affinity for cAMP, but cannot dimerize or bind PKA catalytic subunits (Lefkimmiatis et al., 2009). In such way, *sponges* are designed to act as scavengers of cAMP molecules that limit availability of free cAMP to its effectors, but do not interfere with cAMP generation or degradation. *Sponge* proteins can also be routed to certain subcellular compartment (cell membrane, cytoplasm, or cell nucleus) by means of addition of appropriate localization signals and were shown to blunt cAMP oscillations triggered by either GPCRs activation or FSK. Besides, *cAMP-sponges* successfully attenuated PKA activity, measured in living cells by AKAR sensors. These features make sponges potentially useful tools for complex experimental perturbations of cAMP signaling in different microdomains of living cells.

All in all, modern genetically encoded proteins for cAMP modulation provide hitherto unattainable opportunities for experimental interference and modeling of such major aspects of cAMP life cycle as cAMP generation, trafficking and hydrolysis. The genuine power of these tools obviously dwells in synergy with biosensors for cAMP. Flexibility of design and targetability to desired subcellular compartments, inherent to both modern probes and instruments for cAMP modulation, combined with excellent spatiotemporal resolution of selected biosensors, in principle allow for real-time studies of precisely induced cAMP waves across several domains of a single living cell. Apart from this, introduction of a *second opinion* (e.g., FRET bioprobes for Ca^{2+}) shall expand research potential of a living system even further, opening avenues for insightful studies of cAMP interaction with other intracellular players. Recent results, obtained with the help of combinatorial applications involving several distinct genetically-encoded proteins, support the bright perspectives of this methodology—that is coming of age and shaping the vectors of future research endeavors (Sample et al., 2012; Tsvetanova and von Zastrow, 2014).

Currently, genetically-encoded tools for cAMP fuel the transitional phase of the whole filed of cyclic nucleotide research, shifting the focus from the unresolved and single time point measurements in cellular populations toward the pinpoint real-time modulation and probing of cAMP in discrete subcellular domains of a given cell. And with the present pace of biotechnology the newer and even more advanced instruments are quite likely to come, giving us the possibility to challenge established ideas and pursuit even bolder hypotheses.

A Perfect Sensor for cAMP in Living Systems—Prospects for the Future

If we could think of a *perfect sensor for cAMP*, what principle features should it have? Firstly, and in contrast to all of the biosensors discussed in this review, a perfect sensor should be totally independent of physiological/pathophysiological processes, occurring in a living system, including transcription, translation and post-translational modifications. Obviously, in order to make this possible, a sensor has to be delivered to a system in a ready-to-use state, thus obviating the need of conversion of a gene template into a functional sensor protein by recipient cell's protein synthesis machinery, which is the case with all genetically-encoded sensors for cAMP presently available. Only then it would be possible for a sensor to produce the most objective readout of cAMP dynamics in living cells or tissues under virtually any possible condition, including such extremes as pronounced stress or advancing cell death. In such fashion, a sensor that is based on a biological cAMP—responsive domain, might still be considered a genetically-encoded tool, but transcription and translation events from a coding sequence should happen in a dedicated expression system (e.g., bacteria, plant cells, or yeast) and not in the living system to be analyzed.

Secondly, a *perfect* sensor should be easy to deliver to living cells, with the procedure of delivery *per se* affecting cellular well-being as minimally as possible. This will also facilitate the measurements of cAMP levels in *delicate* primary cells or even tissues (*in vivo*). Targetability of a sensor to selected intracellular compartments, such as nucleus and mitochondria, would be highly desirable as well. Lastly, an ideal sensor designed for measurement of absolute values of cAMP should directly bind and sense cAMP molecules, with binding event being immediately coupled to signal generation and rapidly reversible, producing flash ON–OFF responses and minimizing cAMP buffering. Apart from the favorable kinetics of response to cAMP, allowing for real-time measurements, the signal from a sensor should be reasonably strong, providing sufficient spatial resolution for studies of cAMP oscillations in subcellular domains of single living cells.

For the time being, we could not think of any possible unimolecular sensor meeting the above criteria. The solutions might possibly lie in the interface of synthetic biology and nanotechnology, culminating in engineering of such *smart* devices, as nanoparticulate carriers equipped with cAMP-sensing elements. Nanocarriers already hold a strong position in the field of drug delivery and might make an excellent vehicle for cAMP sensors. A huge variety of different platforms, from liposomes and nanorods to silica- or metal-based nanoparticles, offers immense design flexibility—by means of decoration with various functional elements (antibodies, ligands to endogenous receptors expressed in particular cell types, *smart* bioshells, degradable by selected enzymes or in response to pH change), nanocarriers can be conceptually engineered for highly selective transportation of cAMP sensors to desired cellular population or tissue types in living multicellular organisms, let alone cell lines or primary cultures *in vitro* (Sahay et al., 2010; Sikorski et al., 2015). With the current advances in the nanoscience, we are fully justified to expect development of dedicated nanocarriers for cAMP sensor delivery in the nearest future. Of note, alternative solutions for a delivery of ready-to-use cAMP sensors might well arrive from a different direction—for instance, one could envision that a biosensor could be delivered ready-to-use by means of one of the protein transduction domains, also called cell-penetrating peptides, such as TAT, PEP-1, Antennapedia, or Arg-9 (Heitz et al., 2009). The delivery of sensors by such peptides could be coupled with a protein localization signal and even a *switch* that becomes activated only in specific cell compartments and/or cell types. This approach, to the best of our knowledge, has not been yet explored.

As for the principal component of any cAMP sensor, that is a unit responsible for binding of cAMP molecules and relaying the binding events into a traceable signal, we envision that the future generations of these elements will primarily stem from cAMP binding molecules originally evolved in nature and subsequently modified for the purpose, just like as already happened to all genetically-encoded cAMP sensors engineered so far. However, apart from the established biological cAMP effectors, such as PKA, Epac proteins and CNGCs, new *scaffolds* for cAMP sensor elements are likely to emerge, with ribozymes (RNA molecules with enzymatic activity) being a good and promising example (Koizumi et al., 1999). Combinatorial use of the above natural cAMP effectors with purely synthetic tools, e.g., cAMP-avid RNA aptamers, might lead to the creation of principally new classes of cAMP sensors with improved capabilities (Koizumi and Breaker, 2000; Paige et al., 2012; Kellenberger et al., 2013). Finally, further studies in the fundamental principles of cAMP signaling organization might well uncover hitherto unrecognized cAMP counterparts, opening new horizons for cAMP biosensors design.

Author Contributions

VMP wrote the initial draft; VM, CS, and ARM corrected the draft and provided feedback. All authors read and approved the final version of the manuscript.

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Targeting brain tumor cAMP: the case for sex-specific therapeutics

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A relationship between cyclic adenosine 3′, 5′-monophosphate (cAMP) levels and brain tumor biology has been evident for nearly as long as cAMP and its synthetase, adenylate cyclase (ADCY) have been known. The importance of the pathway in brain tumorigenesis has been demonstrated *in vitro* and in multiple animal models. Recently, we provided human validation for a cooperating oncogenic role for cAMP in brain tumorigenesis when we found that SNPs in *ADCY8* were correlated with glioma (brain tumor) risk in individuals with Neurofibromatosis type 1 (NF1). Together, these studies provide a strong rationale for targeting cAMP in brain tumor therapy. However, the cAMP pathway is well-known to be sexually dimorphic, and SNPs in *ADCY8* affected glioma risk in a sex-specific fashion, elevating the risk for females while protecting males. The cAMP pathway can be targeted at multiple levels in the regulation of its synthesis and degradation. Sex differences in response to drugs that target cAMP regulators indicate that successful targeting of the cAMP pathway for brain tumor patients is likely to require matching specific mechanisms of drug action with patient sex.

Keywords: sex differences, cAMP, PDE, primary cilia, brain tumors

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Brain Tumors and Camp: Some Basics

Soon after its discovery, it became clear that cAMP was a ubiquitous second messenger and that normal physiology was dependent upon precise regulation of its synthesis and degradation. As a corollary, investigations rapidly ensued to determine whether pathology was associated with dysregulation of cAMP levels. In 1971, multiple studies were published that associated differences in cAMP levels with differences between normal and cancer cells (Heidrick and Ryan, 1971; Johnson et al., 1971; Otten et al., 1971). Elevated levels of adenylate cyclase (ADCY) activity and cAMP levels were associated with normalization of cell morphology, restoration of contact inhibition and reduced growth rates in neoplastic cells. Subsequently, an inverse relationship between cAMP levels and tumor grade was established for several types of brain tumors (Furman and Shulman, 1977). High ADCY activity and cAMP levels were found in benign brain tumors, while lower ADCY activity and cAMP levels were correlated with greater degrees of malignancy.

Today, there is good reason to think that elevating cAMP will be an important therapeutic for brain tumors, and here, we will review key data validating cAMP as a target in the treatment of brain tumors. We will also discuss the significant sexual dimorphism in this pathway. Evidence for sex-specific responses to drugs that target cAMP indicate that knowledge of sex differences must be incorporated into preclinical and clinical investigations if cAMP is to be successfully targeted in the treatment of brain tumors. It is our perspective that targeting cAMP in the treatment of brain tumors will be a critical test case for the importance of sex-specific treatment of cancer.

Adenylate Cyclase and Tumorigenic Mechanisms

Differences in cAMP synthesis arise through differences in expression, subcellular localization and activation of nine different membrane bound ADCYs and one soluble ADCY (Cooper and Tabbasum, 2014). Variation in stimulation and inhibition by heterotrimeric G protein subunits, calcium, multiple protein kinase C isoforms and calcium/calmodulin-dependent protein kinase results in unique regulatory "codes" for activation of different ADCY isoforms (Sunahara and Taussig, 2002; Cooper and Tabbasum, 2014). These non-overlapping mechanisms of regulation potentially create alternate landscapes for the interaction between primary oncogenic events in brain tumorigenesis and total ADCY activity. Medulloblastoma and the localization of ADCY3 to the base of the primary cilia (**Figure 1A**), provides a compelling illustration (McIntyre et al., 2015).

Medulloblastoma is the most common malignant brain tumor of childhood. There are currently four recognized subtypes of medulloblastoma, and one involves mutational activation of the sonic hedgehog (SHH) pathway (Gibson et al., 2010; Northcott et al., 2010; Cho et al., 2011; Kool et al., 2012). Sonic hedgehog signaling involves the activation of a receptor and signaling complex within the primary cilium (**Figure 1B**). Activation of the pathway results in translocation of the transcription factor Gli2 to the nucleus (Ruat et al., 2012). This key event is blocked by cAMP and protein kinase A (PKA), possibly through stabilization of the complex between Gli2 and Suppressor of Fused (Sufu) (Tuson et al., 2011; Mukhopadhyay et al., 2013). The localization of ADCY3 and cAMP generation to the base of the primary cilium provides a potent and localized mechanism for inhibition of sonic-induced transcription.

A number of experimental findings suggest that the positioning of ADCY3 in primary cilia anatomy may support a cooperative role for cAMP regulation in SHH pathway dependent medulloblastoma-genesis. Sonic hedgehog driven medulloblastoma arises from the cerebellar granule neuron lineage (Oliver et al., 2005). Two key G protein-coupled receptors, PAC1 and CXCR4, are expressed in normal and neoplastic derivatives of this lineage, and function to elevate and suppress cAMP levels, respectively (Klein et al., 2001; Nicot et al., 2002; Rubin et al., 2003). Each pathway is known to powerfully regulate SHH signaling in a cAMP dependent fashion. Mice with combined deficiency of the genes for the SHH receptor, *Patched* (*Ptc*), and the PAC1 ligand, *Pituitary Adenylate Cyclase Activating Peptide (PACAP)*, have increased incidence of medulloblastoma compared to mice with *Ptc* deficiency alone (Lelievre et al., 2008). The effect of PAC1 activation was recently demonstrated to involve inhibition of ciliary translocation of Gli2 in a PKA-dependent manner (Niewiadomski et al., 2013). In related studies, deletion of the alpha subunit of the stimulatory heterotrimeric G protein G_s also resulted in SHH-driven medulloblastoma (He et al., 2014). Together these genetically engineered mouse models indicate that diminution in the ability to elevate cAMP levels within the granule neuron lineage is permissive for tumorigenesis in a SHH-dependent fashion.

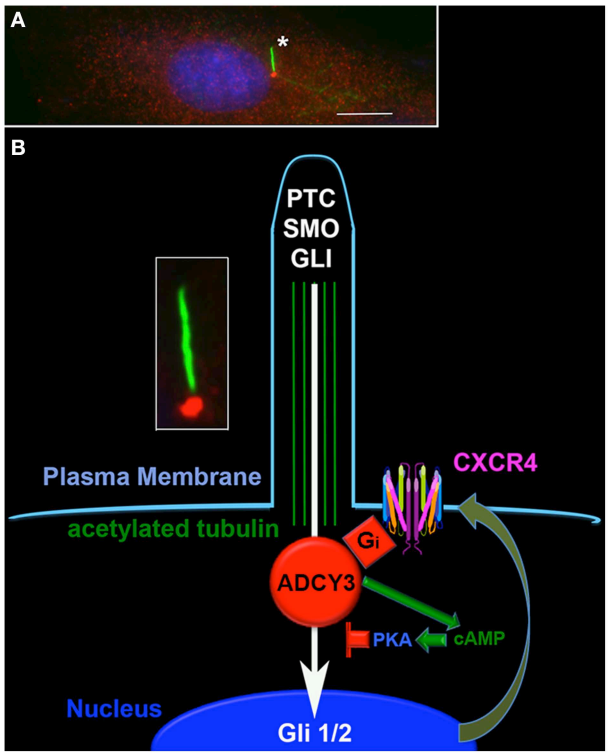

FIGURE 1 | Cyclic AMP signaling at the primary cilium regulates sonic hedgehog signaling. (A) The primary cilium (asterisk, green) in a medulloblastoma cell is clearly visible under direct immunofluorescence using an antibody directed against acetylated tubulin (Sigma). An antibody directed against ADCY3 (red, Santa Cruz) reveals its localization at the base of the primary cilium. The nucleus is counterstained blue with DAPI. Scale bar equals 10 microns. **(B)** Schematic of the primary cilium indicating the potential interactions between the sonic hedgehog and the CXCR4 pathways. The inset is the cilium from **(A)**. In the schematic are shown the plasma membrane (pale blue) with surface localized CXCR4, acetylated tubulin within the cilium (green), ADCY 3 at the base of the cilium (red), sonic hedgehog signaling components (white), CXCR4 (multi-colored), and the nucleus (dark blue). Activation of CXCR4 results in inhibition of ADCY3, local decreases in cAMP levels, decreased PKA activation and enhanced GLI localization to the nucleus. Increased sonic hedgehog signaling results in increased surface localization of CXCR4.

Consistent with these findings are studies exploring the relationship between the SHH pathway and the $G\alpha_i$-coupled receptor, CXCR4. Levels of CXCR4 expression identify two subgroups within the SHH subtype of medulloblastoma (Sengupta et al., 2012). Maximal tumor growth in murine and human models of SHH subtype medulloblastoma is dependent upon co-activation of SHH and the CXCR4 pathway. Surface localization of CXCR4 and consequently, CXCR4-mediated $G\alpha_i$ activation and cAMP suppression, is stimulated by the SHH pathway, creating a positive feedforward loop for suppression of cAMP levels and activation of Gli-mediated transcription (Sengupta et al., 2012). Blockade of CXCR4 signaling, and elevation of cAMP levels, with specific small molecule antagonists has potent anti-tumor effects in

intracranial xenograft models of SHH driven medulloblastoma (Rubin et al., 2003; Yang et al., 2007; Barone et al., 2014).

Phosphodiesterases and Brain Tumors

While modulation of ADCY activity coordinates cAMP production with activity in other signaling pathways and provides some spatial localization for cAMP production, finer aspects of the compartmentalization of cAMP signaling occur through subcellular localization and regulated activity of phosphodieseterases (PDE) (Bender and Beavo, 2006). Overexpression of the cAMP specific PDEs (PDE4, 7, and 8) is a frequent event in cancer, including brain tumors (Goldhoff et al., 2008; Brooks et al., 2014; Dong et al., 2015). An examination of 37 human pediatric and adult brain tumor specimens including astrocytomas, medulloblastomas, oligodendrogliomas, ependymomas, and meningiomas all exhibited high levels of PDE4A expression, primarily in tumor cells (Goldhoff et al., 2008). Furthermore, overexpression of a super-short, brain specific isoform of PDE4A, PDE4A1, accelerated brain tumor growth in mice bearing intracranial xenografts of human glioma cells (Goldhoff et al., 2008). Inhibition of PDE4 activity with the drug Rolipram inhibited growth in those tumors and in mouse xenografts of human medulloblastoma cells (Yang et al., 2007; Goldhoff et al., 2008). Gene expression profiling of medulloblastoma indicates that cAMP signaling and altered expression of *PDE1C* and *PDE4B* is a characteristic feature of Group D tumors (Northcott et al., 2010).

PDE7B is another cAMP specific phosphodiesterase that is frequently upregulated in glioblastoma (GBM) and is negatively correlated with survival (Brooks et al., 2014). Among the four molecular subtypes of GBM, PDE7B is expressed at the highest levels in Classical, followed by Neural, Mesenchymal, and Proneural subtypes (Brooks et al., 2014). Increased expression of PDE7B was observed in a subset of tumor cells with enhanced tumor initiating capacity, and overexpression of PDE7B in a U87 intracranial xenograft model of GBM transformed the typical circumscribed pattern of intracranial U87 growth into a highly invasive one. These observations suggest that similar to the case of PDE4A1, PDE7B, and cAMP suppression may be critical mediators of tumorigenic mechanisms in GBM, particularly in the Classical and Neural subtypes (Brooks et al., 2014).

Phosphodiesterase expression can be regulated by microRNAs (mirs) and this mechanism has been correlated with tumor biology and therapeutic responses. In diffuse large B cell lymphoma, decreased mir-124 expression led to increased PDE4B expression and subsequent insensitivity to glucocorticoid treatment (Kim et al., 2015). In GBM, mir-33a expression has a negative prognostic effect and is necessary for maintenance and self-renewal of the tumor-initiating cell population. This critical function of mir-33a was dependent upon its direct regulation of PDE8A and UV Radiation Resistance Associated Gene (UVRAG) and their downstream mediators, PKE, and Notch, respectively (Wang et al., 2014). Together with data regarding PDE7B functions in GBM, these findings indicate that the cAMP pathway is essential for tumor initiating cell function. This is a key consideration for promoting cAMP elevating approaches for brain tumor treatment.

The large number of PDE isoforms that are generated from 21 different genes in 11 different families provides for exquisite specialization in cAMP signaling through the formation of diverse PDE signalosome complexes (Azevedo et al., 2014). Phosphodiesterase signalosome complexes are comprised of PDEs in association with scaffolding proteins such as AKAPs, and regulators of cAMP signaling like EPACs at specific subcellular sites that allow for the precise localization of cAMP gradients and subcellular compartmentalization of cAMP signaling. For example, PDE8A complexes with AKAP and Raf-1, a potent activator of MAPK signaling to inhibit PKA mediated inactivation of Raf-1 and MAPK signaling (Brown et al., 2013). PDE4 isoforms are targeted to specific subcellular compartments by unique amino termini. For example, PDE4A1 contains an amino terminal TAPAS-1 domain that localizes it to the trans Golgi complex (Baillie et al., 2002), suggesting that modulation of cAMP levels and its downstream effectors in this domain are critical for PDE4A1's role in brain tumor growth. The peri-Golgi domain is in close proximity to the centrosome and these regions are thought to share a cAMP pool (Verde et al., 2001; Terrin et al., 2012). Modulation of this pool by displacement of localized PDE4D3 has been shown to directly induce cell-cycle arrest (Terrin et al., 2012). Interestingly, protein kinase A is localized to this PDE4D3 signalosome by interacting with AKAP9, which is altered in expression in medulloblastoma (Northcott et al., 2010).

Cyclic AMP Plays a Cooperating Role in Brain Tumorigenesis

The work of He et al. (2014) and Lelievre et al. (2008) indicate that AC-mediated cAMP regulation plays an important cooperating role in the genesis of medulloblastoma. The use of genetically engineered mouse models (GEMM) of other brain tumors has also confirmed the importance of PDE activity to brain tumorigenesis. In a GEMM of Neurofibromatosis type 1 (NF1), we found that gliomas could be induced by creating foci of PDE-driven reductions in levels of cAMP (Warrington et al., 2010). NF1 predisposes affected individuals to a number of neoplasms in the central and peripheral nervous systems and other tissues. The most common central nervous system tumor is a low-grade astrocytoma of the optic pathway that most commonly affects children less than 10 years of age (Rubin and Gutmann, 2005). The factors that dictate the temporal and spatial distribution of these tumors are not completely understood. However, there is a clear requirement for the combined effects of: (1) homozygous loss of neurofibromin function in tumor progenitors, (2) heterozygous loss of neurofibromin function in stromal cells of the microenvironment, and (3) other factors specific to the most commonly affected brain regions, such as differences in stem cell populations or growth regulatory/differentiation pathways. Neurofibromin functions as a negative regulator of RAS, and increased RAS activity is accepted as the primary driver of low-grade gliomagenesis through its activation of ERK and AKT pathways (Ratner and Miller, 2015). However, the additional

requirements for tumorigenesis indicate that complete loss of *NF1* and hyperactivation of RAS in tumor progenitors is not sufficient for gliomagenesis and that cooperating molecular events must be at play. We described an "oncogenic" mode of CXCR4 signaling that we hypothesized would be a cooperating event in NF1-associated gliomagenesis (Rubin, 2009). We defined this mode of CXCR4 signaling as a loss of receptor desensitization and capacity for sustained suppression of cAMP levels (Warrington et al., 2007; Sengupta et al., 2012; Woerner et al., 2012). The loss of desensitization was the result of ERK dependent phosphorylation and inhibition of GRK2, which we showed resulted in decreased CXCR4 phosphorylation and sustained suppression of cAMP in response to receptor ligation with CXCL12 (Warrington et al., 2007). As we had also shown that CXCL12 was abundant in low-grade gliomas in tumor-associated endothelial cells, microglia, and entrapped neurons, we hypothesized that low levels of cAMP would promote tumor formation. We tested this hypothesis by forcing expression of PDE4A1 in the brains of NF1 GEMM. This model has a highly stereotypical pattern of tumor formation involving the optic pathway alone, and we postulated that if cAMP suppression was a key cooperating event, then expression of PDE4A1 in the cortex would result in the formation of "ectopic" cortical tumors. We chose PDE4A1 for these experiments because the PDE4 family has been shown to be responsible for of the bulk of the cAMP hydrolyzing activity in cells (Conti et al., 2003), and PDE4A1 is a brain specific, super short isoform of PDE4A (Huston et al., 2006). This super short form lacks the regulatory and protein-complexing domains present in longer PDE4 isoforms. Therefore, the effects of PDE4A1 overexpression can be directly attributed to increased cAMP hydrolysis. We found that PDE4A1 expression resulted in foci of decreased cAMP levels that were significantly correlated with the genesis of cortical "ectopic" tumors (Warrington et al., 2010). Furthermore, inhibition of PDE4 with Rolipram blocked the growth of spontaneous tumors in this GEMM (Warrington et al., 2010). Thus, in both medulloblastoma and NF1-associated glioma, primary oncogenic events are mechanistically complemented by decreased levels of cAMP.

Abnormal PDE expression and activity has also been implicated in chronic lymphocytic leukemia (Zhang et al., 2008), lung cancer (Pullamsetti et al., 2013) and colon cancer (McEwan et al., 2007). Similar to the case for polymorphisms in ADCY and cancer risk, single nucleotide variations in PDE8A and PDE11A have been associated with adrenocortical carcinoma (Oliver et al., 2005) as well as cancer of the testes (Klein et al., 2001) and prostate (Nicot et al., 2002).

Cyclic AMP as a Target for Brain Tumor Therapy

The clear correlation between low levels of cAMP and enhanced brain tumorigenesis, brain tumor grade and brain tumor growth, has naturally prompted efforts to develop cAMP elevating approaches to brain tumor treatment. Early efforts utilizing cAMP analogs like 8-chloro-cAMP were associated with dose-limiting toxicities (Propper et al., 1999). Subsequent clinical trials of targeted agents with potential to modulate cAMP levels include G protein coupled receptor (GPCR) agonists and antagonists, as well as stimulators and inhibitors of ACs and PDEs. Notable among the potential clinically available GPCR antagonists that can elevate cAMP are the CXCR4 antagonists AMD3100 (Plerixafor, Genzyme) and POL6326 (Polyphor). AMD3100, AMD3465, and POL5551, also CXCR4 antagonists, have all been shown to block intracranial brain tumor growth in several experimental models, and this activity was correlated with the elevation of cAMP levels in models of GBM and medulloblastoma (Rubin et al., 2003; Yang et al., 2007; Barone et al., 2014). Key to CXCR4 antagonism is the blockade of critical tumor-stromal interactions that occur within the specialized perivascular stem cell niche (PVN) (Rao et al., 2012). In combination with Avastin, POL5551 was shown to block both the formation of new PVN and the function of existing PVN to maintain brain tumor stem cells (Barone et al., 2014).

More specific manipulation of cAMP levels is possible through the use of agents and strategies to increase cAMP synthesis or decrease its degradation. Fundamental principles of pharmacology indicate that inhibition of degradation can have more potent and stable effects on cAMP levels than stimulation of its synthesis (Bender and Beavo, 2006). Consequently, PDE inhibition as a strategy for the therapeutic elevation of cAMP has a long history (Sengupta et al., 2011). Early efforts involved general PDE inhibitors like pentoxyphylline or caffeine, which have both pan-PDE inhibitory and adenosine agonist activities. Non-specific PDE inhibition for cancer applications was complicated by excessive toxicity. As the full breadth of PDE genetics, biochemistry and cell biology became apparent, non-specific approaches gave way to targeted inhibition of specific PDE families. Among the most productive efforts has been the development of a repertoire of PDE4 antagonists. In fact effective PDE4 antagonists for brain tumor therapy may already be clinically available for other applications such as COPD (Sengupta et al., 2011).

Sex Differences in the cAMP Pathway Demand a Sex-specific Approach to Its Targeting

Drug selection for cAMP elevation should proceed carefully and patient selection should be precise. In this regard it will be critically important to recognize the growing body of evidence for significant sex differences in the pathophysiology of human disease and how these might translate into sex differences in therapeutic responses to targeted agents. Sexual dimorphism in the cAMP pathway, and in PDE activity specifically, is likely to impact on the efficacy and appropriateness of specific inhibitors for individual patients.

There is ample evidence that sex differences exist in cAMP levels. In multiple experimental systems, sex differences have been measured in the expression and activity of cAMP regulators and resultant cAMP levels. For example, neurons in the pontine nucleus of the rat exhibit sexually dimorphic response to

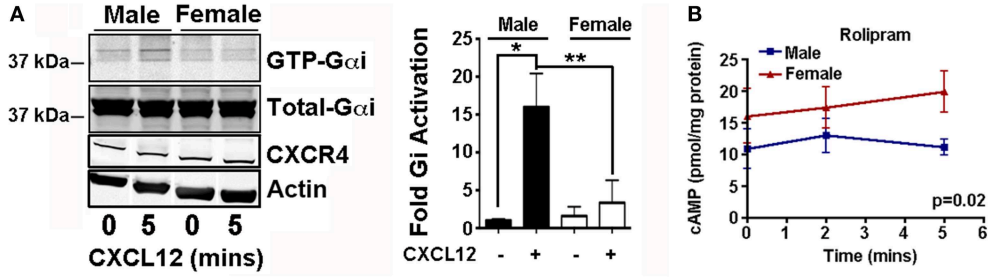

FIGURE 2 | Sex differences in the cAMP pathway. (A) Activation of Gαi was measured by Western blot for the GTP loaded and total Gαi forms using a kit from Neweast Biosciences as described (Sengupta et al., 2012). Male and female *Nf1*$^{-/-}$ astrocytes prepared as described (Warrington et al., 2015), serum starved for 48 h, and treated with vehicle (PBS) or CXCL12 (0.1 ug/ml, 5 min). Shown is a representative Western blot and a plot of the fold differences relative to the basal male condition, in the mean and SEM of the fraction of GTP loaded (active) to total Gαi. $N = 4$. $^{*}p < 0.05$, $^{**}p < 0.005$ as determined by two-tailed *t*-test. Accompanying Western blots of CXCR4 with actin loading control indicate that differences in Gαi activation are not the result of differences in CXCR4 expression. **(B)** Cyclic AMP levels were measured by ELISA as previously described (Warrington et al., 2015) in male and female *Nf1*$^{-/-}$ astrocytes treated with Rolipram (20 uM). $N = 3$. *P*-value was determined by Two-Way ANOVA.

corticotropin-releasing factor (CRF). When treated with CRF, female neurons exhibit both greater cAMP elevation and greater PKA activation compared to male neurons. This has been proposed to be the consequence of greater coupling between Gαs and the CRF receptor in female neurons (Valentino et al., 2013). We have made similar observations about the coupling between CXCR4 and Gαi in murine astrocytes. Male and female *Nf1*$^{-/-}$ astrocytes treated for 5 min with CXCL12 (0.1 μg/ml) exhibit significantly different levels of GTP loading of Gαi despite equal expression of CXCR4 (**Figure 2A**).

In rat myocytes, greater PDE4B expression leads to lower baseline cAMP levels in female cells. This results in decreased activation of PKA, smaller calcium transients and less forceful contractions than in their male counterparts (Parks et al., 2014). Additionally, PDE3B expression is higher in female endothelial cells rendering them more responsive to treatment with a PDE3 inhibitor than male endothelial cells (Wang et al., 2010).

Sex differences in expression and function of cAMP regulators have also been demonstrated in glioma precursor cells derived from a mouse model of NF1. In this model, astrocytes with targeted deletion of neurofibromin *Nf1*$^{-/-}$ are poised to be tumorigenic in the appropriate setting, which includes requirements for heterozygous loss of neurofibromin in tumor stromal elements and other, as yet to be fully defined factors, that stereotypically promote tumorigenesis in the optic nerves and optic chiasm. Male *Nf1*$^{-/-}$ astrocytes expressed more *Gnai3* and less *Gnas* transcript than female cells (Warrington et al., 2015). Female *Nf1*$^{-/-}$ astrocytes expressed significantly lower levels of *Adcy3* and more *Adcy5* levels, and higher *Pde4a1* levels than male astrocytes. In response to treatment with forskolin, a pan activator of AC, in the presence of IBMX, a pan PDE inhibitor, female cells exhibited greater cAMP synthetic capacity, while in cells treated with forskolin alone, males showed greater capacity to activate phosphodiesterases and block forskolin-induced cAMP elevation. These data indicate that sex differences in expression of cAMP regulators render male and female cells differentially sensitive to the effects of drugs that target the activity of cAMP regulators.

Evidence for sexual dimorphism in the cAMP pathway is also evident in human data. A single nucleotide polymorphism (SNP) array analysis of polymorphisms in the cAMP pathway with DNA from individuals with NF1 with and without optic pathway gliomas revealed that polymorphisms in *ADCY8* (AC8) increase glioma risk in female patients with NF1, but are protective against glioma in male patients (Warrington et al., 2015). Reports of sex specific effects of SNPs in *ADCY7* (rs2302717) in alcohol dependence (Desrivieres et al., 2011) and SNPs in *PDE4B* in schizophrenia (Pickard et al., 2007) suggest there is a broader importance to sex differences in cAMP regulation to human disease.

We would propose that therapeutic cAMP elevation is an ideal setting to test the hypothesis that sexual dimorphism in the cAMP pathway will render males and females differentially sensitive to specific cAMP modulating agents. We previously reported that PDE4 inhibition with Rolipram had significant anti-brain tumor effect in multiple brain tumor models (Yang et al., 2007; Goldhoff et al., 2008; Warrington et al., 2010). We performed a preliminary determination of whether the reported sex differences in PDE4 isoform expression in *Nf1*$^{-/-}$ astrocytes would render male and female *Nf1*$^{-/-}$ astrocytes differentially sensitive to Rolipram. Male *Nf1*$^{-/-}$ astrocytes exhibit lower basal levels of cAMP and are insensitive to Rolipram (**Figure 2B**). In contrast, female *Nf1*$^{-/-}$ astrocytes exhibit higher baseline levels of cAMP and these levels further increase with Rolipram treatment. Thus, female brain tumor patients may be more responsive to Rolipram treatment than male brain tumor patients.

The multiplicity of agents that can target different levels of the cAMP regulatory pathways from GPCR through AC and PDE should allow for exhaustive determination of how significant the effects of sex are on cAMP regulation and therapeutic responses to cAMP modulation. Only with this kind of knowledge can we hope to harness the full power of cAMP elevating agents to treat cancer.

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Different toxic effects of YTX in tumor K-562 and lymphoblastoid cell lines

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Yessotoxin (YTX) modulates cellular phosphodiesterases (PDEs). In this regard, opposite effects had been described in the tumor model K-562 cell line and fresh human lymphocytes in terms of cell viability, cyclic adenosine 3',5'-cyclic monophosphate (cAMP) production and protein expression after YTX treatment. Studies in depth of the pathways activated by YTX in K-562 cell line, have demonstrated the activation of two different cell death types, apoptosis, and autophagy after 24 and 48 h of treatment, respectively. Furthermore, the key role of type 4A PDE (PDE4A) in both pathways activated by YTX was demonstrated. Therefore, taking into account the differences between cellular lines and fresh cells, a study of cell death pathways activated by YTX in a non-tumor cell line with mitotic activity, was performed. The cellular model used was the lymphoblastoid cell line that represents a non-tumor model with normal apoptotic and mitotic machinery. In this context, cell viability and cell proliferation, expression of proteins involved in cell death activated by YTX and mitochondrial mass, were studied after the incubation with the toxin. Opposite to the tumor model, no cell death activation was observed in lymphoblastoid cell line in the presence of YTX. In this sense, variations in apoptosis hallmarks were not detected in the lymphoblastoid cell line after YTX incubation, whereas this type I of programmed cell death was observed in K-562 cells. On the other hand, autophagy cell death was triggered in this cellular line, while other autophagic process is suggested in lymphoblastoid cells. These YTX effects are related to PDE4A in both cellular lines. In addition, while cell death is triggered in K-562 cells after YTX treatment, in lymphoblastoid cells the toxin stops cellular proliferation. These results point to YTX as a specific toxic compound of tumor cells, since in the non-tumor lymphoblastoid cell line, no cell death hallmarks are observed.

Keywords: Yessotoxin, apoptosis, autophagy, K-562, lymphoblastoid line

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Introduction

Under unfavorable conditions, cells develop different strategies to survive and autophagy is the main mechanism; if survival is not possible, they activate autophagy cell death (Codogno and Meijer, 2005). Autophagy, also known as type II programmed cell death is activated after nutrient deprivation or with other stimulus, such as rapamycin treatment (Jung et al., 2010; Ouyang et al., 2012). In both cases, the target is the protein mTOR. This protein inhibits the autophagy through its active phosphorylated form (pmTOR). Under unfavorable conditions, the protein is dephosphorylated and inactivated, and as a result the autophagy cascade is triggered

(Jung et al., 2010). Then, a complex network of metabolic pathways is activated to develop digestive vacuoles, called autophagosomes. These vacuoles are covered by type II of Light Chain 3 (LC3-II) protein (Codogno and Meijer, 2005; Stern et al., 2012). Three different human isoforms are described of LC3 protein: LC3A, LC3B, and LC3C (Wu et al., 2006). These types of LC3 protein are synthesized as the cytosolic type I LC3 protein (LC3A-I, LC3B-I, or LC3C-I) under normal conditions. When autophagy is activated, LC3-I is metabolized in type II (LC3A-II, LC3B-II, or LC3C-II) that binds to autophagosomes membrane (Lazova et al., 2012). Different parts of the cell and damaged organelles are digested by autophagosomes and fused with lysosomes (Klionsky and Emr, 2000). In this way, the basic elements to survive under unfavorable conditions are obtained (Cuervo et al., 2005). However, sometimes, autophagy degenerates in the digestion of the whole cell leading to the autophagy cell death (Tsujimoto and Shimizu, 2005). Another type of programmed cell death is apoptosis, also known as the type I of programmed cell death (Ouyang et al., 2012). Apoptosis can be activated through the intrinsic or mitochondrial apoptotic cell death or through the extrinsic or death receptor (DR) pathway (Elmore, 2007). The first one, known as mitochondrial apoptosis, is triggered by different stimulus that destabilizes mitochondrial membrane potential. As a result, the mitochondrial permeability transition pore (MPTP) is opened and pro-apoptotic proteins are released from the intermembrane space to the cytosol (Debatin et al., 2002). The pro-apoptotic proteins such as cytochrome c, smac/DIABLO, apoptotic inductor factor (AIF), and endonuclease G, activate first caspase 9 and then caspase 3 along with the rest of the apoptotic pathway (Javadov and Karmazyn, 2007). The extrinsic apoptotic cell death is triggered after the binding of a ligand to the DR that belongs to the tumor necrosis factor receptor family (TNFR), in the plasmatic membrane. Then, the death inducing signaling complex (DISC) is developed in the cytosolic region of the plasma membrane. This complex recruits and cleaves procaspases 8 and 10 into the active forms caspases 8 and 10. Caspase 8 also activates caspase 3 and converges at this point with the intrinsic apoptotic pathway (Korsnes and Espenes, 2011).

Yessotoxins (YTXs) are sulfated polyether compounds isolated for the first time from the scallops *Patinopecten yessoensis* (Murata et al., 1987). However, this group of toxins are synthesized by the dinoflagellates *Protoceratium reticulatum, Lingulodinium polyedrum, and Gonyaulax spinifera* (Satake et al., 1997; Paz et al., 2004; Rhodes et al., 2006). YTXs are modulators of phosphodiesterases (PDEs) and consequently affect the levels of cyclic adenosine 3′,5′-cyclic monophosphate (cAMP) (Alfonso et al., 2003, 2004, 2005; Pazos et al., 2006). The final effect is different depending on the cellular model studied, human fresh lymphocytes or human leukemic K-562 cell line (Alfonso et al., 2003; Tobío et al., 2012). Moreover, YTX has been described as a mitochondrial apoptosis inducer (Korsnes and Espenes, 2011; Korsnes, 2012). On the other hand, the structural protein A kinase anchoring protein 149 (AKAP149) binds PDE4A and protein kinase A (PKA) to the outer mitochondrial membrane (Asirvatham et al., 2004; Carlucci et al., 2008). These three components make a complex that is regulated by cAMP levels, since this second messenger activates PKA, and the whole complex moves around the cell depending on cAMP gradients (Baillie et al., 2005; Sample et al., 2012). Since YTX modulates PDEs, the complex was studied after toxin treatment in the tumor K-562 cell line. In this sense, a close relation between the complex expression and cell death activated by the toxin was discovered (Tobío et al., 2012; Fernandez-Araujo et al., 2014). This was supported by the fact that silencing the expression of PDE4A, the effect of YTX on K-562 cell viability is avoided and changes in the cytosolic expression of the rest of the proteins of the complex is observed (Fernandez-Araujo et al., 2014). In addition, a key role of PDE4A in apoptosis and autophagy cell death activated by YTX in the K-562 cell line has been observed (Fernández-Araujo et al., 2015). As mentioned, large differences, in terms of YTX toxicity, cAMP levels and AKAP149 expression, were found depending on the cellular model studied. In this sense, while no effect on cell viability was observed in human fresh lymphocytes, high cell death was detected in leukemic K-562 cells after YTX treatment (Tobío et al., 2012). Later on, the effect in the K-562 line was studied in depth and YTX was described as apoptotic and autophagy inductor in these cells (Fernandez-Araujo et al., 2014). As fresh lymphocytes have no mitotic capacity while leukemia cells are tumor cells, the aim of this work was to study the effect of YTX in a non-tumor cellular model with mitotic and apoptotic intact machinery in order to elucidate whether the toxic effects of YTX are exclusively for tumor cells or if they depend on the mitotic machinery. For this objective a non-tumor cell line, a lymphoblastoid cell line, was chosen. This cell line is a result of human B lymphocytes immortalized with the Epstein Barr virus, hence without tumor features (Sugimoto et al., 2004; Sie et al., 2009; Hussain and Mulherkar, 2012).

Materials and Methods

Reagents and Solutions

YTX was obtained from CIFGA Laboratories (Lugo, Spain). Anti-β-tubulin I, Bovine serum albumin (BSA), $CaCl_2$, NaH_2PO_4, Trizma hydrochloride, Triton X-100, glycine, trizma base, SDS (sodium dodecyl sulfate) and Tween®20 were from Sigma-Aldrich (Madrid, Spain). NaCl, $MgSO_4$, $NaHCO_3$, and glucose were from Panreac (Barcelona, Spain). Anti-PDE4A and anti-LC3B was from ABCAM (CA, USA). Anti-Histone 1, anti-β-Actin, anti-pmTOR, anti-caspase 8 (active form), anti-cytochrome C, anti-rabbit IgG peroxidase conjugated, and Polyvinylidene fluoride (PVDF) membrane was from Millipore (Temecula, USA). Anti-Mouse IgG horseradish peroxidase-linked species-specific whole antibody was purchased from GE Healthcare (Barcelona, Spain).

Polyacrylamide gels and molecular weight marker Precision Plus Protein™ Standards Kaleidoscope™ were purchased from BioRad® (Barcelona, Spain). Protease Inhibitor Complete Tablets and Phosphatase Inhibitor Cocktail Tablets were from Roche (Spain). Free calcium and magnesium PBS used in flow cytometry assays was purchased from Gibco, Life Technologies (UK).

Physiological saline solution composition was (in mM): Na^+ 142.3; K^+ 5.94; Ca^{2+} 1; Mg^{2+} 1.2; Cl^- 126.2; HCO_3^- 22.85;

HPO_4^{2-} 1.2, SO_4^{2-} 1.2; glucose 1 g/L was added to the medium giving an osmotic pressure of 290 ± 10 mOsm/kg of H_2O and pH was adjusted to 7.2 with HCl 0.1 N and CO_2. PBS used to wash the western blotting membranes consisted of NaCl 137 mM; Na_2HPO_4 10.14 mM; KH_2PO_4 1.76 mM; KCl 2.68 mM; pH was adjusted to 7.2 with NaOH.

Cell Culture

K-562 cell line was purchased from the National Cancer Institute (NCI's, USA) and maintained in the Roswell Park Memorial Institute 1640 (RPMI 1640) medium supplemented with 10% fetal bovine serum (FBS) and 50 units/ml penicillin and 50 μg/ml streptomycin. All these reagents were from Gibco, Invitrogen (Spain). Cells were growing at 37°C in a humidified 5% CO_2 atmosphere. Incubations with 30 nM YTX were carried out under these conditions of temperature, humidity and percentage of CO_2.

Lymphoblastoid cell line was obtained from the Banco Nacional de ADN Carlos III (Spain). This cell line is a result of human B lymphocytes immortalized with the Epstein Barr virus, without tumor features and can be used for genetic or functional studies since these cells preserve the genetic characteristics of the lymphocyte B donor (Sugimoto et al., 2004; Sie et al., 2009; Hussain and Mulherkar, 2012). Lymphoblastoid cell line is maintained in the Roswell Park Memorial Institute 1640 (RPMI 1640) medium with HEPES and glutamine (from Biowest, France), supplemented with 15% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin. All these reagents were from Gibco (Invitrogen, Spain). Cells were growing at 37°C in a humidified 5% CO_2 atmosphere. Incubations with 30 nM YTX were carried out under these conditions of temperature, humidity, and percentage of CO_2.

Subcellular Fractionation

3×10^6 cells per condition were incubated for 24 and 48 h with and without 30 nM YTX and then centrifuged and washed with saline solution. Cells were resuspended in lysis buffer with the following composition: 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1X Complete Protease Inhibitor and 1X Phosphatase Inhibitor Cocktail. The extract was sonicated and centrifuged (9300 g, 10 min, 4°C). The supernatant with the cytosolic fraction was transferred to a new tube and stored at −20°C for protein quantification.

Western Blotting Analysis

Direct Detect Spectrometer from Millipore (Germany) was used to know sample protein concentration and BSA was used as protein standard. The electrophoresis run conditions were 200 V for 35 min. The cytosolic fraction was blotted to PVDF membrane with reduced SDS-PAGE. To determine the protein size and also to monitor the progress of the electrophoretic runs, a Precision Plus ProteinTM Standards Kaleidoscope™ molecular weight marker was used. After blockage with 0.5% BSA the membranes were incubated for 10 min with anti-cytochrome C, anti-PDE4A, anti-caspase 8, and anti-pmTOR, then were washed three times with PBS and 0.1% Tween®20 and incubated for 10 min with secondary anti-mouse or anti-rabbit IgG horseradish peroxidase-linked species-specific whole antibody. After three washes, chemiluminescence was visualized with SuperSignal® West Pico (low intensity) or with SuperSignal® West Femto (high intensity) both from Pierce (ThermoScientific, USA) and it was also used a Clarity Western ECL substrate (medium intensity) from BioRad®. The chemiluminiscent signal was detected with the Diversity GeneSnap software and analyzed by the Diversity 4 gel documentation and analysis system. Relative protein expression was calculated in relation to β-actin expression for each experiment in the cytosolic fraction. Experiments were carried out at least three times by duplicate. The purity of the subcellular fraction was tested by measuring control proteins from the cytosol fraction: Histone 1 was the negative control, as it is only present in the nuclear fraction and β-tubulin was the positive control because it is located in the cytosolic fraction.

Cell Viability

After the treatment with the toxin, 5×10^5 cells per condition, cells were centrifuged (250 × g, 4 min, 4°C) and pellet and supernatant were separated. The pellets were first washed with saline solution and centrifuged (1100 × g, 5 min, 4°C), and then re-suspended in saline solution with MTT (250 μg/mL) (M2128, Sigma) and incubated for cell viability (Tobío et al., 2012). The supernatants were utilized to measure LDH release by using the *in vitro* Toxicology Assay Kit (TOX7, Sigma) following the commercial protocol.

Mitochondrial Mass

After the treatment with the toxin, cells were washed with saline solution, centrifuged (1200 × g, 5 min, 4°C) and incubated with 200 nM MitoTracker® Deep Reed FM (Invitrogen, USA) for 30 min, at 37°C in dark. Then the cells were washed and fixed with 4% paraformaldehyde (PFA) for 20 min at 4°C. Next, the cells were washed twice with PBS free of calcium and magnesium and finally resuspended in 80 μL PBS for flow cytometry. The data were analyzed with the IDEAS 4.0 Cell Image Analysis software. The assays were performed with 1×10^6 cells per condition.

Cell Proliferation

Cell proliferation was measured by using three methods, the Scepter™ Handheld automated cell counter (Millipore, Spain) and two methods for total cellular protein determination, Bradford (Bio-Rad protein Assay) and the Direct Detect™ Spectrometer (Millipore, Germany). The number of cells was corrected in each experiment with the cell viability data by using the MTT assay. After the treatment with 30 nM of YTX for 24 and 48 h, 6×10^5 cells per condition, cells were centrifuged (600 × g, 5 min, 4°C), and supernatants were eliminated. The pellets were first washed with saline solution and centrifuged (600 × g, 5 min, 4°C), and then re-suspended in saline solution 250 μL. Hundred microliter of cellular suspension were used to count the number of cells with the Scepter™ Handheld automated cell counter. One hundred and fifty microliter were centrifuged, diluted in 50 μl of water and sonicated. Then, the concentration of protein was determined both, by Bradford assay and Direct

Detect™ (Millipore, Spain). A calibration curve of number of cells (750,000 from 50,000) vs. concentration of protein was done in each experiment and the linearity was always higher than 0.99 for both cellular lines.

Statistical Analysis

All the experiments were carried out at least three times by duplicate. ANOVA was used to examine statistical significance, assumed for $p < 0.05$. Results were expressed as the means ± SEM.

Results

Opposite effects of YTX were reported in tumor K-562 cell line and fresh human lymphocytes (Tobío et al., 2012). This may be due to the differences between these cellular models, since fresh human lymphocytes do not have the ability to grow by themselves. Therefore, it would be interesting to compare the effect of YTX in two cellular lines with similar mitotic machinery. For this purpose, the lymphoblastoid cell line was chosen as a non-tumor model, and compared with the K-562 tumor cell line model. The lymphoblastoid cell line can grow and has normal apoptotic machinery, while the K-562 cell line has not (Hussain and Mulherkar, 2012). **Figure 1** shows cellular proliferation, measured by MTT metabolization, and plasma membrane integrity measured by LDH release (**Figures 1A,B**, respectively) in both cell lines after 24 and 48 h of toxin exposure. A 32% decrease in cell viability was observed in K-562 cell line after 24 h with YTX. However, no effect was observed in lymphoblastoid line under the same conditions. After 48 h of treatment, K-562 cell viability was decreased by 59% while the reduction in lymphoblastoid cell line was 27%. On the other hand, after 24 h of YTX treatment, LDH release was 78% increased in the K-562 cell line, while at this time YTX did not induce any effect in LDH release in lymphoblastoid cells. After 48 h in the presence of YTX, the increase in LDH release was 52% in K-562 cells, while no effects were observed in lymphoblastoid cells.

The result obtained after 48 h of YTX treatment in lymphoblastoid cells with MTT, show a decrease in cellular proliferation but not in LDH release. Since the MTT dye is metabolized by the mitochondria, the fall of MTT signal observed may be caused either by a decrease in mitochondrial mass or by a decrease in the cell number. To find out which of the two options was going on, a dye to measure mitochondrial mass was used and in this way to know the direct effect of YTX in this organelle by using imaging flow cytometry. Therefore, in **Figure 2** mitochondrial mass is shown both in K-562 cell line and in lymphoblastoid line. When the MitoTracker® Deep Reed FM dye is metabolized by the mitochondria, the intensity in red channel is increased, therefore this signal is proportional to mitochondria quantity. **Figures 2A,B** represent mitochondrial mass of K-562 cells and lymphoblastoid cells, respectively, after 48 h of YTX incubation. Higher X-axis values represent more dye intensity, hence more quantity of the mitochondria. **Figure 2C** represents the mean of three experiments. This graphic shows a significant 6% decrease of mitochondrial intensity after 48 h of incubation with YTX in a K-562 cell line, while the intensity is 9% increased in the lymphoblastoid line. Therefore, the decrease in MTT signal obtained in **Figure 1A** with the lymphoblastoid line after 48 h of treatment with YTX is probably not due to a decrease in mitochondrial mass but probably due to a decrease in the cell number with respect to the control without treatment.

It has been described the key role of PDE4A in the mechanism of action of YTX (Fernandez-Araujo et al., 2014). Therefore,

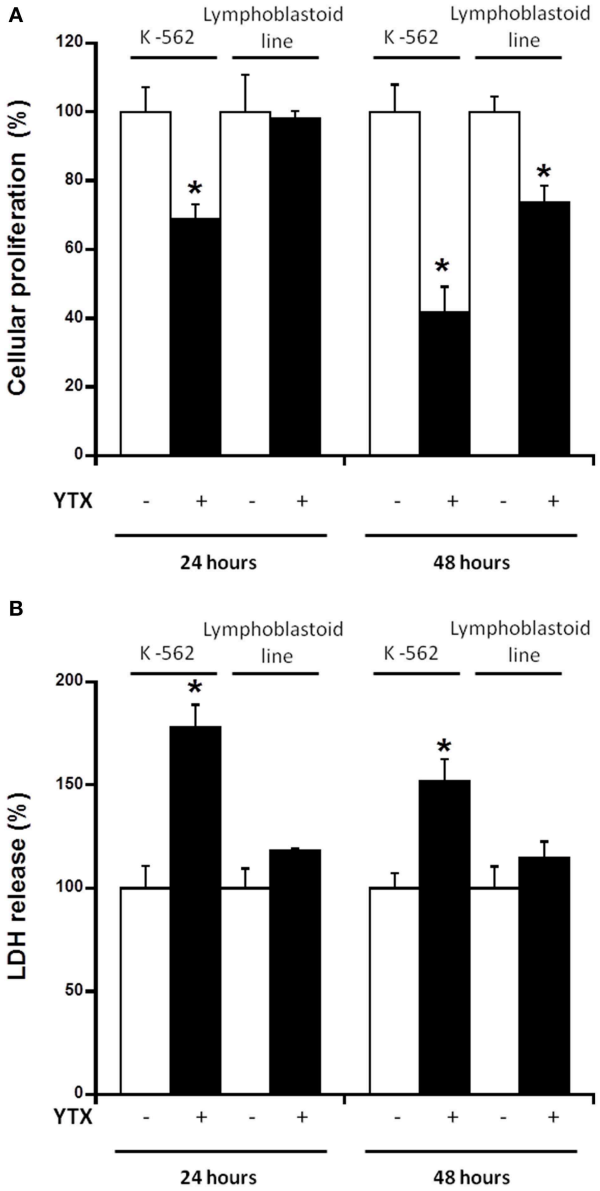

FIGURE 1 | Effect of YTX on cellular MTT signal and LDH release in K-562 and lymphoblastoid cell lines. Cells were incubated with 30 nM YTX for 24 and 48 h (37°C and 5% CO_2 atmosphere). **(A)** Percentage of cellular proliferation measured by MTT assay in K-562 and lymphoblastoid cell lines after 24 and 48 h of YTX incubation. **(B)** Percentage of LDH release of K-562 and lymphoblastoid cell lines after 24 and 48 h of YTX incubation. Mean ± SEM of three experiments. *Significant differences between untreated and YTX-treated cells in each case by ANOVA test.

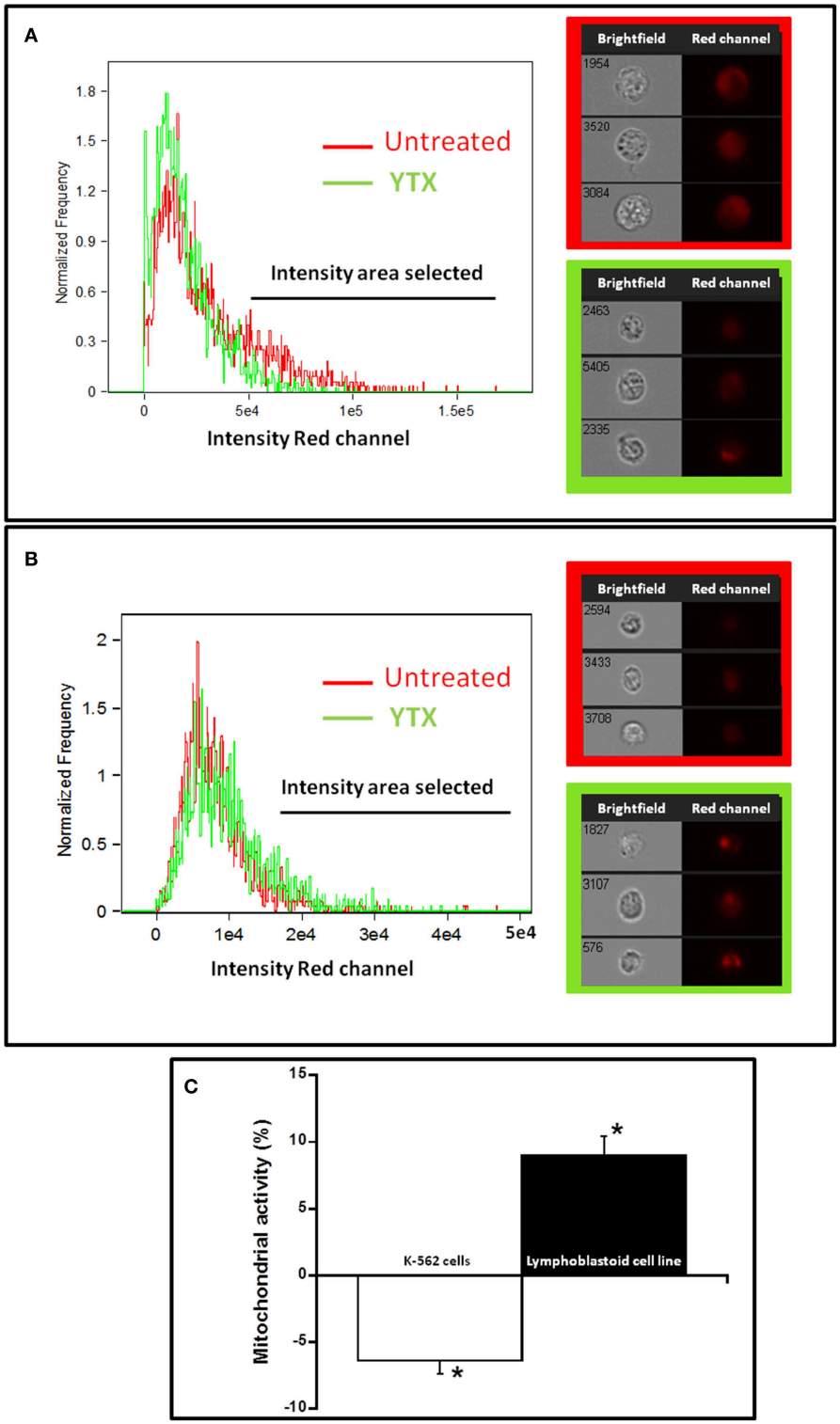

FIGURE 2 | Effect of YTX on total mitochondrial mass of K-562 and lymphoblastoid cell lines. Representative cellular images and histograms of the mitochondrial mass intensities in K-562 and lymphoblastoid cell lines after 48 h (**A,B,** respectively) of YTX incubation (37°C and 5% CO_2 atmosphere). Brightfield images and red channel images with MitoTracker®Deep Reed FM intensity are represented to each cell model on the right of the histograms. **(C)** Percentage of cells with mitochondrial mass intensities selected of K-562 and lymphoblastoid cell lines after 48 h of YTX treatment. Data referred to untreated cells. Mean ± SEM of three experiments (5000 cells were analyzed in each experiment). *Significant differences between YTX-treated and untreated cells by ANOVA test.

this protein was studied in the lymphoblastoid cell line and compared to the tumor K-562 cell line after YTX treatment. **Figure 3** shows the PDE4A expression in both cell models after 24 and 48 h (**Figures 3A,D**, respectively) of exposure to the toxin. While a 25% decrease in PDE4A expression was observed in K-562 cells after 24 h of treatment, no variations were detected in the lymphoblastoid cell line (**Figure 3A**). However, as **Figure 3D** shows, a decrease of 34 and 22%, in PDE4A levels was observed in K-562 and lymphoblastoid lines, respectively, after 48 h of toxin treatment. Therefore, cytosolic PDE4A levels are also involved in YTX effect in lymphoblastoid cell line. Surprisingly, according to the western blot experiments, different molecular weights for PDE4A proteins were observed depending on cellular model use. **Figure 3G** shows the PDE4A band with a lower molecular weight, around 80 KDa, in the case of K-562 cells, and 98 KDa band in lymphoblastoid cells. This is the normal molecular weight that targets the anti-PDE4A used in these experiments.

Either, the activation of both intrinsic and extrinsic apoptosis processes through PDE4A modulation in K-562 cells after 24 h YTX incubation, as well as the autophagy cell death triggered through the PDE4A modulation in the same cell line after 48 h of YTX treatment were extensively studied (Fernandez-Araujo et al., 2014; Fernández-Araujo et al., 2015). Therefore, the activation of these pathways was checked in the lymphoblastoid cell line. First, different apoptotic hallmarks were studied under these conditions. **Figure 4** shows the expression of the mitochondrial apoptotic marker cytochrome c. While in the K-562 cell line a 34% increase in cytosolic cytochrome c levels after 24 h YTX incubation, no effects were observed in lymphoblastoid cells. The same happens when the typical extrinsic apoptotic hallmark, the active form of caspase 8, was measured. As **Figure 5** shows, caspase 8 levels were 75% increased in the tumor K-562 cell line in the presence of YTX for 24 h, while in these conditions no effect was observed in the lymphoblastoid line. Therefore, contrary to K-562 cell line, after 24 h of incubation in the presence of YTX apoptosis was not activated in the lymphoblastoid cell line. Since autophagy was trigged in the K-562 cell line after 48 h of YTX incubation, next the active form of mTOR (pmTOR), as one representative autophagy hallmark, was checked in the lymphoblastoid cell line after 48 h incubation with the toxin (Codogno and Meijer, 2005). Surprisingly, similar results were obtained in both K-562 and lymphoblastoid cell lines. A decrease of 39 and 40% was observed after 48 h of YTX treatment in the tumor and in the non-tumor cell line, respectively (**Figure 6**). Furthermore, **Figure 7** shows the other autophagic hallmark studied in both cell lines, the LC3B-II/LC3B-I ratio. Again very similar results were obtained in both cellular models, since after 48 h of YTX incubation an increase of 208% in LC3B-II/LC3B-I ratio was observed in K-562 cells and 190% in lymphoblastoid cell line. Therefore, these results suggest the activation of an autophagic pathway in the lymphoblastoid cell line, but it does not imply the induction of cell death since no LDH release was observed as **Figure 1B** shows. However, to confirm this theory, the proliferation of both cell lines was studied, **Figure 8**. K-562 cells grow up from near to 6×10^5 cells at the beginning of the experiment, time 0, to around 7.3×10^5 after 24 h and to around of 11×10^5 after 48 h (**Figure 8A**). However, after 24 h of YTX incubation, the number of cells has decreased to 4.7×10^5 cells, and after 48 h of YTX treatment, the number of cells has fallen to near 3.7×10^5 cells. These results mean a decrease in cells number after YTX incubation and significant differences were obtained in untreated and YTX-treated cells. On the other hand, lymphoblasoid cell line shows another pattern of proliferation (**Figure 8B**). The total number of cells in control population was around 5.6×10^5, 7.4×10^5, and 9×10^5 at time 0, after 24 and 48 h, respectively. However, after 24 h of YTX treatment, the number of cells was near to 6.64×10^5, and after 48 h of

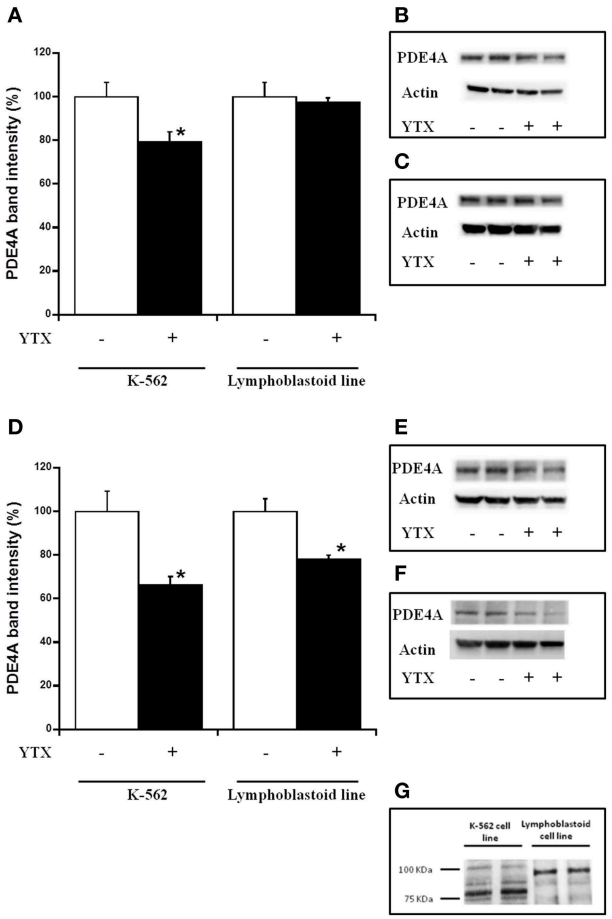

FIGURE 3 | Effect of YTX on cytosolic PDE4A expression in K-562 and lymphoblastoid cell. Cells were incubated for 24 and 48 h with 30 nM YTX (37°C and 5% CO_2 atmosphere). **(A,D)** Percentage of cytosolic PDE4A levels in K-562 and lymphoblastoid cell lines after 24 and 48 h of YTX incubation, respectively. Mean ± SEM of three experiments. Cytosolic PDE4A values were calculated respect to β-actin band intensity. 3×10^6 cells per condition were lysed and 20 μg of total protein per condition was charged in the electrophoresis gel. *Significant differences between untreated and YTX-treated cells by ANOVA test. **(B,C)** Representative experiments of western blot band intensity of cytosolic PDE4A in K-562 (~80 KDa) and lymphoblastoid (~98 KDa) cell lines after 24 h of treatment, respectively. **(E,F)** Representative experiments of western blot band intensity of cytosolic PDE4A in K-562 (~80 KDa) and lymphoblastoid (~98 KDa) cell lines after 48 h of treatment, respectively. **(G)** PDE4A band of K-562 and lymphoblastoid cell lines in western blot membranes. PDE4A antibody targets to a PDE4A protein with a molecular weight of 98 KDa.

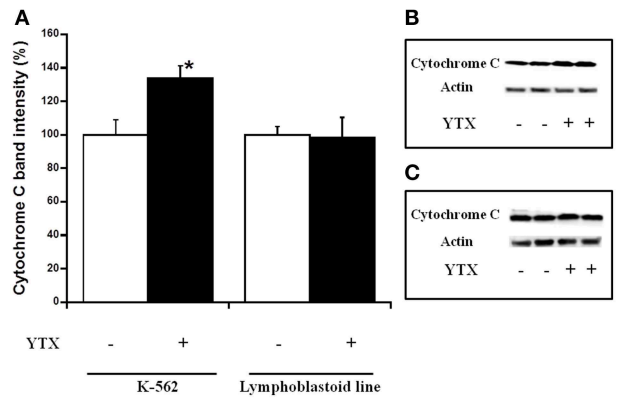

FIGURE 4 | Effect of YTX on cytosolic cytochrome c expression in K-562 and lymphoblastoid cell lines after 24 h of incubation. Cells were incubated for 24 h with 30 nM YTX (37°C and 5% CO_2 atmosphere). **(A)** Percentage of cytosolic cytochrome c levels in K-562 and lymphoblastoid cell lines after 24 h of YTX incubation. Mean ± SEM of three experiments. Cytosolic cytochrome c values were calculated respect to β-actin band intensity. 3×10^6 cells per condition were lysed and 20 μg of total protein per condition was charged in the electrophoresis gel. *Significant differences between untreated and YTX-treated cells by ANOVA test. **(B,C)** Representative experiments of western blot band intensity of cytosolic cytochrome c in K-562 and lymphoblastoid cell lines after 24 h of treatment, respectively.

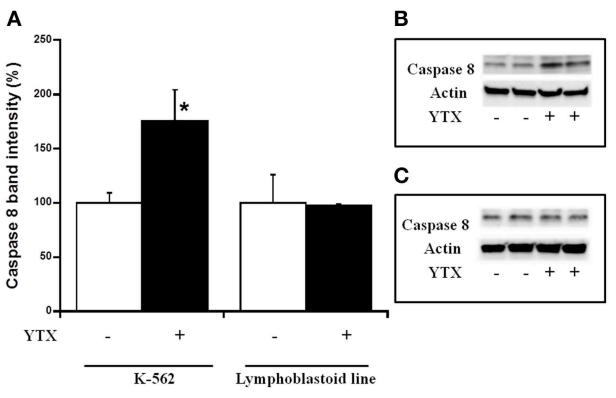

FIGURE 5 | Effect of YTX on cytosolic caspase 8 expression in K-562 and lymphoblastoid cell lines after 24 h of incubation. Cells were incubated for 24 h with 30 nM YTX (37°C and 5% CO_2 atmosphere). **(A)** Percentage of cytosolic caspase 8 levels in K-562 and lymphoblastoid cell lines after 24 h of YTX incubation. Mean ± SEM of three experiments. Cytosolic caspase 8 values were calculated respect to β-actin band intensity. 3×10^6 cells per condition were lysed and 20 μg of total protein per condition was charged in the electrophoresis gel. *Significant differences between untreated and YTX-treated cells by ANOVA test. **(B,C)** Representative experiments of western blot band intensity of cytosolic caspase 8 in K-562 and lymphoblastoid cell lines after 24 h of treatment, respectively.

YTX incubation, the number of cells was around 5.5×10^5. No significant differences in the number of cells were observed after 24 and 48 h of YTX treatment compared to time 0, therefore neither cell death nor growth was detected. Also, no differences were detected between untreated and YTX-treated cells after 24 h of YTX incubation. However, the number of cells after 48 h of YTX treatment was significantly lower compared to untreated cells at this time. Therefore, the proliferation of lymphoblastoid cell line was arrested by YTX effect.

Discussion

YTX activates different cell death types depending on the cellular model (Korsnes, 2012). Moreover, opposite effects in terms of cell viability were obtained after the treatment with YTX of the tumor K-562 cell line and fresh human lymphocytes (Tobío et al., 2012). These observations point to YTX as an interesting compound to target tumor cell lines but not normal cells (Botana et al., 2011). However, there are several differences between an immortalized cellular line and fresh primary cells (Baserga, 1965). In this context, the comparative study of YTX effect in a tumor cell line and a non-tumor cell line could give interesting information about the mechanism of action of YTX. The pathways activated in the leukemic K-562 cell line by this toxin were widely studied. It was described that PDE4A shows a key role in YTX effect that leads to the activation of different programmed cell death types (Tobío et al., 2012; Fernandez-Araujo et al., 2014; Fernández-Araujo et al., 2015). The lymphoblastoid cell line used in this paper is a result of human B lymphocytes immortalized with the Epstein Barr virus, hence without tumor features and with intact apoptotic and mitotic machineries (Sugimoto et al., 2004; Sie et al., 2009; Hussain and Mulherkar, 2012). Therefore, this line is a good tool to study the effects of the toxin in a non-tumor cellular model with mitotic ability to grow up (fresh cells do not have this property), and normal apoptotic machinery (tumor cells do not have it) (Sugimoto et al., 2004; Sie et al., 2009; Hussain and Mulherkar, 2012).

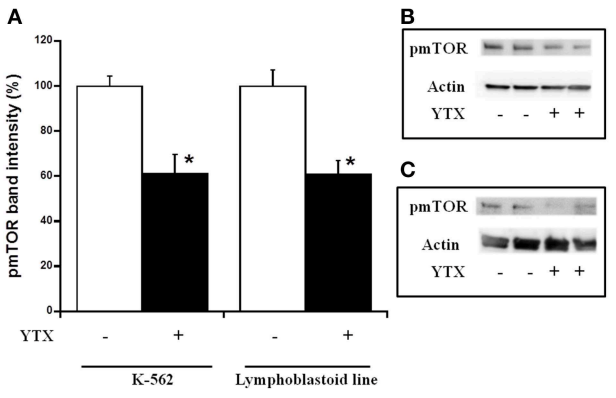

FIGURE 6 | Effect of YTX on cytosolic pmTOR expression in K-562 and lymphoblastoid cell lines after 48 h of incubation. Cells were incubated for 48 h with 30 nM YTX (37°C and 5% CO_2 atmosphere). **(A)** Percentage of cytosolic pmTOR levels in K-562 and lymphoblastoid cell lines after 48 h of YTX incubation. Mean ± SEM of three experiments. Cytosolic pmTOR values were calculated respect to β-actin band intensity. 3×10^6 cells per condition were lysed and 20 μg of total protein per condition was charged in the electrophoresis gel. *Significant differences between untreated and YTX-treated cells by ANOVA test. **(B,C)** Representative experiments of western blot band intensity of cytosolic pmTOR in K-562 and lymphoblastoid cell lines after 48 h of treatment, respectively.

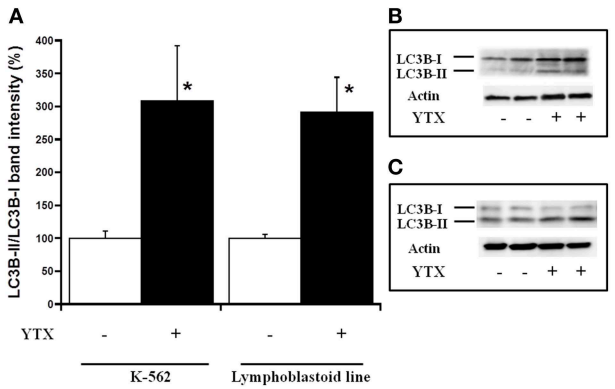

FIGURE 7 | Effect of YTX on cytosolic LC3B-II/LC3B-I expression in K-562 and lymphoblastoid cell lines after 48 h of incubation. Cells were incubated for 48 h with 30 nM YTX (37°C and 5% CO_2 atmosphere). **(A)** Percentage of cytosolic LC3B-II/LC3B-I levels in K-562 and lymphoblastoid cell lines after 48 h of YTX incubation. Mean ± SEM of three experiments. Cytosolic LC3B-I and LC3B-II values were calculated respect to β-actin band intensity and the ratio between LC3B-II and LC3B-I was calculated in order to quantify the autophagosomal LC3B-II isotype 3 × 10^6 cells per condition were lysed and 20 μg of total protein per condition was charged in the electrophoresis gel. *Significant differences between untreated and YTX-treated cells by ANOVA test. **(B,C)** Representative experiments of western blot band intensity of cytosolic LC3B-I and LC3B-II in K-562 and lymphoblastoid cell lines after 48 h of treatment, respectively.

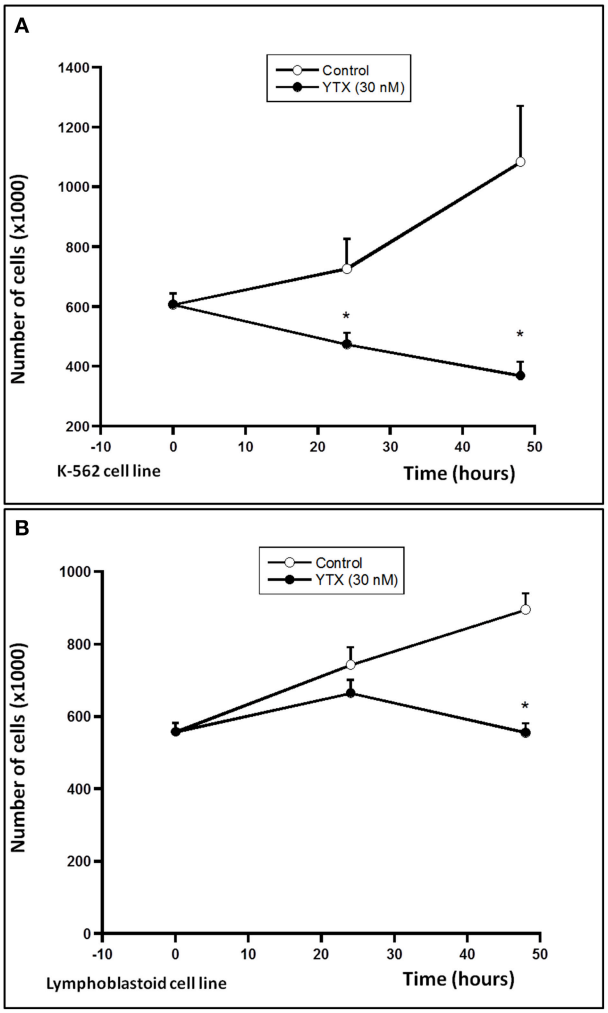

FIGURE 8 | Effect of YTX on cellular proliferation in K-562 and lymphoblastoid cell lines. Cells were incubated with 30 nM YTX for 24 and 48 h (37°C and 5% CO_2 atmosphere). Cells were counted with the Scepter™ Handheld automated cell counter and confirmed as final concentration of protein by Direct Detect™ and Bradford assay. **(A)** Number of K-562 cells after 24 and 48 h of YTX incubation. **(B)** Number of lymphoblastoid cells after 24 and 48 h of YTX incubation. Mean ± SEM of four experiments. *Significant differences between untreated and YTX-treated cells in each case by ANOVA test.

The first difference observed between K-562 and lymphoblastoid lines was the effect of YTX on cell viability. Cellular proliferation is usually measured by MTT assay. This assay informs about the percentage of viable cells capable to reduce the MTT dye within the mitochondria (Mosmann, 1983; Loveland et al., 1992; Verma et al., 2010). The quantification of LDH release is a simple assay to know cell membrane integrity, since after the lysis of the cells, LDH is released to the extracellular medium and the released amount is proportional to the number of broken cells (Lobner, 2000). In this regard, the K-562 cell line did show a decrease in cell proliferation accompanied by an increase in LDH release after both 24 and 48 h of YTX incubation. That is, after YTX treatment the number of K-562 cells was decreasing because cell death was triggered, since apoptosis and autophagy activation was observed, the same as it was described in previous studies (Fernandez-Araujo et al., 2014; Fernández-Araujo et al., 2015). However, in the lymphoblastoid cell line, no effect in cell viability was observed in the first 24 h of treatment, and after 48 h, cell proliferation was decreased without any LDH release, that is without cellular lysis. These results suggest no cell death activation but probably proliferation arrested in the lymphoblastoid line after a long term of YTX exposure. The decrease in the signal observed in MTT assay in the lymphoblastoid line after 48 h YTX treatment comparing with cells without treatment is due to a non-increase in the cell number in the same way than the control where the cells can proliferate. In addition at this time, an increase in the mitochondrial mass, studied by flow cytometry, was observed. In this sense, while the lymphoblastoid cell line usually duplicate its population in 24 h (Sie et al., 2009), YTX could block this increase in the population without cell death activation.

Interesting, the increase in caspase 8 activity was previously observed in K-562 cell line after 24 h of YTX treatment (Fernandez-Araujo et al., 2014). In the present paper, the expression of the active form of caspase 8 is also observed in K-562 cells. This active hallmark is typical from extrinsic apoptotic pathway, and has never been discussed after YTX incubation (Korsnes and Espenes, 2011; Korsnes, 2012; Fernandez-Araujo et al., 2014). In addition, the levels of cytochrome c demonstrate that YTX is also triggering the intrinsic apoptosis (Fulda and Debatin, 2006). Therefore, YTX activates both intrinsic and extrinsic apoptotic cell death. However, none of these hallmarks

were observed in lymphoblastoid cells, showing different YTX effects depending on the cellular model, as it was previously pointed out (Tobío et al., 2012).

PDE4A is involved in the mechanism of action of YTX in the K-562 cell line (Fernandez-Araujo et al., 2014). However, after 24 h of YTX treatment, the levels of cytosolic PDE4A were not modified in the lymphoblastoid cell line, and at this time apoptotic hallmarks and cell viability were not modified either. Moreover, when PDE4A was studied by western blot, different molecular weights for both K-562 cell line and lymphoblastoid cells were observed. This fact can be relevant since any difference in the structure of the PDE4A protein could be a key point of the mechanism of action of YTX. The molecular structure of proteins is a critical parameter for the affinity or interaction between proteins and other molecules, as it was reported in previous studies (Funder et al., 1974; Yatime et al., 2011; Tubaro et al., 2014). These differences can lead to different YTX-PDE4A affinity or to different PDE4A functions depending on the PDE4A structure. In this sense, tumor cells have protein mutations that could lead to the change in the protein function, and these mutations can be used as tumor biomarkers (Wang et al., 2011). Therefore, the PDE4A on the K-562 cell line seems to be the specific and maybe a potential target to tumor therapies in this type of leukemia.

After 48 h of YTX treatment, lymphoblastoid cells showed similar features to those observed in the K-562 cell line (cytosolic PDE4A and pmTOR decrease and LC3B-II/LC3B-I ratio increase). However, these effects did not imply cell death in the lymphoblastoid cell line, as LDH results have shown. At this time, 48 h of YTX treatment, the autophagy activated in K-562 leads to cell death. However, in the lymphoblastoid cell line, autophagy pathway is activated, as the pmTOR and LC3B-II/LC3B-I expression shows. Under unfavorable conditions cells develop mechanisms to start the digestion or recycling of different parts of the cell in order to obtain elements to survive under these critical conditions. Sometimes, the cell cannot survive to this process, and autophagic cell death is activated (Fulda, 2012). But in other times, the aim of the autophagy, which is to survive in extreme conditions, is performed (Hu et al., 2012). Contrary to the K-562 cell line, in lymphoblastoid cells, although autophagy is activated, cell death is not induced while a decrease in cell proliferation rate is observed. One possibility could be the activation of the autophagic survival mechanism through the cell growth regulator mTOR protein, the same as it was described after nutrient deprivation in different type of cells (Jung et al., 2010). A decrease in cell proliferation by autophagic activation was also observed in colon cells and in the human mammary epithelial cell line after treatment with rapamycin and hydrogen sulfide, respectively (Wu et al., 2012; Chen et al., 2013). Also, rapamycin has shown cell cycle arrest in G1 phase of the mitosis in endometrial carcinomas (Bae-Jump et al., 2010). Therefore, the activation of autophagy in the lymphoblastoid cell line leads to a proliferation rate arrest due to the YTX treatment. This theory is corroborated by the results shown in **Figure 8**, since K-562 cells number is decreased by YTX effect, while lymphoblastoid cells number is not modified after YTX incubation, suggesting cellular proliferation arrest by the toxin.

It was described that the point where cell proliferation, apoptosis, and autophagy converge are the mitochondria (Filippi-Chiela et al., 2011). Therefore, a close relationship between MPTP and autophagy or apoptosis was established (Lemasters et al., 1998). MPTP leads to mitochondrial membrane depolarization that finally induces the opening transition pore to release pro-apoptotic proteins to the cytosol (Weiss et al., 2003). When the MPTP process is activated only in some mitochondria, the autophagy is triggered, while apoptosis is activated when a high number of mitochondria have MPTP activation (Lemasters et al., 1998). But the exact linking mechanism between MPTP and autophagy is unknown (Elmore et al., 2001). In his work, after 48 h YTX treatment, when autophagic cell death is activated in the K-562 line, mitochondrial mass was decreased. In this context, mitophagy was defined as the mitochondrial autophagy to describe the mitochondrial removal, characteristic of autophagy activation (Youle and Narendra, 2011). On the other hand, after the incubation with YTX for 48 h, the mitochondrial mass was increased in the lymphoblastoid cell line. Therefore, this is another hallmark to guarantee that the autophagic process activated after 48 h in the K-562 cell line is not occurring in the lymphoblastoid cell line. In the first case, the cells activate autophagy accompanied by mitophagy and mitochondrial signal decrease, while the lymphoblastoid cells could activate autophagy as a survival process accompanied by an increase of mitochondrial mass.

In conclusion, YTX modulates different pathways with different final result depending on the cellular model. In the tumor model, K-562 cells, the type II of programmed cell death is triggered after YTX treatment. While in a non-tumor model, the lymphoblastoid cell line, a survival autophagic process is activated after toxin incubation. Furthermore, apoptosis is only triggered in the tumor model, while in lymphoblastoid cell line, this type of cell death was not activated after YTX incubation.

TABLE 1 | Summary of the viability, apoptotic and autophagic features of K-562 and lymphoblastoid cell line after YTX incubation studied in this paper.

Features	K-562 tumor cell line		Lymphoblastoid cell line	
	24 h	48 h	24 h	48 h
MTT viability	↓	↓	=	↓
LDH release	↑	↓	=	=
PDE4A expression	↓	↓	=	↓
Mitochondrial mass	—	↓	—	↑
Cell death	YES	YES	NO	NO
Apoptotic hallmarks	YES	NO	NO	NO
Autophagic hallmarks	NO	YES	NO	YES
Number of cells	↓	↓	=	=
YTX EFFECT	Apoptosis cell death	Autophagic cell death	No effect	Cell proliferation arrested by autophagy cell survive

This fact could be interesting in order to study the potential effect of YTX in different anti-tumor therapeutics treatments. All these results are summarized in **Table 1**.

Acknowledgments

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Effect of cAMP signaling on expression of glucocorticoid receptor, Bim and Bad in glucocorticoid-sensitive and resistant leukemic and multiple myeloma cells

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Stimulation of cAMP signaling induces apoptosis in glucocorticoid-sensitive and resistant CEM leukemic and MM.1 multiple myeloma cell lines, and this effect is enhanced by dexamethasone in both glucocorticoid-sensitive cell types and in glucocorticoid-resistant CEM cells. Expression of the mRNA for the glucocorticoid receptor alpha (GR) promoters 1A3, 1B and 1C, expression of mRNA and protein for GR, and the BH3-only proapoptotic proteins, Bim and Bad, and the phosphorylation state of Bad were examined following stimulation of the cAMP and glucocorticoid signaling pathways. Expression levels of GR promoters were increased by cAMP and glucocorticoid signaling, but GR protein expression was little changed in CEM and decreased in MM.1 cells. Stimulation of these two signaling pathways induced Bim in CEM cells, induced Bad in MM.1 cells, and activated Bad, as indicated by its dephosphorylation on ser112, in both cell types. This study shows that leukemic and multiple myeloma cells, including those resistant to glucocorticoids, can be induced to undergo apoptosis by stimulating the cAMP signaling pathway, with enhancement by glucocorticoids, and the mechanism by which this occurs may be related to changes in Bim and Bad expression, and in all cases, to activation of Bad.

Keywords: cAMP signaling, glucocorticoid signaling, Bim, Bad, leukemia, multiple myeloma, glucocorticoid receptors, phosphodiesterases

INTRODUCTION

Glucocorticoids remain a central component of all therapeutic regimens used to treat leukemia and multiple myeloma (Ploner et al., 2005; Sionov et al., 2008; Bhadri et al., 2012). However, about 20% of patients demonstrate resistance to glucocorticoids and do not respond to treatment, and invariably, of those that do respond, many develop resistance to glucocorticoids later during treatment, causing them to relapse, with a very poor prognosis (Haarman et al., 2003; Frankfurt and Rosen, 2004; Schmidt et al., 2004; Ploner et al., 2005; Sionov et al., 2008; Bhadri et al., 2012). The molecular basis of glucocorticoid resistance is not fully understood. A number of studies using

in vitro model systems have suggested that it may be associated with a decrease in the expression or alteration of the glucocorticoid receptor (GR), such that the steps normally carried out by the GR that lead to therapeutic benefit are muted (Moalli and Rosen, 1994; Gaynon and Carrel, 1999; Schmidt et al., 2004, 2006; Ploner et al., 2005); however, at least one study finds no correlation with GR expression or function, but finds instead a correlation with the profound attenuation of the induction of the BH3-only pro-apoptotic protein, Bim (Bachmann et al., 2005). Studies using acute lymphocytic leukemic (ALL) cells obtained from patients, as well as 15 T-ALL cell lines grown directly from patients' cells without prior drug exposure in culture, also indicated that resistance could not be attributed to mutations in GR or variations in levels of its expression (Tissing et al., 2006; Bachmann et al., 2007; Beesley et al., 2009). We found that stimulation of the cAMP signaling pathway can overcome glucocorticoid resistance in chronic lymphocytic leukemia (CLL) cells, and in the ALL cell line, CCRF-CEM (Tiwari et al., 2005; Lerner and Epstein, 2006; Dong et al., 2010). The mechanism by which this synergistic effect between stimulation of the cAMP and glucocorticoid signaling pathways occurs, to induce apoptosis of glucocorticoid resistant cells, is, however, still not fully understood. The purpose of this study is to examine the mechanism(s) by which cAMP and glucocorticoid signaling synergize to induce apoptosis of leukemic and multiple myeloma cells.

With respect to leukemia, it appears that the BH3-only pro-apoptotic proteins, Bim and Bad, may be very critical regulators of apoptosis of these cells. In a DNA microarray analysis to uncover genes important in glucocorticoid-induced apoptosis of leukemic cells, Bim was identified as one of the proteins whose expression was most upregulated (Wang et al., 2003). Additionally, studies with mice made deficient for the production of Bim indicate that Bim plays a key role in mediating apoptosis of B and T lymphocytes (Hildeman et al., 2002; Mouhamad et al., 2004). And silencing of the Bim gene with RNAi inhibits glucocorticoid-induced apoptosis of leukemic cells (Abrams et al., 2004). Bim exists as three alternate spliced forms, a short form, BimS, a long form, BimL, and an extra long form, BimEL. Both the turnover and activation of BimEL have been shown to be regulated by its phosphorylation by the MAP Kinases, ERK 1/2 (Ley et al., 2004) and JNK (Putcha et al., 2003). Additionally, studies have shown that the expression of Bim at the gene level is under direct control of the Forkhead transcription factor, FOXO (FKHR; Dijkers et al., 2000). FOXO itself can be phosphorylated and inhibited by the growth promoting kinase PKB/Akt (Burgering and Medema, 2003). PKB/Akt was shown to be inhibited in lymphoma cells by stimulating the cAMP pathway with phosphodiesterase4 (PDE4) inhibitors (Smith et al., 2005), and a similar effect was also seen in mouse embryo fibroblasts (Kuiperij et al., 2005). Hence, stimulating the cAMP pathway and inhibiting PKB/Akt, would be expected to disinhibit FOXO and drive the expression of Bim. And indeed, it was shown that stimulation of the cAMP and glucocorticoid pathways in mouse S49 lymphoma and human CCRF-CEM leukemia cells resulted in a synergistic increase in the expression of Bim (Zhang and Insel, 2004). Bad also appears to be a key player in the regulation of lymphoid cell apoptosis. The activity of Bad is largely controlled by its state of phosphorylation. Studies with interleukin-3 dependent lymphoid cells have shown that when Bad is phosphorylated it is sequestered into an inactive complex with the chaperone protein, 14-3-3. Upon stimulation of its dephosphorylation, it dissociates from 14-3-3 and can then act to initiate apoptosis (Chiang et al., 2001). In cells from patients with CLL, stimulation of the cAMP pathway with the PDE4 inhibitor rolipram led to activation of the protein phosphatase 2A, which dephosphorylated Bad, on its ser112 residue, resulting in increased apoptosis of the leukemic cells (Moon and Lerner, 2003).

We showed previously that inhibitors of PDE4 induce apoptosis of primary CLL cells and synergize with glucocorticoids in doing so (Tiwari et al., 2005). Using glucocorticoid-sensitive and resistant CCRF-CEM leukemic cell lines that we established previously (Tiwari et al., 2005), and established glucocorticoid-sensitive and resistant multiple myeloma cell lines (Greenstein et al., 2003), we examined the effects of stimulation of these two signaling pathways on these glucocorticoid-sensitive and resistant cell types, in relation to their effects on the expression of the mRNA for the GR alpha promoters 1A3, 1B and 1C, expression of mRNA and protein for GR, and the BH3-only proapoptotic proteins, Bim and Bad, and the phosphorylation state of Bad, as a means to examine the mechanism for the synergy between stimulation of the cAMP and glucocorticoid signaling pathways on apoptosis of these cells.

MATERIALS AND METHODS

Materials

Dexamethasone, hydrocortisone, rolipram, forskolin, and 1,9-dideoxyforskolin were obtained from Biomol (Plymouth Meeting, PA, USA). Phenazine methosulfate (PMS) and protease inhibitor cocktail for use with mammalian cell and tissue extracts were from Sigma-Aldrich (St. Louis, MO, USA). [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] (MTS) was from Promega (Madison, WI, USA). Primary rabbit polyclonal antibodies directed against GR, Bim, Bad, and S112 phospho-Bad were obtained either from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Cell Signaling Technologies (Danvers, MA, USA), Biosource International (Camarillo, CA, USA), or Zymed Laboratories (South San Francisco, CA, USA). The specific antibodies used were: GR, Santa Cruz sc-8992; Bim, Santa Cruz sc-11425 and Zymed 38-6500; Bad, Santa Cruz sc-7869 and Cell Signaling Technologies 9292; phospho-Bad, Santa Cruz sc-7998-R, Cell Signaling Technologies 9291 and Biosource International 44-522. Primary rabbit monoclonal antibody directed against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from Cell Signaling Technologies. Anti-Rabbit IgG-horseradish peroxidase was obtained from GE Healthcare (Piscataway, NJ, USA).

Cell Culture

Glucocorticoid-sensitive (CEM-S2) and glucocorticoid-resistant (CEM-R8) CCRF-CEM T leukemic cell lines were isolated as described previously (Tiwari et al., 2005). Established glucocorticoid-sensitive (MM.1S) and glucocorticoid-resistant (MM.1R) cell lines (Greenstein et al., 2003) were also obtained for this study. CEM and MM.1 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen, Carlsbad, CA, USA), at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

MTS Assay

MTS assays were conducted as described previously (Dong et al., 2010, 2015). CEM and MM.1 cells were plated in triplicate at a density of 3×10^4 cells/well in 96-well flat-bottom tissue culture plates in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ ml streptomycin, in a total volume of 0.1 ml of fresh medium containing the test reagents or vehicle as indicated. Following incubation at 37°C for 72 h, 20 ul of a combined solution of MTS (2 mg/ml)/PMS (0.92 mg/ml; 20:1, mixed immediately before use) was added to each well, and the plates incubated for an additional 2 h at 37°C, protected from light, following which the absorbency (OD) of the formazan product formed was determined at 492 nm using a microtiter plate reader (Titertek Multiscan Plus model MK II from Labsystems). The assay was optimized by seeding different amounts of cells at zero time, and determining changes in cell number over 24, 48, and 72 h times to establish ranges of linearity. All assays were done under conditions that maintained them in these linear ranges. With the exception of dexamethasone, all reagents tested were dissolved in DMSO and diluted into the cell culture medium such that the final concentration of DMSO in the assay was 0.1%. Percent cell viability is proportional to the amount of formazan product formed and was calculated as follows: (OD test sample – OD blank)/(OD control – OD blank) × 100, where blank refers to plate wells where media, vehicle, and test reagents were added, as appropriate, but cells were omitted.

Quantitative Real-Time RT-PCR

Quantitative real-time RT-PCR (qRT-PCR) was performed as described previously (Dong et al., 2010, 2015). Total RNA was isolated from cells incubated at different times as indicated, using RNeasy mini kits (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. cDNA was synthesized using M-MLV reverse transcriptase (Promega, Madison, WI, USA). Primers were designed using ABI Primer Express Software v3.0. and synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA) The primers used for the different mRNA expressions analyzed are presented in **Table 1**. qRT-PCR was performed using an ABI 7500 fast system and data analyzed using 7500 fast system SDS software v3.0.

Western Immunoblot Analysis

Western immunoblot analysis was performed as described previously (Dong et al., 2010, 2015; Vang et al., 2013). CEM and MM.1 cells were centrifuged at 300 × *g* for 5 min at the appropriate time points after treatments, washed twice with ice-cold PBS, and lysed in 100 μl RIPA buffer (50 mM Tri-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% Na-deoxycholate, and 1:100 protease inhibitor cocktail). Protein concentration was determined using a Micro BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Equal amounts of protein were loaded and run on 12% SDS-PAGE gels. Proteins were then transferred onto Immobilon-p Transfer Membrane (Millipore). Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline for 1 h at room temperature and probed with primary antibody overnight at 4°C, washed three times with TBS-T buffer, and incubated with horseradish peroxidase-conjugated secondary antibody at a final dilution of 1:5000 and then washed three more times. Proteins were visualized with SuperSignal West Femto maximum Sensitivity Substrate (Pierce, Rockford, IL, USA) and densities of the bands determined using either a UVP BioImaging system (St. Upland, CA, USA) or a Gene-snap Bioimaging system (Syngene, Frederick, MD, USA) with associated software. Blots were stripped and reprobed with GADPH antibody for normalization. Quantitative comparisons of protein expression following different treatments were calculated by determining the ratio of the target protein band density/GAPDH band density for each given treatment and time, divided by the ratio of the target protein band density/GAPDH band density for the untreated control for that given time.

TABLE 1 | Sequences of primers used for quantitative real-time RT-PCR.

Name	Sequence
GR	Forward: AGCCATTGTCAAGAGCGAAC
	Reverse: TGATTGGTGATGATTTCAGCTA
GR1A3	Forward: GCCTGGCTCCTTTCCTCAA
	Reverse: CAGGAGTTAATGATTCTTTGGAGTCC
GR1B	Forward: GCCCAGATGATGCGGTG
	Reverse: TCTACCAGGAGTTAATGATTCTTTGGA
GR1C	Forward: GGGAACTGCGGACGGTG
	Reverse: GGAGTTAATGATTCTTTGGAGTCCA
Bim	Forward: ACAGAGCCACAAGACAGGAG
	Reverse: CCATTGCACTGAGATAGTGGTTG
Bad	Forward: CGGAGGATGAGTGACGAGTT
	Reverse: CCACCAGGACTGGAAGACTC
RPL19	Forward: GAGAAACGGCTGGATGATAGC
	Reverse: TGGTTAGGCTCTTGTACTACTGG

RESULTS

cAMP Signaling Induces Apoptosis of Both Glucocorticoid-Sensitive and Glucocorticoid-Resistant CEM and MM.1 Cell Lines

Established glucocorticoid-sensitive and glucocorticoid-resistant CEM and MM.1 cell lines were maintained in culture and the effects of stimulation of the glucocorticoid and cAMP

signaling pathways on viability of these cells was examined. As shown in **Figure 1A**, stimulation of the glucocorticoid signaling pathway by dexamethasone (1 μM) decreased the viability of the glucocorticoid-sensitive CEM-S2 cells by 78% after 72 h, but had no effect on the viability of the glucocorticoid-resistant CEM-R8 cells (**Figure 1B**). In contrast, stimulation of the cAMP signaling pathway by the adenylyl cyclase activator forskolin (10 μM) plus the PDE4 inhibitor rolipram (10 μM) decreased the viability of both CEM-S2 and CEM-R8 cells (40 and 30% inhibition, respectively, **Figures 1A,B**). Additionally, these effects of forskolin and rolipram on cell viability were greatly potentiated by dexamethasone, resulting in 98 and 95% decrease in cell viability of CEM-S2 and CEM-R8 cells respectively when both the cAMP and glucocorticoid signaling pathways were stimulated concurrently (**Figures 1A,B**). Very similar to results seen with CEM leukemic cells, stimulation of the glucocorticoid signaling pathway by dexamethasone (1 μM) also decreased the viability of the glucocorticoid-sensitive MM.1S multiple myeloma cells by 78% after 72 h (**Figure 1C**), and had no effect on the viability of the glucocorticoid-resistant MM.1R cells (**Figure 1D**). Additionally, also similar to CEM cells, stimulation of the cAMP signaling pathway by forskolin (10 μM) and rolipram (10 μM) greatly decreased the viability of both MM.1S cells (78% inhibition) and MM.1R cells (56% inhibition). The effects of forskolin and rolipram on cell viability were potentiated by the addition of dexamethasone in MM.1S cells, where inhibition of cell viability was increased from 78 to 92% (**Figure 1C**), although, unlike glucocorticoid-resistant CEM-R8 cells, there was only a small potentiation in glucocorticoid-resistant MM.1R cells where inhibition of cell

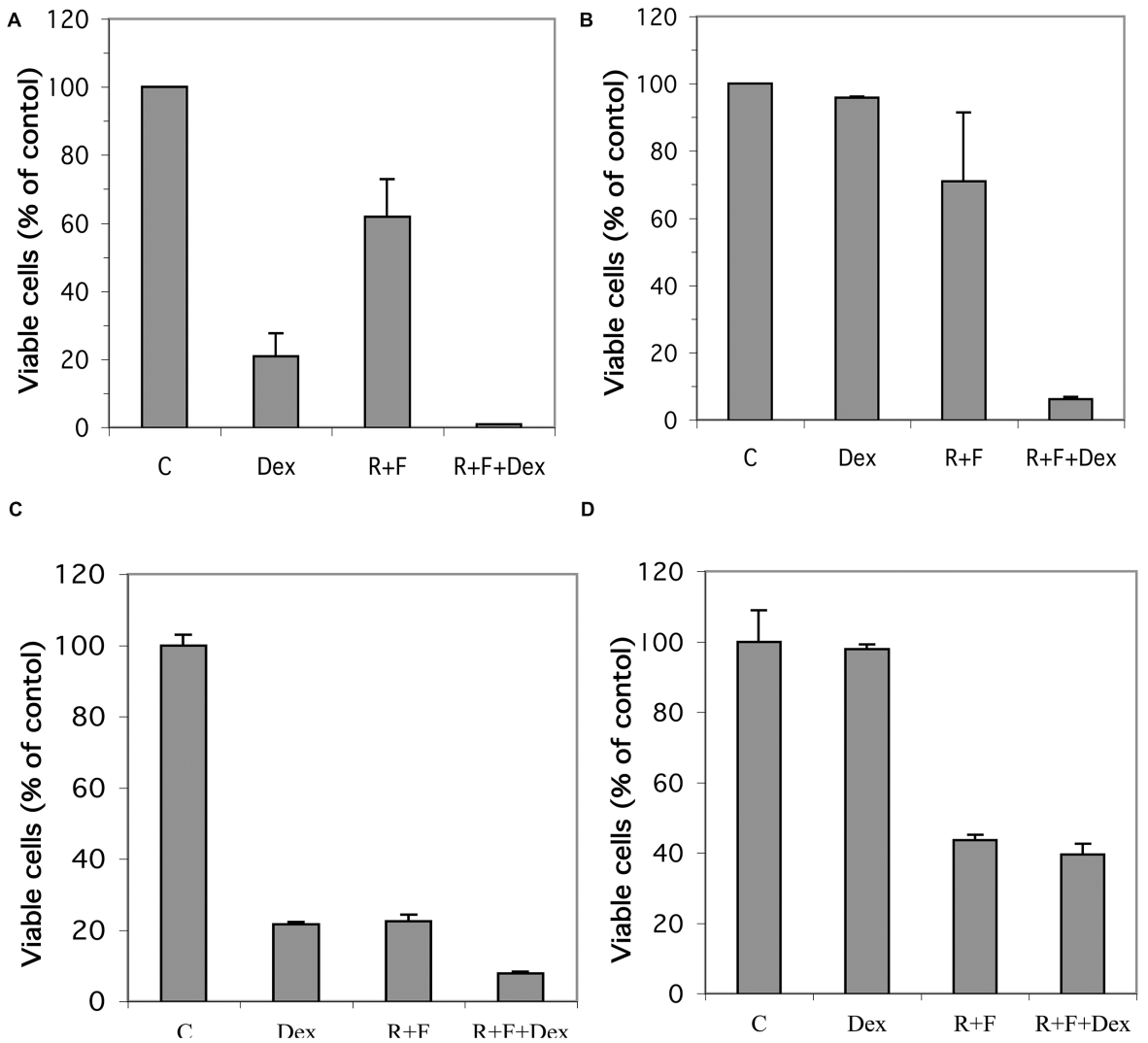

FIGURE 1 | Effect of dexamethasone, rolipram, and forskolin on viability of glucocorticoid-sensitive and glucocorticoid-resistant CEM and MM.1 cells. (A) CEM-S2 cells, **(B)** CEM-R8 cells, **(C)** MM.1S cells, and **(D)** MM.1R cells were treated with 1 μM dexamethasone (Dex), 10 μM rolipram plus 10 μM forskolin (R+F), or 1 μM dexamethasone plus 10 μM rolipram plus 10 μM forskolin (R+F+Dex) for 72 h. Cell viability was then determined by the MTS method. Data represent the mean ± SD of at least two independent experiments assayed in triplicate.

viability was increased from 56 to 60% (**Figure 1D**). Analysis of the cells following these treatments by direct microscopic visualization revealed cell loss and cell shrinking, and by gel analysis of isolated genomic DNA revealed DNA ladder patterns, indicating that loss of cell viability is consistent with apoptosis of the cells (data not shown). These results indicate that the glucocorticoid-resistant CEM-R8 leukemic and MM.1R multiple myeloma cell lines, while completely resistant to the effects of glucocorticoids, are still able to be killed by stimulation of the cAMP signaling pathway. Further, stimulation of the cAMP and glucocorticoid signaling pathways together act synergistically to decrease viability of both glucocorticoid-sensitive and resistant CEM cells and glucocorticoid-sensitive MM.1 cells.

Forskolin Acts through Stimulation of the cAMP Signaling Pathway

Inasmuch as forskolin has been reported to produce effects independent of its activation of adenylyl cyclase (Ding and Staudinger, 2005; Riddell et al., 2013; Angel-Chavez et al., 2015), we compared forskolin with 1,9-dideoxyforskolin, an analog of forskolin that does not stimulate adenylyl cyclase, but maintains many of the other pleiotropic effects of forskolin, for their ability to synergize with glucocorticoids to induce cell death in CEM cells. As seen in **Figure 2**, neither forskolin nor 1,9-dideoxyforskolin by themselves have any effect on viability of CEM-S2 or CEM-R8 cells; however, forskolin synergizes with hydrocortisone to induce cell death in CEM-S2 and CEM-R8 cells, whereas 1,9-dideoxyforskolin does not. This indicates that forskolin is most likely acting through stimulation of the cAMP signaling pathway, and not through other pleiotropic effects.

Expression of the Glucocorticoid Receptor Alpha (GR) Gene Product is Little Changed by Stimulation of the cAMP and Glucocorticoid Signaling Pathways in CEM cells and Greatly Downregulated in MM.1 Cells

RNA transcript and protein expression for the alpha form of the GR was examined by qRT-PCR and Western blot analysis in response to stimulation of the cAMP and glucocorticoid signaling pathways. As shown in **Figures 3A,B**, stimulation of the cAMP signaling pathway by forskolin and rolipram and the glucocorticoid pathway by dexamethasone induced transcription of the mRNA for GR in CEM cells. When protein product for GR was analyzed by Western blot, however, little change was seen in its expression following stimulation of the cAMP signaling pathway, except for some (1.3–2.3-fold) induction at the late, 24 h time point, for both glucocorticoid-sensitive and resistant CEM cells, and this induction of GR was attenuated by simultaneous stimulation of the glucocorticoid pathway with dexamethasone (**Figures 3C,D**). The doublets seen in the Western blot immunostaining for GR alpha (**Figures 3C,D,F**) of Mr 94 kDa and Mr 91 kDa, represent the two forms of GR alpha, termed GR-A and GR-B, produced by alternative translation of the GR alpha gene, with the 91 kDa form produced from an internal ATG codon corresponding to met27 (Yudt and Cidlowski, 2001). In MM.1S cells, cAMP and glucocorticoid signaling produced either no change or small increases in mRNA for GR (**Figure 3E**), but GR protein was greatly diminished in response to dexamethasone treatment, and further diminished to the point of being nearly absent at the 48 and 72 h time points, when both the glucocorticoid and cAMP signaling pathways were stimulated together (**Figure 3F**). The MM.1R cell line has been reported to mainly express truncated forms of the GR with non-functional hormone binding domains, and as such, produces very little full length GR product (Moalli et al., 1992). Consistent with this, we were unable to detect any expression for GR in MM.1R cells under any conditions (data not shown).

Stimulation of the cAMP Signaling Pathway Induces the 1A, 1B, and 1C Promoters for GR in CEM and MM.1 Cells

In humans, the GR is transcribed from at least three different promoters, termed 1A, 1B, and 1C, with 1A further exhibiting three different splice sites designated 1A1, 1A2, and 1A3 (Yudt and Cidlowski, 2002). Using primers specific for the 1A3, 1B, and 1C promoter regions, we examined the effect of stimulation of the cAMP signaling pathway on expression of the three GR promoters. As seen in **Figures 4Ai,Bi**, expression of the 1A3 promoters for CEM-S2 and CEM-R8 cells were highly induced by stimulation of cAMP signaling, and this was potentiated by glucocorticoid signaling to yield inductions of the 1A3 promoter of as much as ≈30–120-fold by 24 h. The 1B and 1C promoters in CEM cells were also induced 6–8-fold at 24 h by combined cAMP and glucocorticoid signaling (**Figures 4Aii,iii,4Bii,iii**). The 1A3 promoter was transiently induced by forskolin and rolipram in MM.1S cells. A 150-fold induction of the 1A3 promoter was seen in MM.1S cells at 2 h after forskolin and rolipram treatment, after which the enhanced expression diminished to only sixfold by 6 h (**Figure 4Ci**), and expression of 1A3 was then undetectable at 24 and 48 h (not shown). Dexamethasone by itself had little effect on the expression of the 1A3 promoter in MM.1S cells, but, as with protein expression of the GR itself, dexamethasone attenuated the induction of the 1A3 promoter by forskolin and rolipram (**Figure 4Ci** insert). The effect of cAMP signaling on expression of the 1B and 1C promoters in MM.1S cells was quite different from that of the 1A3 promoter. Forskolin and rolipram had no effect on expression of the 1B and 1C promoters until 48 h, at which time they induced expression of both of these promoters by 4–5-fold. Dexamethasone by itself also had no effect on expression of the 1B and 1C promoters until 48 h, at which time it induced expression of both of these promoters also by about fivefold. However, whereas dexamethasone attenuated the induction of the 1A3 promoter by forskolin and rolipram, in contrast, it greatly potentiated the induction of the 1B and 1C promoters, resulting in 19–26-fold induction of both of these promoters (**Figure 4Cii,iii**).

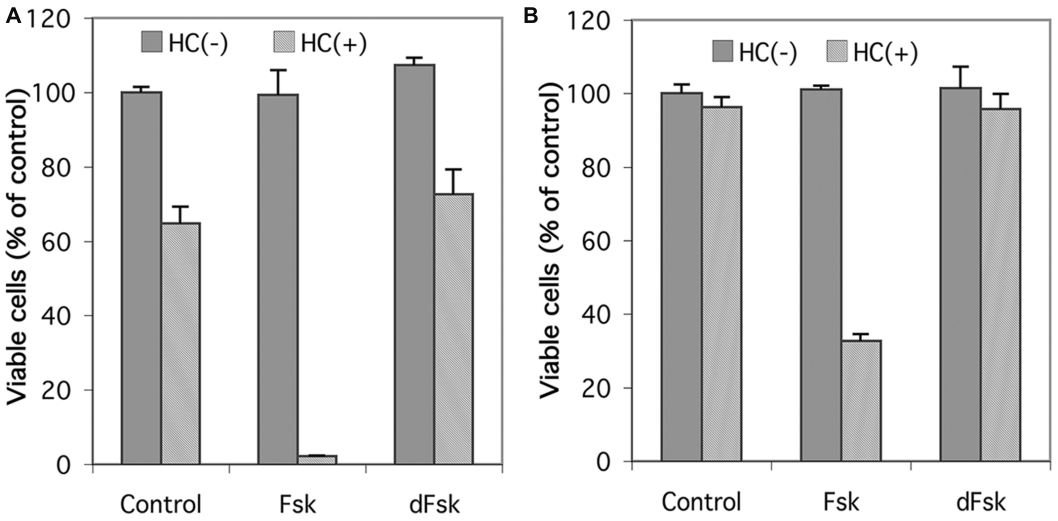

FIGURE 2 | Effect of 1,9-dideoxyforskolin on CEM cells. Cell viability of CEM-S2 cells **(A)** and CEM-R8 cells **(B)**. Cells were treated with 10 μM forskolin (Fsk) or 10 μM 1,9-dideoxyforskolin (dFsk) in the presence or absence of 10 μM hydrocortisone (HC) for 72 h. After treatment, cell viability was determined by MTS assay. Data represent the mean ± SD of at least two independent experiments assayed in triplicate.

Bim Expression is Induced by Stimulation of the cAMP and Glucocorticoid Signaling Pathways in Glucocorticoid-Sensitive and Resistant CEM Cells and in Glucocorticoid-Sensitive MM.1 Cells, but not in Glucocorticoid-Resistant MM.1 Cells

RNA transcript and protein expression for the BH3-only proapoptotic protein, Bim, was examined by qRT-PCR and Western blot analysis in response to stimulation of the cAMP and glucocorticoid signaling pathways. As shown in **Figures 5A,B**, mRNA expression for Bim is induced several fold by cAMP and glucocorticoid signaling in both CEM-S2 and CEM-R8 cells, at all time points examined, and this induction is greatly potentiated, yielding inductions of ≈20–60-fold, when both signaling pathways are stimulated together.

Expression of Bim protein in response to stimulation of these signaling pathways reflected the changes seen in Bim mRNA levels, for the most part. Bim can be expressed as three different splice variants, an extra long form of Mr ≈24 kDa (BimEL), a long form of Mr ≈21 kDa (BimL), and a short form of Mr ≈19 kDa (BimS), and CEM cells express all three of these Bim protein products. As seen in **Figures 5A,B**, all three Bim protein products are induced to some extent following stimulation of cAMP or glucocorticoid signaling, and the induction is more pronounced (1.6–4.2-fold stimulation of BimEL) when both signaling pathways are stimulated together.

As shown in **Figure 5C**, stimulation of the cAMP signaling pathway with forskolin and rolipram induced expression of the mRNA for Bim in MM.1S cells by sevenfold at 4 h, threefold at 24 h, and fivefold at 48 h. Stimulation of the glucocorticoid pathway by dexamethasone also induced expression of Bim mRNA in MM.1S cells by 2–3-fold at each of these time points. When both signaling pathways were activated by adding forskolin, rolipram, and dexamethasone together, induction of Bim mRNA was potentiated, resulting in induction of 12-fold at 4 h, eightfold at 24 h, and ninefold at 48 h (**Figure 5C**). Analysis of the effects of these agents on Bim mRNA transcript in MM.1R cells showed that stimulation of the cAMP signaling pathway with forskolin and rolipram also induced Bim mRNA expression in these cells, by 13-fold at 4 h, twofold at 24 h, and fourfold at 48 h (**Figure 5D**). However, in contrast to MM.1S cells, and in contrast to the effects of cAMP signaling, stimulation of the glucocorticoid signaling pathway with dexamethasone in MM.1R cells had no effect at all on the expression of Bim mRNA, and little or no effect on the induction seen in response to forskolin and rolipram (**Figure 5D**).

In contrast to CEM (**Figures 5A,B**) and Hut78 (data not shown) T leukemic cell lines, which visibly express all three forms of Bim protein, expression of BimS in MM.1 cells was far less than that of BimEL and BimL and was only detectable if the Western blots were greatly overexposed (data not shown). In MM.1S cells, expression of the BimEL and BimL splice variants were induced at most of the time points measured, 4, 24, 48, and 72 h, in response to dexamethasone, forskolin plus rolipram, or all three agents added together (**Figure 5C**). However, induction of Bim protein in MM.1S cells was somewhat higher following stimulation of the cAMP signaling pathway alone (2–4.7-fold) than following concurrent stimulation of the cAMP and glucocorticoid signaling pathways (1.2–3.6-fold), and at all time points examined stimulation of the glucocorticoid signaling pathway actually attenuated the induction effect of cAMP on Bim protein expression. In contrast to MM.1S cells, in MM.1R cells, there was little change in the expression of

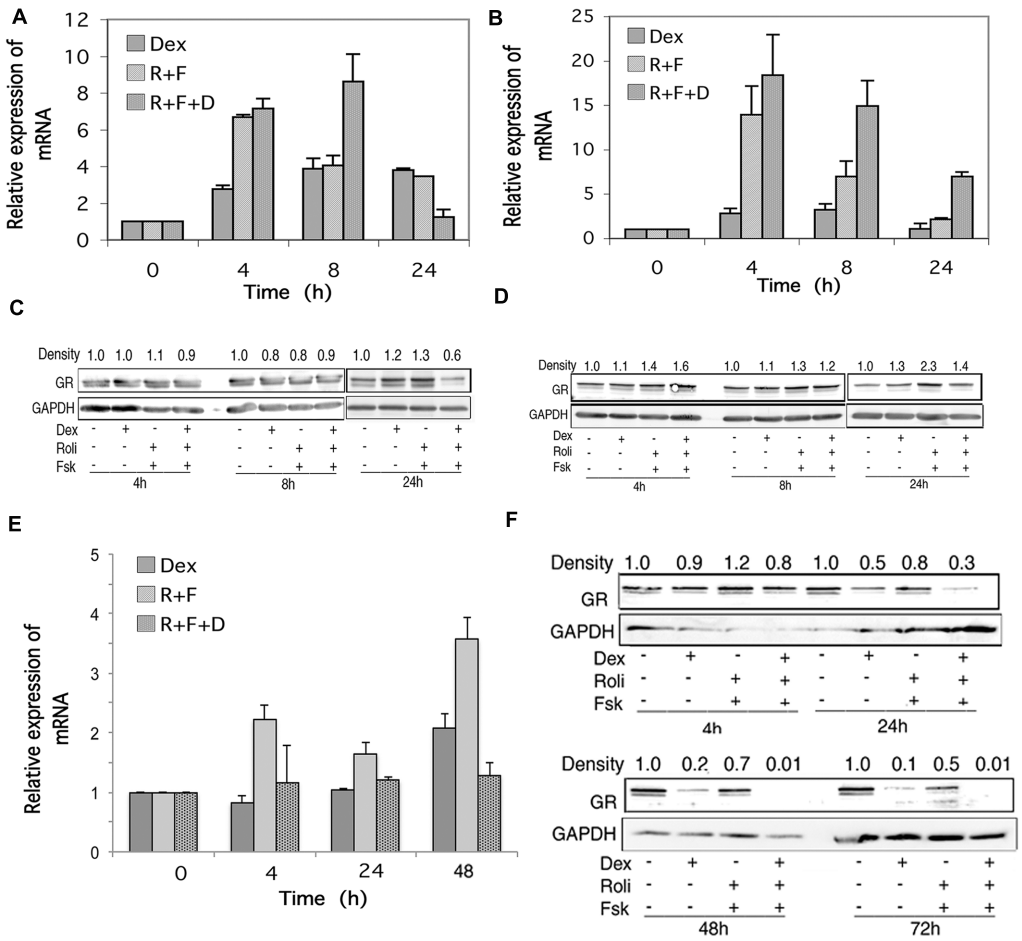

FIGURE 3 | Glucocorticoid receptor (GR) expression in CEM and MM.1 cells. (A,B,E) Quantitative real-time PCR analysis of GR mRNA expression in CEM-S2 **(A)**, CEM-R8 **(B)**, and MM.1S **(E)** cells, following treatment with 1 μM dexamethasone (Dex), 10 μM rolipram plus 10 μM forskolin (R+F), or 1 μM dexamethasone plus 10 μM rolipram plus 10 μM forskolin (R+F+D) at different times as indicated. Data represent the mean ± SD of at least two independent experiments assayed in triplicate, shown as fold change relative to control. **(C,D,F)** Western blot analysis of GR protein expression in CEM-S2 **(C)**, CEM-R8 **(D)**, and MM.1S **(F)** cells following treatment at different times with dexamethasone and/or rolipram and forskolin as indicated. Equal amounts of protein (20 μg) from whole cell lysates were added per lane. Data shown represents one of at least two independent experiments with similar results. The numbers at the top of the Western blot images represent the GR band densities following different treatments relative to control, normalized based on the density of the GAPDH housekeeping protein, calculated as described in Section "Materials and Methods."

BimEL and BimL following stimulation of the cAMP and glucocorticoid signaling pathways, either alone or together (**Figure 5D**).

Bad Expression is Little Changed in CEM Cells in Response to Stimulation of the cAMP and Glucocorticoid Signaling Pathways, but is Highly Induced in MM.1 Cells

RNA transcript and protein expression for the BH3-only proapoptotic protein, Bad, was examined by qRT-PCR and Western blot analysis in response to stimulation of the cAMP and glucocorticoid signaling pathways. As shown in **Figure 6A**, in CEM-S2 cells, stimulation of the glucocorticoid signaling pathway with dexamethasone produced no effect on Bad mRNA expression at any time point examined, and stimulation of the cAMP signaling pathway with forskolin and rolipram produced a small increase in Bad mRNA expression, about 1.5-fold, only at the 24 h time point, with or without dexamethasone. Similarly, as shown in **Figure 6B**, in CEM-R8 cells, stimulation of either the cAMP or glucocorticoid signaling pathways produced little change in Bad mRNA expression, although combined stimulation of the glucocorticoid and cAMP signaling pathways produced a 5–6-fold increase in the expression of Bad mRNA at the 4 and 6 h time points. Also as shown in **Figures 6A,B**, expression of total Bad protein in CEM cells was also little affected by these signaling pathways. Stimulation of the glucocorticoid and cAMP signaling pathways produced only very small increases in the expression of total Bad protein, seen mostly at the 4 h time point, where in CEM-S2 cells, Bad protein increased 1.5-fold in response to dexamethasone, 1.7-fold in response to forskolin plus rolipram,

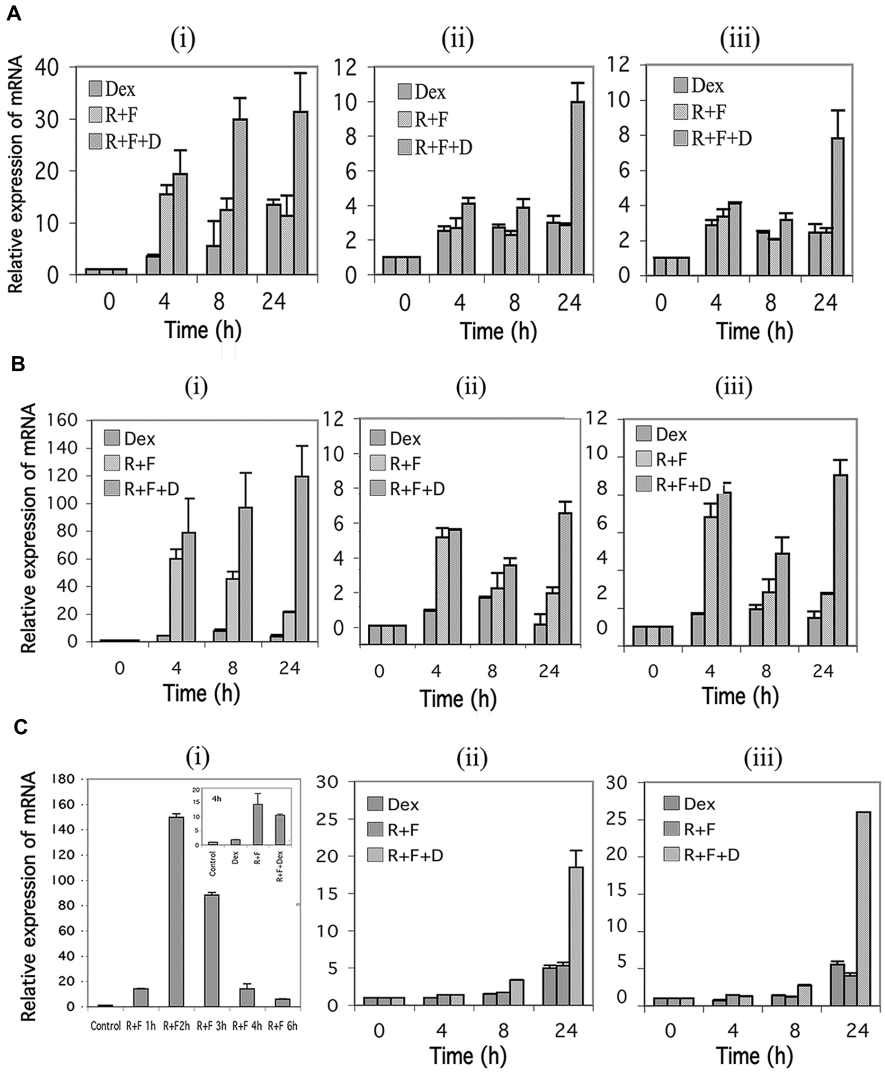

FIGURE 4 | Glucocorticoid receptor alpha (GR) promoter 1A, 1B, and 1C expression in CEM and MM.1 cells. Quantitative real-time PCR analysis of GR1A3, 1B and 1C mRNA expression in both CEM and MM.1 cells. **(A)**, GR1A3(i), 1B(ii), and 1C(iii) expression in CEM-S2 cells. **(B)**, GR1A3(i), 1B(ii), and 1C(iii) expression in CEM-R8 cells. **(C)**, GR1A3(i), 1B(ii), and 1C(iii) expression in MM.1S cells. Points were taken following treatment with 1 μM dexamethasone (Dex), 10 μM rolipram plus 10 μM forskolin (R+F), or 1 μM dexamethasone plus 10 μM rolipram plus 10 μM forskolin (R+F+D) for different times as indicated. Data represent the mean ± SD of at least two independent experiments assayed in triplicate.

and 1.9-fold when all three agents were added together. In CEM-R8 cells at 4 h Bad protein increased 1.2-fold in response to dexamethasone, 1.2-fold in response to forskolin plus rolipram, and 1.4-fold when all three agents were added together.

In MM.1S and MM.1R cells, as shown in **Figures 6C,D**, stimulation of the glucocorticoid signaling pathway alone by dexamethasone had no effect on expression of the mRNA for Bad at any of the time points measured. Stimulation of the cAMP pathway with forskolin and rolipram resulted in a small upregulation of Bad mRNA, ranging from 1.3 to 1.5-fold in MM.1S cells and 1.6–2.2-fold in MM.1R cells at the different time points measured. Addition of dexamethasone to forskolin and rolipram produced no further change in Bad mRNA expression in MM.1R cells, but potentiated the effects of cAMP signaling in MM.1S cells, such that the expression levels of Bad mRNA in MM.1S cells were increased to 2.2-fold at 4 h, twofold at 24 h, and fourfold at 48 h (**Figures 6C,D**).

Western blot analysis of total Bad protein revealed appreciable increases in expression in MM.1S cells in response to stimulation of cAMP and glucocorticoid signaling. Bad protein expression increased 1.1–1.7-fold by independent stimulation of the cAMP or glucocorticoid signaling pathways at 4 h and 24 h, and increased further to 2.2-fold at 24 h when both signaling pathways were stimulated by addition of dexamethasone and forskolin plus rolipram together (**Figure 6C**). At 48 h, expression of Bad protein was increased 1.9-fold by dexamethasone and twofold by forskolin plus rolipram treatment, and its expression was increased further to fourfold when all three agents were

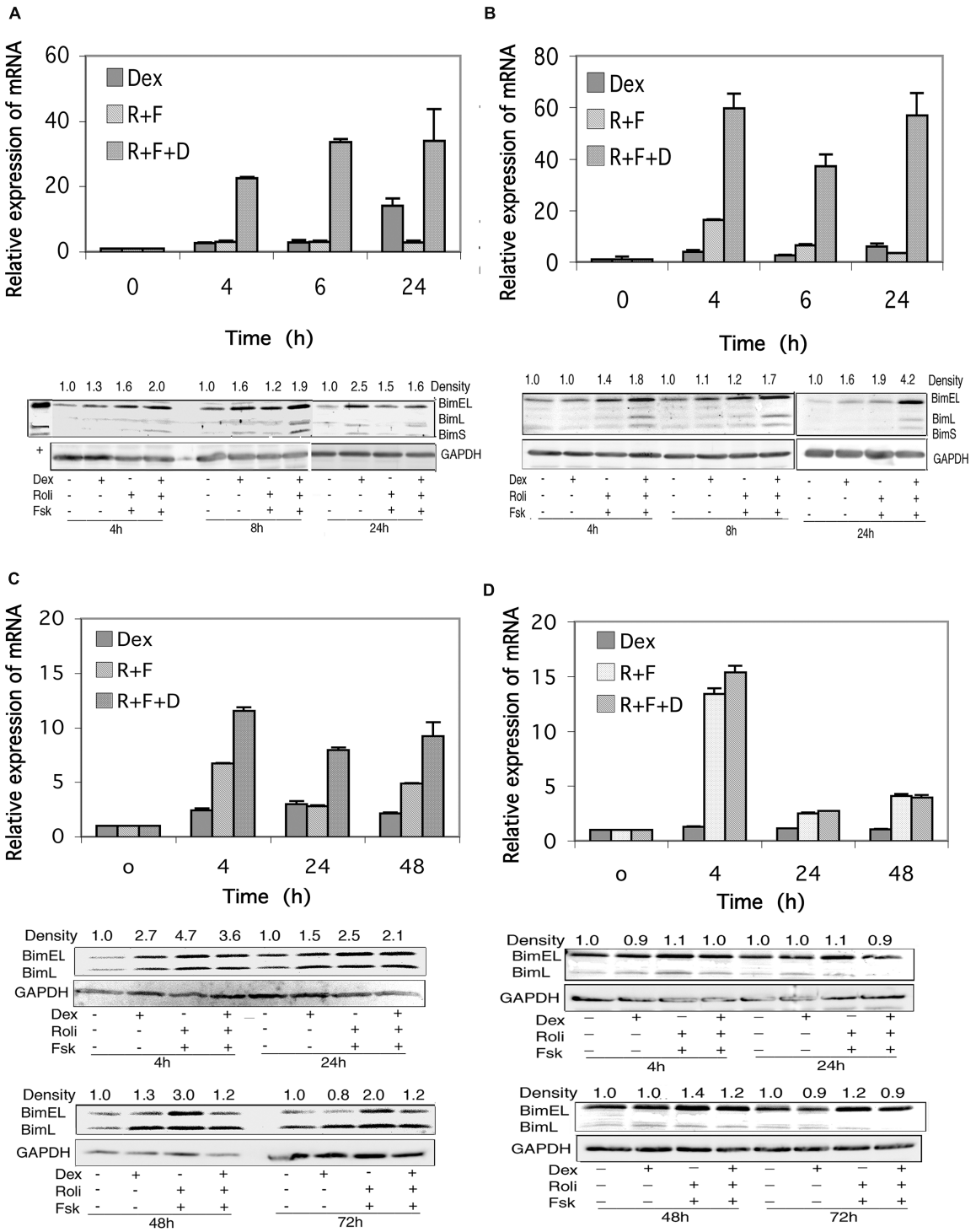

FIGURE 5 | Bim expression in CEM and MM.1 cells. (A) Quantitative real-time PCR analysis of Bim mRNA expression in CEM-S2 cells (top), at different times following treatment with 1 μM dexamethasone (Dex), 10 μM rolipram plus 10 μM forskolin (R+F) or 1 μM dexamethasone plus 10 μM rolipram plus 10 μM forskolin (R+F+D) as indicated. Data represent the mean ± SD of at least two independent experiments assayed in triplicate, shown as fold change relative to control. (bottom), Western blot analysis of expression of Bim protein. Cells were treated at different times with dexamethasone and/or rolipram and forskolin as indicated, and Bim protein expression was determined by immunoblots of whole cell lysates. Equal amounts of protein (20 μg) were loaded per lane. Data shown represents one of at least two independent experiments with similar results. The numbers at the top of the Western blot images represent the BimEL band densities following different treatments relative to control, normalized based on the density of the GAPDH housekeeping protein, calculated as described in Section "Materials and Methods." **(B)** CEM-R8 cell Bim mRNA (top) and protein (bottom) expression. **(C)** Bim mRNA (top) and protein (bottom) expression in MM.1S cells. **(D)** Bim mRNA (top) and protein (bottom) expression in MM.1R cells. Conditions were as in **(A)**.

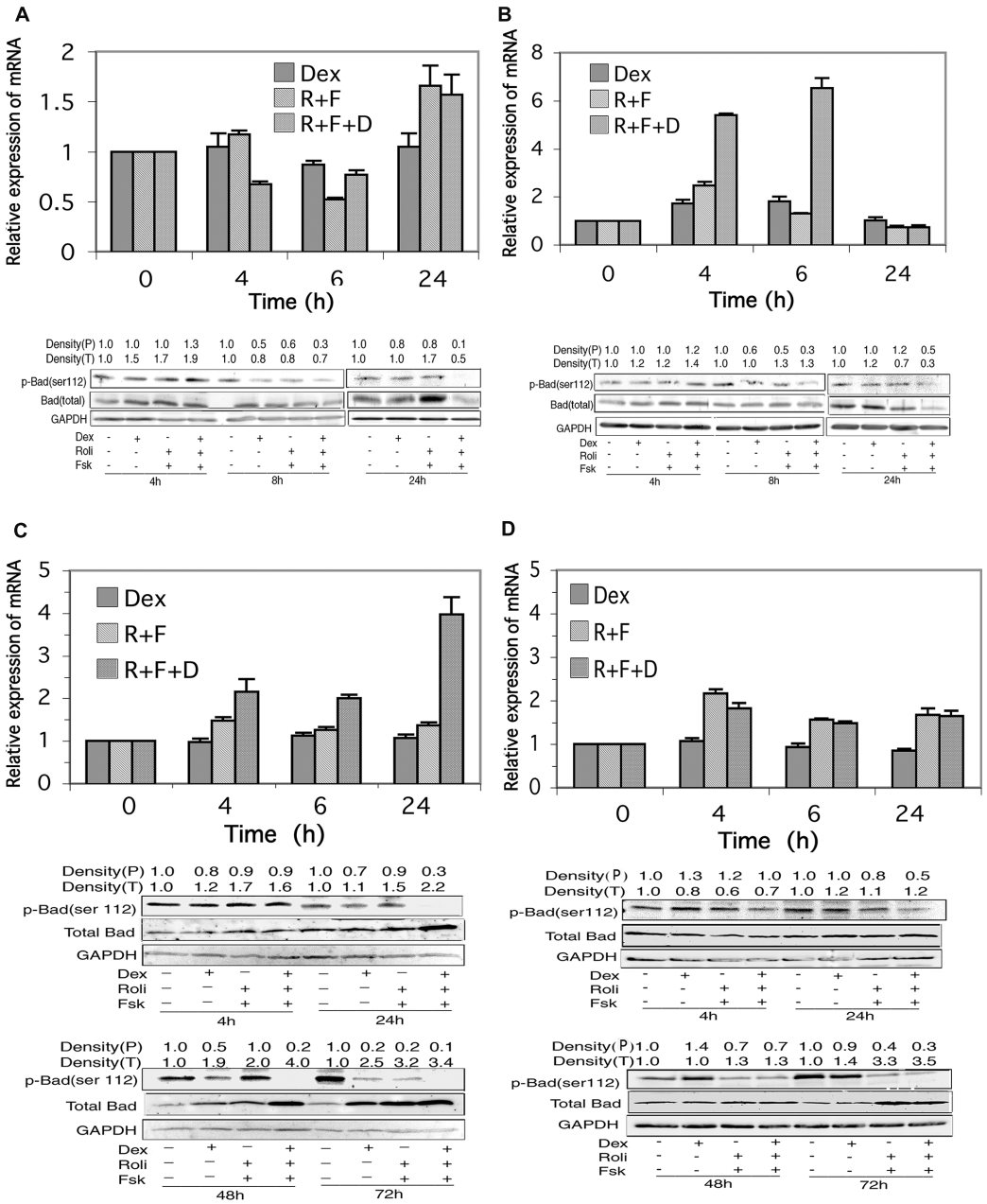

FIGURE 6 | Bad expression in CEM and MM.1 cells. Top panels: quantitative real-time PCR analysis of Bad mRNA at different times after treatment with 1 μM dexamethasone (Dex), 10 μM rolipram plus 10 μM forskolin (R+F), or 1 μM dexamethasone plus 10 μM rolipram plus 10 μM forskolin (R+F+D) as indicated. Data represent the mean ± SD of at least two independent experiments assayed in triplicate, shown as fold change relative to control. Bottom panels: Western blot analysis of expression of total Bad and p-Bad (ser112) protein. Cells were treated at different times with dexamethasone and/or rolipram and forskolin as indicated, and Bad protein expression was determined by immunoblots of whole cell lysates. An equal amount of protein (20 μg) was loaded per lane. Densities of phosphorylated Bad (P) and total Bad (T) are given at the top of the Western blot panels, calculated as described in Section "Materials and Methods." Data shown represents one of at least two independent experiments with similar results. **(A)** CEM-S2 cells; **(B)** CEM-R8 cells; **(C)** MM.1S cells, and **(D)** MM.1R cells.

added together to stimulate both signaling pathways (**Figure 6C**). At 72 h, Bad expression was increased 2.5-fold by dexamethasone, 3.2-fold by forskolin plus rolipram, and 3.4-fold when all three agents were added together (**Figure 6C**). Similarly, in MM.1R cells, Bad expression was also induced by stimulation of these signaling pathways, with the largest effects seen at 72 h, where Bad expression was increased 1.4-fold by dexamethasone, 3.3-fold by forskolin plus rolipram, and 3.5-fold by all three agents added together (**Figure 6D**). Hence, although total Bad protein is little changed in CEM-S2 and CEM-R8 cells in response to stimulation of cAMP and glucocorticoid signaling, it is considerably induced in MM.1 cells, both in the MM.1S sensitive and MM.1R resistant

cells, both by stimulation of the cAMP and glucocorticoid signaling pathways independently, and to an even greater extent when both signaling pathways are stimulated together.

Stimulation of the cAMP Signaling Pathway Alters the Phosphorylation State of Bad in Glucocorticoid-Sensitive and Resistant CEM and MM.1 Cells

The activity of Bad, as a proapoptotic protein, is regulated by its state of phosphorylation. Phosphorylation of Bad on several sites, especially Ser112, promotes its association with 14-3-3 protein and sequesters it in an inactive state. Dephosphorylation of Bad at these sites promotes its dissociation from 14-3-3 allowing it to interact with mitochondria and promote apoptosis (Chiang et al., 2001; Moon and Lerner, 2003). We therefore investigated the state of phosphorylation of Bad following stimulation of the glucocorticoid and cAMP signaling pathways. As shown in **Figures 6A,B**, although there was little change in total Bad protein in CEM cells following stimulation of the cAMP and glucocorticoid pathways, stimulation of these two signaling pathways together resulted in dephosphorylation of Bad at ser112 in both the glucocorticoid-sensitive and resistant CEM cell lines. Additionally, very similar to the effect seen with CEM cells, stimulation of the cAMP and glucocorticoid signaling pathways also led to a dramatic dephosphorylation of Bad on ser112 in both the MM.1S and MM.1R cells (**Figures 6C,D**). Hence, one common result of the stimulation of the cAMP and glucocorticoid pathways in both the glucocorticoid-sensitive and resistant CEM and glucocorticoid-sensitive and resistant MM.1 cells is the activation of Bad via its dephosphorylation on ser112.

Model for the Synergy between cAMP and Glucocorticoid Signaling Pathways on Induction of Apoptosis in Leukemic and Multiple Myeloma Cells

A model for how cAMP and glucocorticoid signaling might synergize with each other to induce apoptosis and overcome glucocorticoid resistance in these cells is presented in **Figure 7**. In this model it is proposed that glucocorticoids upregulate the expression of Bim through a Fox03a-dependent mechanism. Phosphodiesterase inhibitors, such as the PDE4 inhibitor rolipram, or agents that activate adenylyl cyclase through G protein coupled receptors, stimulate cAMP signaling which can lead to activation of the protein phosphatase 2A. The activated protein phosphatase 2A then dephosphorylates Bad on ser112, resulting in its dissociation from 14-3-3 chaperone protein and its translocation to the mitochondria, where it acts as a sensitizer by binding to antiapoptotic proteins such as Bcl-2 in leukemic cells and Mcl-1 in multiple myeloma cells, and releasing Bim from its association with these proteins. The released Bim then binds to Bax and the complex acts to permeabilize the mitochondrial membrane, leading to apoptosis. In some cells, as shown in this study for the MM.1S and MM.1R multiple myeloma cells, the increased cAMP signaling not only activates Bad, but also upregulates its expression. Further, the increased cAMP can also inhibit the growth promoting kinase, Akt, which disinhibits Fox03a, leading to further upregulation of Bim. The increased Bim, as an activator protein, primes the cells for death in response to the translocation of the dephosphorylated, activated Bad sensitizer protein to the mitochondria, leading to a synergistic induction of apoptosis and overcoming glucocorticoid resistance.

DISCUSSION

Resistance to glucocorticoids is a major problem in the treatment of leukemia and multiple myeloma (Haarman et al., 2003; Frankfurt and Rosen, 2004; Schmidt et al., 2004; Ploner et al., 2005; Sionov et al., 2008; Bhadri et al., 2012). In this study we investigated changes in GR, and the BH3-only proapoptotic proteins, Bim and Bad, in cell line models of glucocorticoid-sensitive and resistant leukemia and multiple myeloma. Consistent with other reports (Krett et al., 1997; Medh et al., 1998; Ogawa et al., 2002; Tiwari et al., 2005; Lerner and Epstein, 2006; Dong et al., 2010; Follin-Arbelet et al., 2011) in the cell models we examined here, we found that both the glucocorticoid-sensitive and resistant leukemia and multiple myeloma cell lines could be killed by stimulation of the cAMP signaling pathway, and that stimulation of the cAMP signaling pathway acted synergistically with glucocorticoids to induce cell death of both the glucocorticoid-sensitive and resistant CEM leukemic cell lines and the glucocorticoid-sensitive MM.1S multiple myeloma cell line. A synergistic effect of cAMP and glucocorticoid signaling was not seen with the MM.1R, glucocorticoid-resistant cell line, most likely as a result of the MM.1R cell line expressing a truncated form of GR (Moalli et al., 1992).

Although loss or mutations in GR occur in a number of glucocorticoid-resistant cell lines, this only rarely occurs in primary cells from most patients, and thus appears not to account for the prevalence of glucocorticoid-resistance seen in patients treated with glucocorticoids (Bachmann et al., 2005, 2007; Tissing et al., 2006; Beesley et al., 2009). We examined changes in the expression of GR mRNA, GR promoters, and GR protein in glucocorticoid-sensitive and resistant leukemia and multiple myeloma cells in response to stimulation of cAMP and glucocorticoid signaling in these cells, and examined the correlation of these changes with induction of cell death in these cells following stimulation of these signaling pathways. Most cell types demonstrate down regulation of GR following glucocorticoid exposure, presumably as a feedback mechanism, although GR in some lymphoid leukemia cell lines has been reported to be upregulated (Tissing et al., 2006). It has also been observed in some cell lines that dexamethasone can dramatically alter GR protein levels without changing GR mRNA expression, leading to the suggestion that glucocorticoids may influence GR protein levels by other means besides transcriptional regulation, such as mRNA and GR protein stability (Okret et al., 1991). Effects of cAMP signaling on GR expression are also complex and can lead either to upregulation (Oikarinen et al., 1984; Dong et al., 1988) or down regulation (Sheppard et al., 1991) depending on the cell type. A study from the Lerner laboratory showed

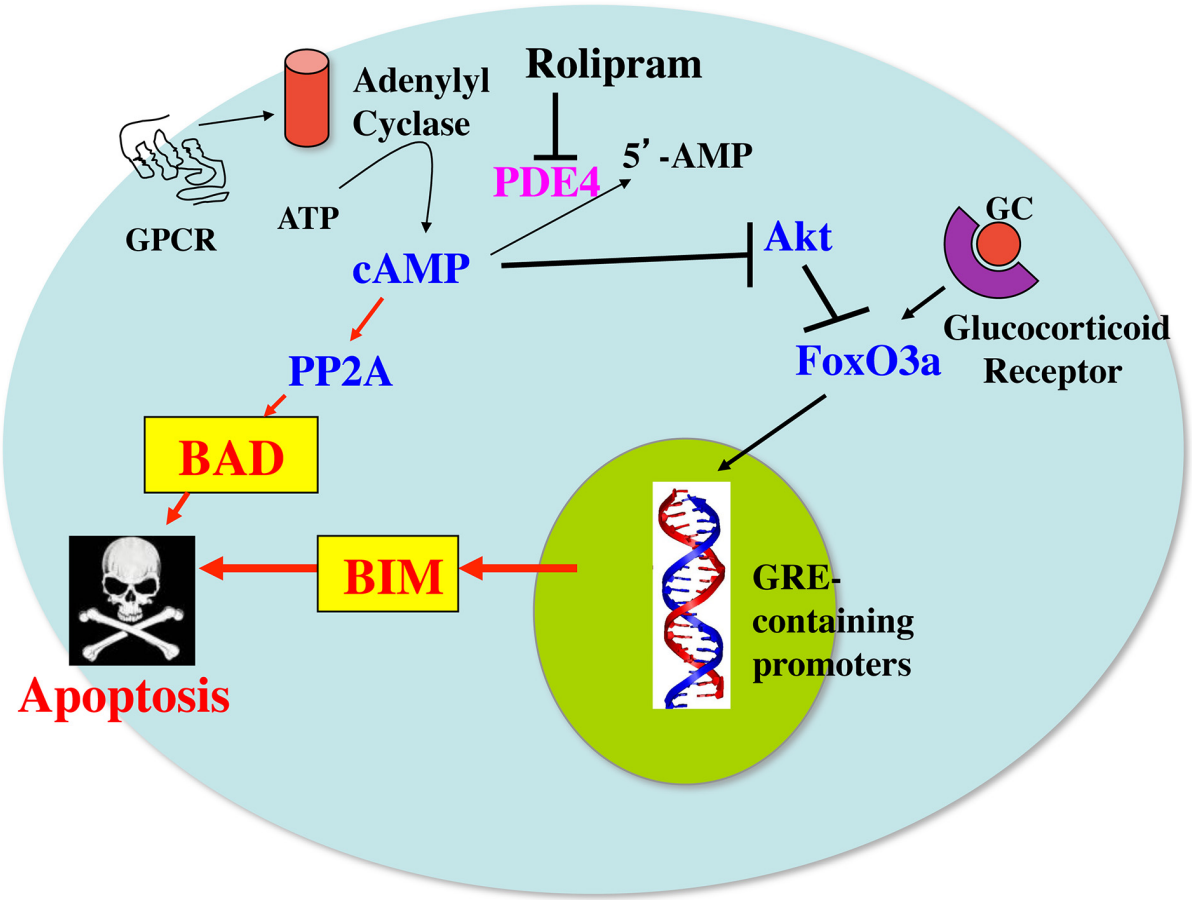

FIGURE 7 | Model for the synergy between cAMP and glucocorticoid signaling pathways on induction of apoptosis in leukemic and multiple myeloma cells. GC, glucocorticoids; GPCR, G protein coupled receptor; PP2A, protein phosphatase 2A; PDE4, phosphodiesterase4; GRE, glucocorticoid-responsive element.

that stimulation of the cAMP signaling pathway with PDE4 inhibitors upregulated GR transcript levels in B-CLL cells but not T-CLL cells, Sezary cells or normal circulating T or B cells, monocytes, or neutrophils (Meyers et al., 2007). In this same study, it was shown that glucocorticoids reduce GR mRNA levels in B-CLL and that co-treatment with PDE4 inhibitors maintains GR mRNA levels above baseline. In our current study, although we see some increased expression of GR mRNA following stimulation of cAMP and glucocorticoid signaling, we see almost no change, only very small modest increases in GR protein at later time points, in both glucocorticoid-sensitive CEM-S2 and glucocorticoid-resistant CEM-R8 cells following stimulation of either cAMP signaling, stimulation of glucocorticoid signaling, or stimulation of both signaling pathways together. In contrast, in the MM.1S multiple myeloma cells, which are of B cell origin, stimulation of glucocorticoid signaling with dexamethasone greatly down regulated GR protein. However, unlike that seen in the B-CLL cells, co-stimulation of the cAMP signaling pathway enhanced this down regulation, rather than preventing it, leading to a profound reduction in the expression of GR.

Results from this study indicate that the cell death induced by cAMP signaling in these cells and the potentiation of glucocorticoid-induced cell death by cAMP signaling does not correlate with increased expression of GR protein, suggesting that the actions of these signaling pathways on viability of these cells is not mediated by changes in expression of GR. However, it is possible that alterations in GR may still play a role in the actions of these signaling pathways at the post-translational level, since GR has been shown to be a substrate for PKA (Haske et al., 1994), and thus its actions could be modified by cAMP signaling. Further, there is some evidence to suggest that translocation of GR from the cytosol to mitochondria may also play a role in mediating cell death (Sionov et al., 2006), and it is possible that this translocation could be influenced by these signaling pathways. Additionally, we found that cAMP signaling induced expression of the GR promoters in glucocorticoid-sensitive and resistant CEM cells and glucocorticoid-sensitive MM.1 cells, with induction of the 1A3 promoter stimulated more than 1B and 1C, and with further potentiation of this effect by dexamethasone in CEM cells. cAMP signaling was also shown to increase levels of the 1A3 promoter to a greater extent than the 1B promoter in

B-CLL cells as well (Meyers et al., 2007). Inasmuch as expression of GR from the 1A promoter correlates best with T lymphocyte sensitivity to glucocorticoid-induced cell death (Purton et al., 2004), a shift to expression of more of the GR from the 1A3 promoter, relative to its expression from the other promoters, might also contribute to the cAMP sensitivity of these cells.

A number of studies have pointed to Bim as being an essential mediator of glucocorticoid-induced apoptosis of leukemic, lymphoma and myeloma cells, in that Bim is upregulated in these cells following glucocorticoid treatment, and down regulation of the expression of Bim inhibits glucocorticoid-induced apoptosis (Wang et al., 2003; Abrams et al., 2004; Bachmann et al., 2005; Ploner et al., 2005; Lu et al., 2006; Iglesias-Serret et al., 2007; Lopez-Royuela et al., 2010; Jiang et al., 2011; Zhao et al., 2011; Heidari et al., 2012; Jing et al., 2015). Additionally, stimulation of the cAMP signaling pathway has also been shown to upregulate Bim and induce apoptosis in leukemic and lymphoma cells (Meyers et al., 2009; Huseby et al., 2011; Zambon et al., 2011). cAMP signaling also upregulates Bim in normal T and B lymphocytes, but of note, in contrast to leukemic cells, the upregulation of Bim in normal lymphoid cells in response to cAMP signaling does not lead to apoptosis (Meyers et al., 2009). It was reported that stimulation of cAMP signaling enhanced dexamethasone upregulation of Bim and promoted increased apoptosis of leukemic and lymphoma cells, suggesting that Bim may be a convergence point for the increased response seen when these two signaling pathways are stimulated (Zhang and Insel, 2004). Our results confirm this and also point to increased upregulation of Bim as being a possible convergence point for the synergistic effects of cAMP and glucocorticoid signaling on inducing apoptosis of the glucocorticoid-sensitive CEM-S2 and glucocorticoid-resistant CEM-R8 cells that we employed in this study. However, we did not see evidence for this in multiple myeloma cells. In the glucocorticoid-sensitive MM.1S myeloma cells, although stimulation of the glucocorticoid and cAMP signaling pathways both stimulated upregulation of Bim protein expression, there was no further enhanced expression of Bim protein when both signaling pathways were stimulated together, even though there was enhanced promotion of apoptosis when both signaling pathways were stimulated. And in the glucocorticoid-resistant MM.1R myeloma cell line, no upregulation of Bim protein expression was seen in response to glucocorticoid signaling, cAMP signaling, or stimulation of both signaling pathways together. Inasmuch as cAMP signaling clearly induced apoptosis of MM.1R cells, our findings suggest that a mechanism(s) other than Bim induction must come into play to account for the apoptosis that results in response to cAMP stimulation in these cells.

Studies from the Lerner laboratory had shown that stimulation of the cAMP signaling pathway with rolipram and forskolin led to activation of protein phosphatase 2A, dephosphorylation of Bad on ser112, and translocation of Bad to mitochondria, suggesting that activation of the proapoptotic protein Bad may also be important for apoptosis induced by cAMP signaling (Moon and Lerner, 2003). In this study we saw little upregulation of total Bad protein in either CEM-S2 or CEM-R8 cells, but we saw considerable upregulation of Bad protein in both the MM1.S and MM1.R multiple myeloma cells. Moreover, we observed dephosphorylation of Bad on ser112 in response to stimulation of cAMP signaling in all cell types studied here, CEM-S2, CEM-R8, MM.1S and MM.1R, and this dephosphorylation was greatly potentiated by stimulation of the glucocorticoid pathway concurrent with the cAMP signaling pathway. These results suggest that Bad may be an important mediator of apoptosis in multiple myeloma cells in response to these signaling pathways, and that dephosphorylation and activation of Bad may also be a convergence point for induction of apoptosis by the glucocorticoid and cAMP signaling pathways.

Studies have shown that the ability of glucocorticoids to induce apoptosis of leukemic cells may depend in part on the relative balance of the proapoptotic protein Bim and the antiapoptotic protein Bcl-2 present in the target cell (Ploner et al., 2005; Jing et al., 2015), and the same may be true for Bim and the antiapoptotic protein Mcl-1 in multiple myeloma cells (Gomez-Bougie et al., 2004; Follin-Arbelet et al., 2013). Models put forth by the Letai laboratory describe Bim as an activator protein, capable of binding directly to Bax, resulting in mitochondrial membrane permeabilization, whereas Bad is described as a sensitizer protein capable of binding to antiapoptotic proteins like Bcl-2 and Mcl-1, which thus frees Bim from its tethered association with Bcl-2 or Mcl-1, allowing it to bind Bax and initiate permeabilization of the mitochondrial membrane (Brunelle and Letai, 2009). Cells in which activator proteins such as Bim are in higher abundance may be primed for death by being particularly sensitive to exposure to sensitizer domains provided by Bad, such that an increase in Bad may tip the balance and induce apoptosis. Inasmuch as Bad was induced in MM.1S and MM.1R cells, as well as dephosphorylated on ser112, in response to cAMP signaling, this may in part explain the mechanism whereby cAMP signaling induces apoptosis in multiple myeloma. Small molecule BH3-mimetics that mimic the BH3 binding domain of proapoptotic proteins such as Bad have been found to be effective inducers of apoptosis, and at least one such BH3-mimetic, ABT-199, has been entered into clinical trials for treatment of multiple myeloma, as well as for lymphomas and leukemias (Correia et al., 2015). Our results presented in this study would suggest that stimulation of the cAMP signaling pathway with PDE inhibitors in conjunction with BH3-mimetics may well provide an even more effective therapeutic strategy for treatment of leukemia and multiple myeloma.

Several studies have shown altered expression of PDEs in leukemic patients to be associated with poorer treatment outcomes, often related to glucocorticoid resistance in the treatment of these patients. For example, CLL patients with higher expression levels of PDE7B have a several-year shorter median time-to-treatment compared to patients with lower levels of PDE7B expression (Zhang et al., 2011). Overexpression of PDE4B in patients with diffuse large B cell lymphoma (DLBCL) was associated with relapse after chemotherapy, and overexpression of PDE4B in DLBCL impinged on the same genes that are normally active in glucocorticoid resistance (Smith et al., 2005; Kim et al., 2011). A study of 2,535 children with ALL showed single nucleotide polymorphisms in the PDE4B gene to be associated with a high risk of relapse in children

newly diagnosed with ALL, and it was suggested that this may be due to the resultant glucocorticoid resistance that develops due to the PDE4B polymorphisms, which further underscores the importance of targeting PDE4 in overcoming glucocorticoid resistance (Yang et al., 2012). When high throughput screening technology was employed to identify agents that synergize with dexamethasone to inhibit proliferation of MM.1S and DLBCL cells, the compounds identified to produce the greatest amount of synergy were adenosine A2A receptor agonists and PDE 2,3,4, and 7 inhibitors (Rickles et al., 2010). In a study examining the *in vitro* antileukemic activity of 20 different anticancer agents against tumor cells from CLL patients aimed at identifying agents active in poor-prognostic subgroups, it was found that prednisolone and rolipram displayed high CLL specificity and high activity in CLL with unmutated IGHV genes and when prednisolone and rolipram were combined they displayed considerable synergy against these CLL cells, thereby identifying rolipram as an agent with high activity in cells from patients with poor prognosis (Lindhagen et al., 2009). Recent studies from the Lerner laboratory showed that rolipram induced apoptosis of both IGHV unmutated and mutated CLL cells, suggesting that cAMP signaling may abrogate a TLR9-mediated survival signal in prognostically unfavorable IGHV unmutated CLL cells, and indicating that PDE4 inhibitors may well be of clinical utility in CLL or autoimmune diseases that are driven by TLR-mediated signaling (Tan et al., 2015). PDE inhibitors are currently under intense development for a wide range of illnesses and are rapidly being approved for clinical use (Houslay et al., 2005; Bender and Beavo, 2006; Lugnier, 2006; Epstein, 2012; Maurice et al., 2014; Ahmad et al., 2015). Of note, two PDE4 inhibitors, roflumilast and apremilast, are already approved and in clinical use for the indications of chronic obstructive pulmonary disease and psoriatic arthritis, respectively. These studies, as well as the results of our study presented here, suggest that PDE inhibitors may provide valuable tools for overcoming glucocorticoid resistance and thereby improve the treatment outcome of patients with lymphomas, leukemias, and multiple myeloma. As such, clinical studies should clearly be undertaken with PDE inhibitors, either as single agents, or in combination with BH3-mimetics and/or established therapeutic agents for treatment of patients with leukemia, lymphoma and multiple myeloma in relation to glucocorticoid resistance.

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The ever unfolding story of cAMP signaling in trypanosomatids: *vive la difference*!

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Kinetoplastids are unicellular, eukaryotic, flagellated protozoans containing the eponymous kinetoplast. Within this order, the family of trypanosomatids are responsible for some of the most serious human diseases, including Chagas disease (*Trypanosoma cruzi*), sleeping sickness (*Trypanosoma brucei* spp.), and leishmaniasis (*Leishmania* spp). Although cAMP is produced during the life cycle stages of these parasites, its signaling pathways are very different from those of mammals. The absence of G-protein-coupled receptors, the presence of structurally different adenylyl cyclases, the paucity of known cAMP effector proteins and the stringent need for regulation of cAMP in the small kinetoplastid cells all suggest a significantly different biochemical pathway and likely cell biology. However, each of the main kinetoplastid parasites express four class 1-type cyclic nucleotide-specific phosphodiesterases (PDEA-D), which have highly similar catalytic domains to that of human PDEs. To date, only TbrPDEB, expressed as two slightly different isoforms TbrPDEB1 and B2, has been found to be essential when ablated. Although the genomes contain reasonably well conserved genes for catalytic and regulatory domains of protein kinase A, these have been shown to have varied structural and functional roles in the different species. Recent discovery of a role of cAMP/AMP metabolism in a quorum-sensing signaling pathway in *T. brucei*, and the identification of downstream cAMP Response Proteins (CARPs) whose expression levels correlate with sensitivity to PDE inhibitors, suggests a complex signaling cascade. The interplay between the roles of these novel CARPs and the quorum-sensing signaling pathway on cell division and differentiation makes for intriguing cell biology and a new paradigm in cAMP signal transduction, as well as potential targets for trypanosomatid-specific cAMP pathway-based therapeutics.

Keywords: ***Trypanosoma cruzi*, *Trypanosoma brucei*, *Leishmania*, phosphodiesterase, cAMP, kinase, adenylyl cyclase, PKA**

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Introduction

Trypanosomatids are protozoan parasites belonging to the order kinetoplastida, family trypanosomatidae, and are characterized by a particular substructure of the mitochondrion, called the kinetoplast, which contains the mitochondrial DNA. They are digenetic flagellated protozoans with similar cellular structures as well as similar genome organization and are all known to undergo morphological transformations during their life cycles (Stuart et al., 2008). Members of this order are

unicellular eukaryotes, and many of them parasitize multicellular organisms and cause medically and economically important diseases in humans, their domestic animals and cash crops (Barrett et al., 2003). Human African trypanosomiasis (HAT), also known as African sleeping sickness, is a vector-borne parasitic disease caused by the protozoan pathogen *Trypanosoma brucei* and transmitted by several *Glossina* species, commonly called tsetse flies (Stich et al., 2002). There are three subspecies of *T. brucei* that infect mammals: *Trypanosoma brucei brucei*, *Trypanosoma brucei gambiense*, and *Trypanosoma brucei rhodesiense*. However, only *T. b. gambiense* (acute infections) and *T. b. rhodesiense* (chronic infections) infect and cause clinical disease in humans whilst *T. b. brucei* infect animals causing the disease known as nagana in cattle (Fevre et al., 2006; Brun et al., 2010). It has been estimated that the core group of neglected tropical diseases (NTDs), the majority of which are caused by trypanosomatids, results in the loss of more than 57 million disability-adjusted life years (DALY), coupled with attendant impacts on poverty (Hotez et al., 2006, 2009). Although there has been dramatic improvement in infections and death in recent years (Simarro et al., 2011), optimism is tempered in the light of previous recurrences, migration and the instability in many endemic regions (Odiit et al., 2005; Picozzi et al., 2005; Mumba et al., 2011; Blum et al., 2012). Although the old, toxic and difficult to administer drugs have helped to combat the disease until the present (Delespaux and de Koning, 2007; Kennedy, 2008; Barrett, 2010; Jacobs et al., 2011), the current increase in resistance to these drugs is very worrying (Vincent et al., 2010; Simarro et al., 2012; Baker et al., 2013). If modern standards in pharmacology were to be applied, the aforementioned issues with trypanosomatid chemotherapy mean that there are effectively no acceptable chemotherapies for these diseases. A quest to produce more clinically effective and less toxic drugs is hampered by the fact that, as eukaryotes, trypanosomatids are genetically and evolutionarily much closer to their human hosts than bacteria, resulting in problems with selectivity and toxicity (Seebeck et al., 2011). In the context of cAMP metabolism, the kinetoplastid phosphodiesterases (PDEs) are highly similar to that of most of the well-studied human homologs. However, PDEs are highly amenable to selective inhibition, due to small differences in their binding pockets that can be exploited by structure-based inhibitor design, even when using the pharmacologically well explored scaffolds of human PDE inhibitors. Moreover, downstream effectors of cAMP are very different in human and trypanosomatid cells, potentially providing further drug targets, this time without mammalian counterparts.

Signal Transduction in Trypanosomatids

Adenylate Cyclases

Signal cascades exist for the amplification of a small signal into a large response, leading to significant cellular changes such as expression of specific genes, the activity of certain proteins, or changes in cell cycle progression. Many disease processes, such as diabetes, heart disease, autoimmunity and cancer, arise from defects in signal transduction pathways, further highlighting the critical importance of signal transduction to biology as well as the development of medicine (Huang et al., 2010). Cyclic AMP levels in most eukaryotes are increased by stimulation of adenylyl cyclases (ACs), whilst cyclic nucleotide PDEs degrade the phosphodiester bond in cAMP, thereby limiting or abrogating signal transduction. A putative kinetoplastid AC gene was first identified in *T. brucei* when the active gene expression site of a variant surface glycoprotein (VSG) was sequenced, revealing that there were multiple genes in the site that were co-expressed with VSG. These genes were termed expression site-associated genes (ESAGs), and one of them, ESAG4, showed homology with an AC from yeast (Pays et al., 1989). Further copies of apparent ACs were identified in the genome and named GRESAG4.1 and GRESAG4.2 (genes related to ESAG4; Pays et al., 1989). Related genes were also found in *T. b. gambiense*, *Trypanosoma congolense*, *Trypanosoma mega*, *Trypanosoma equiperdum*, and *Trypanosoma vivax*. These apparent AC genes were proven to actually code for functional AC enzymes by complementing AC-deficient yeast mutants (Ross et al., 1991; Paindavoine et al., 1992).

Since then similar multigene families with high homology to ESAG4 and GRESAG4.1 have also been identified in *Leishmania donovani* and *T. cruzi*, and these ACs share the same predicted protein architecture (Sanchez et al., 1995). *T. brucei* encodes up to 20 telomeric ESAG4 AC genes and approximately 65 GRESAG4 proteins (Salmon et al., 2012a,b) and at least some of these are localized along the flagellum both in the mammalian-infective bloodstream forms (BSFs) and in procyclic (fly midgut stage) cells (Paindavoine et al., 1992; Saada et al., 2014). Their similarity results in cross-reactivity with some antibodies raised against ESAG4 (Paindavoine et al., 1992; Oberholzer et al., 2011). Whereas knocking out ESAG4 from the expression site does not affect parasite proliferation, a knockdown of all the AC family that includes ESAG4 and the two GRESAG4 genes led to a total decrease in AC activity, resulting in a phenotype that is defective in cytokinesis (Salmon et al., 2012a).

Trypanosomatid ACs contain a single *trans*-membrane domain, a conserved intracellular C-terminal domain, and a large variable extracellular domain. The N-terminal domains may function as receptors, similar to mammalian receptor-type guanylyl cyclases (Garbers et al., 2006). Whilst the catalytic domain is structurally very similar to those of mammalian ACs, it is not activated by forskolin. The purified proteins form homodimers *in vitro* (Bieger and Essen, 2001; Naula et al., 2001; Gould and de Koning, 2011) and dimerization was recently also shown *in vivo* (Saada et al., 2014). The possibility of the N-terminal extracellular domain of ACs acting as a receptor for signaling due to the lack of G-protein-coupled receptor (GPCR) in the kinetoplastid genome has been strongly speculated (Seebeck et al., 2004; Laxman and Beavo, 2007). Indeed, the recently revealed relationship of the N-terminal of a representative AC from *T. brucei* with an *Escherichia coli* L-leucine-binding protein (LBP; Emes and Yang, 2008) and a similar LBP that acts as an amide receptor in *Pseudomonas aeruginosa* (O'Hara et al., 2000) have lend support to the hypothesis that AC activity could be directly regulated by extracellular stimuli. Analysis of the AC gene clusters also showed that the variation in the extracellular domains, specifically in areas predicted to come into close

contact with putative ligands, appear to be significantly driven by positive selection. This is an indicator of adaptive evolution and consistent with a receptor or sensory function for at least some of the cyclases (Emes and Yang, 2008). Although no putative ligand has been identified as yet, some extracts from *T. cruzi* and *T. brucei* insect vectors have been shown to activate ACs, whilst a low-molecular-weight molecule, stumpy induction factor (SIF), probably secreted by the trypanosome itself, was inferred to trigger the differentiation of long slender bloodstream *T. brucei* to the non-replicating stumpy form via the cAMP signaling cascade (Garcia et al., 1995; van den Abbeele et al., 1995; Vassella et al., 1997). In addition it has been shown that ACs influence host parasite interactions through the modulation of tumor necrosis factor alpha (TNF-α) and that AC activity of lysed trypanosomes contributes to establishing an infection of these parasites in a host (Salmon et al., 2012b).

The above suggests that diversity in ACs provides an adaptive advantage to the extracellular *T. brucei* enabling host immune evasion and modulation, and thus survival. It is postulated that the high number of ACs of *T. brucei* species, compared with the intracellular *T. cruzi* and *Leishmania*, has to do with the evasion of the host immune system by continuously switching its VSG expression in the various telomeric sites, which resulted in the duplication of ESAGs, including ACs (ESAG4). Other forces of selection may then have resulted in alternative specificities for the individual receptor-cyclases to arise. Thus, the large numbers of ACs found in the *T. brucei* genome may allow more specific responses to the multiple ligands found in its extracellular environment, compared with the relatively sheltered intracellular lifestyle of *Leishmania* and *T. cruzi* (Gould and de Koning, 2011).

The cellular localization of the ACs of kinetoplastids is also consistent with them acting as a receptor. Antibodies against ESAG4 were shown to specifically bind to the cell surface along the flagellum in both BSFs and procyclic trypanosomes (Paindavoine et al., 1992). Similarly, in *T. cruzi* epimastigotes, the calcium-stimulatable AC was found to be associated with the flagellum (D'Angelo et al., 2002).

Proteomic analysis of bloodstream *T. brucei* flagella and plasma membrane fractions have identified receptor and transport-like proteins that likely play important roles in signaling and parasite-host interactions (Bridges et al., 2008; Oberholzer et al., 2011). Recently, several receptor-type flagellar ACs have been shown to be specifically expressed in the procyclic stage, glycosylated, surface-exposed and catalytically active. Interestingly, these cyclases were differentially distributed: either along the entire flagellum or localized to just the tip of the flagellum (Saada et al., 2014). This indicates a microdomain flagellar cyclic AMP signaling in *T. brucei*, and that ACs have specific subdomains. These possibilities were further strengthened by the findings that one of these insect stage specific ACs (adenylate cyclase 6) is responsible for social motility and that functional mutation or RNAi knock-down results in a hypersocial phenotype (Lopez et al., 2015), again demonstrating the involvement of cAMP signaling in response to extracellular stimuli. All these observations together, coupled with the fact that cAMP levels are significantly increased during the differentiation of *T. brucei* BSFs to procyclic forms (Vassella et al., 1997), suggests a probable role of cAMP involvement in parasite behavior and differentiation through ACs.

Similarly, *T. cruzi* ACs form dimers (D'Angelo et al., 2002) and have been implicated in the conversion of epimastigotes in the insect midgut, and later in the hindgut, into human-infectious non-proliferative metacyclic trypomastigotes (Gonzales-Perdomo et al., 1988; Fraidenraich et al., 1993; Garcia et al., 1995), a process known as metacyclogenesis that is akin to cellular differentiation of BSF *T. brucei* to procyclic forms. This event can reportedly be triggered *in vitro* by a proteolytic fragment of α-D-globin from the insect host's hindgut (Fraidenraich et al., 1993), confirming a role for cAMP in mediating parasite responses to environmental changes. *In vitro* metacyclogenesis triggered by nutritional stress also caused an increase in cAMP production (and cellular content) in two phases (Hamedi et al., 2015), with a first peak rapidly following the initiation of differentiation, and a second phase of elevated cAMP associated with the adhesion of the epimastigotes that is a prerequisite for their final differentiation to metacyclic trypomastigotes (Boker and Schaub, 1984).

Protein Kinase A

The cAMP-dependent protein kinase (PK) family or protein kinase A (PKA) is a collection of serine/threonine kinases whose activity is dependent on levels of cAMP in the cell and is one of the most studied and best known members of the PK family (Huang, 2011). The kinetoplastid genomes contain reasonably well conserved genes for catalytic and regulatory domains of PKA (Huang et al., 2006). In *T. brucei*, a 499-amino acid protein with high homology to eukaryotic regulatory subunits of PKA was identified and named TbRSU. This led to the first actual measurement of the cyclic nucleotide-dependent kinase activity in *T. brucei*. The protein has the usual two cyclic nucleotide-binding domains, which are predicted to retain all the conserved residues necessary for function, as well as a pseudo-inhibitor site, which interacts with the catalytic subunit (Shalaby et al., 2001). However, further research on the kinase activity co-immunoprecipitated with TbRSU showed that although it displayed phosphorylation activity and was also inhibited by the protein kinase inhibitor peptide (PKI), both characteristics of PKA, it was not stimulated by cAMP but was instead stimulated by cyclic guanosine monophosphate (cGMP; Shalaby et al., 2001). There is to date scant evidence of cGMP production in any of the kinetoplastid parasites, although a soluble, cytosolic guanylate cyclase activity was described in *L. donovani* (Karmakar et al., 2006), and MacLeod et al. (2008) reported that feeding cGMP (but not cAMP) to tsetse flies resulted in higher trypanosome infection rates.

The finding that cAMP signaling mediates *T. cruzi* differentiation (Flawia et al., 1997) and the fact that PKAs are the major effectors in most eukaryotic cells, led to the need to identify PKA activity in *T. cruzi*. A cAMP-stimulatable PK fraction was identified and displayed a half-maximal effect at approximately 1 nM cAMP. As expected for a cAMP-dependent kinase its activity was not affected by cGMP; moreover, its phosphate-acceptor profile (including histones and kemptide, but not casein and phosvitin) was consistent with other PKA

activities (Ulloa et al., 1988). The holoenzyme appeared to consist of two regulatory and two catalytic subunits (Ochatt et al., 1993). Expression of both the *T. cruzi* PKA catalytic subunit (TcPKAc) and the *T. cruzi* PKA regulatory subunit (TcPKAr) is similarly regulated and leads to coordinated expression in the life cycle stages, indicating that the two subunits are associated *in vivo*, as also shown by immunoprecipitation of the holoenzyme (Huang et al., 2002; Huang, 2011). TcPKAc activity was inhibited by the PKA-specific inhibitor PKI and both TcPKAc and TcPKAr localized to the plasma membrane and the flagellar region (Huang et al., 2002, 2006; Bao et al., 2010). TcPKAr was found to interact with several P-type ATPases, which suggests that these P-type ATPases may play a role in anchoring PKA to the plasma membrane and could play a role in compartmentalization of the kinase (Bao et al., 2009), as reported for some mammalian P-type ATPases (Xie and Cai, 2003).

The functional importance of the TcPKAc in *T. cruzi* was examined by introducing a gene encoding a PKI peptide containing a specific PKA pseudo-substrate, Arg-Arg-Asn-Ala, into epimastigotes. Expression of this PKI has a lethal effect on the parasite. Similarly, a pharmacological inhibitor, H89, killed epimastigotes at a concentration of 10 μM proving that PKA enzymatic activity is essential for the survival of the parasites (Bao et al., 2008). A yeast two hybrid screen for the substrates of PKA identified 38 candidate proteins that interact with TcPKAc, including eight genes with potential regulatory functions with respect to environmental adaptation and differentiation. These included a type III PI3 kinase (Vps34), a putative PI3 kinase, a MAPK, a cAMP-specific phosphodiesterase (PDEC2), a hexokinase, a putative ATPase, a DNA excision repair protein and an aquaporin. PKA phosphorylated the recombinant proteins of these genes (Bao et al., 2008). Additional findings also suggest TcPKAc may play a role in invading cells by mediating protein trafficking that enables parasite adhesion to cells, enabling the invasion thereof, as *trans*-sialidases were found to be substrates of TcPKA (Bao et al., 2010). As discussed by Huang (2011), there seems to be co-incidence of cAMP production, PKA activity and *trans*-sialidase expression enabling the differentiation from late stage epimastigotes to invasive trypomastigotes—all consistent with a role for cAMP signaling in differentiation and invasion by *T. cruzi*.

A *Leishmania* catalytic subunit of PKA (LdPKA) was first isolated and characterized from *L. donovani* promastigotes by column chromatography and found to be similarly inhibited by PKI as in *T. brucei* and *T. cruzi*, indicating that the kinetoplastid enzymes are likely to be structurally related, as well as topologically similar to mammalian PKA. Indeed, LdPKAc was able to make a functional holoenzyme when combined with the regulatory subunit of a mammalian cAMP-dependent kinase (Banerjee and Sarkar, 1992). In *Leishmania major*, a gene encoding a protein with high homology to other PKA catalytic subunits (LmPKA-C1) was cloned. Analysis of the sequence and structural modeling showed the protein to have all the conserved domains of eukaryotic PKAs involved in ATP and substrate binding. However, some structural and functional differences were observed with other PKA-C subunits, such as a unique 8-residue C-terminal extension (Siman-Tov et al., 1996, 2002). Expression of LmPKA-C1 was developmentally regulated with expression barely detectable in intracellular amastigotes, in contrast to a high expression level in insect-stage promastigotes (Siman-Tov et al., 1996; Duncan et al., 2001).

The role of cyclic nucleotide-regulated PK activities in promastigote proliferation and infectivity was confirmed in *Leishmania amazonensis*, with PKA activity particularly high in metacyclic promastigotes, which are primed for macrophage invasion (Genestra et al., 2004). PKA inhibitors PKI and H89 affected both replication and macrophage infection. Smaller effects were observed with the PDE inhibitors dipyridamole, rolipram and isobutyl-methyl-xanthine (IBMX) but to date we are not aware of confirmation that these effects were mediated by one of the leishmanial PDEs, or which one. These effects were temporary and did not affect intra-macrophage growth (Malki-Feldman and Jaffe, 2009).

Phosphodiesterases

It is long been self-evident that increased knowledge of cyclic nucleotide signaling pathways can lead to the development of therapeutic agents against human diseases (Maurice et al., 2014). General pharmacological principles particularly support the potential of PDEs as therapeutic targets, as regulating the degradation of a second messenger or ligand offers a more effective intervention in cellular levels than through regulation of the rate of synthesis. Moreover, endogenous levels of the substrates (cAMP and cGMP) are not very high within the cells (between submicromolar and at most 10 μM) and competitive inhibitors can therefore be much more effective than against, for instance, PKs, where inhibitors must compete against millimolar levels of ATP (Bender and Beavo, 2006).

In mammals, PDEs exist as a superfamily and are classified into 11 families on the basis of their sequence identity, biochemical and pharmacological properties, regulation, and substrate specificity (Maurice et al., 2014). PDEs have their well-conserved catalytic domain located near their C-terminus and may contain various regulatory domains at the N-terminal end (Laxman and Beavo, 2007; Shakur et al., 2011) which presents extensive variations (Gancedo, 2013). PDEs may contain allosteric cyclic nucleotide binding sites in addition to their catalytic sites.

Phosphodiesterases have been grouped into three classes based on their different catalytic domains. Class I PDEs are found in all eukaryotes and they are the only forms of PDEs in higher eukaryotes (Beavo, 1995). Class I PDEs are the only enzymes that are capable of efficiently hydrolysing cyclic nucleotides. The genome of known kinetoplastids encodes four different class I PDEs (PDE-A to PDE-D) and does not contain members of the other PDE classes (Beavo, 1995) just as is the case in the human genome (Seebeck et al., 2011). At least one copy of each of the four PDE genes is present in the genome database of *T. brucei*, *T. cruzi*, and *L. major* (Vij et al., 2014). Class II PDEs are found in certain prokaryotes (e.g., *Vibrio fischeri*) or fungi (e.g., *Saccharomyces*, *Candida*) and in many lower eukaryotes (e.g., *Dictyostelium discoideum*). These PDEs also catalyze the hydrolysis of phosphodiester bonds but they do not show the same substrate selectivity as the class I enzymes (Bender and Beavo,

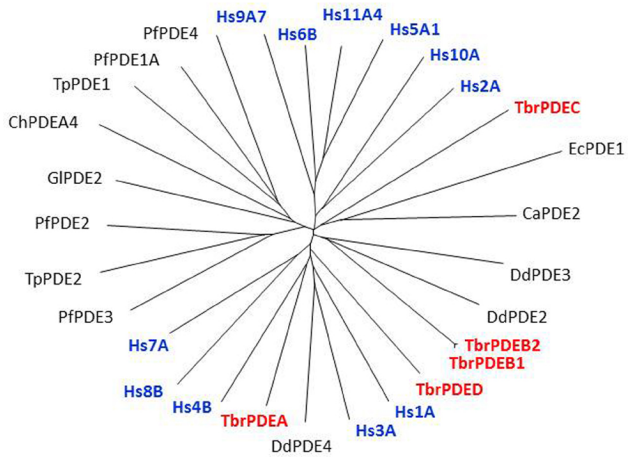

FIGURE 1 | Non-rooted tree of Class I protozoan and human PDEs. The catalytic domains of many protozoan PDEs, including *T. brucei* (in red), are as closely related to the human PDEs (in blue) as these are among themselves. Hs, *Homo sapiens*; Pf, *Plasmodium falciparum*; Ca, *Candida albicans*; Tp, *Theileria parva*; Gl, *Giardia lamblia*; Ch, *Chilomastix hominis*; Ec, *Encephalitozoon cuniculi*; Dd, *Dictyostelium discoideum*. Figure courtesy of Professor T. Seebeck, University of Bern, Switzerland.

2006). Class III PDEs are restricted to the bacteria (Gancedo, 2013).

Phosphodiesterases are regulated at multiple levels and by a number of factors, such as at the genetic level (transcriptional control), through biochemical mechanisms (e.g., phosphorylation and dephosphorylation), binding of Ca^{2+}, various protein–protein interactions, and by binding of cAMP or cGMP to allosteric sites (Bender and Beavo, 2006). The field of PDE research has greatly advanced and moved from basic identification of PDE enzymes and characterization of their kinetic and regulatory properties to more recent work on their structure and activity regulation. Major efforts, and important successes, are ongoing in the pharmacological exploitation of human PDEs (Azam and Tripuraneni, 2014; Chen et al., 2015; Fallah, 2015). In contrast, after 30 years of work in the area of cAMP signaling and its role in the cell biology and virulence of kinetoplastids, many of the fundamental questions remain unanswered. Shakur et al. (2011) argue that the implicit assumption that cAMP signaling in kinetoplastids would be organized as in mammals substantially delayed progress. Although this assumption has proven to be far from true, the catalytic domains of trypanosomatid PDEs, at least, are as highly conserved in relation to their human homologs as the 11 human PDEs are among themselves and similarly are suitable targets for drug screening and development (Seebeck et al., 2011; Shakur et al., 2011).

The kinetoplastid genomes all code for the same set of cyclic nucleotide-specific class 1-type PDEs with catalytic domains that are highly similar to those of the human PDEs (**Figure 1**; Beavo, 1995; Kunz et al., 2006). PDEs are hydrolases that convert cAMP or cGMP into the corresponding 5′-monophosphates (5′-AMP and 5′-GMP; Alonso et al., 2006). This makes them important players in signaling pathways as they regulate the (rate of) degradation of these cyclic nucleotides, and are thus an important factor in determining cyclic nucleotide concentrations at the cellular and subcellular levels (Bender and Beavo, 2006). Through their own cellular distribution PDEs can be instrumental in directing or containing a cyclic nucleotide signal in a particular location, thereby preventing its diffusion throughout the cell (Johner et al., 2006; Maurice et al., 2014).

Although protozoan PDEs are valid targets for the development of antiparasitic drugs, one must not ignore the potential of side-effects arising from inhibition of human PDEs. Early work, testing mammalian PDE inhibitors against kinetoplastid PDEs was encouraging in that these displayed no significant activity against the parasite enzymes (Johner et al., 2006; Laxman et al., 2006; de Koning et al., 2012), suggesting that they are pharmacologically distinct from mammalian PDEs and that structure-assisted design of selective inhibitors should be possible, just as it has been for single human PDEs. Although the fine-tuning of an inhibitor to a single therapeutic target could aid in the development of drug resistance by single point mutations in the target enzyme, this is an unfortunate reality in all target-based drug design (Seebeck et al., 2011). However, as the inhibitors are targeted to the active site of essential enzymes (protozoan PDEs), mutations that also reduce substrate binding or catalytic activity would be lethal to the parasites. *In vitro* induction of resistance to the PDE inhibitor CpdA in *T. brucei* did not result in PDE mutations (Gould et al., 2013).

In *T. brucei*, PDEA is a single-copy gene; apart from the Class I active domain it shows almost no similarity to the mammalian PDEs, placing it in a separate gene family, and appears to be expressed throughout the life cycle (Kunz et al., 2004). It has been characterized but does not appear to be essential for BSFs of *T. brucei*, as genetic deletion mutants were viable and did not display reduced proliferation rates *in vitro* (Gong et al., 2001; Kunz et al., 2004). TbrPDEB, on the other hand, exists as a small family of genes that are much more closely related to the mammalian PDEs. The TbrPDEB family was the first kinetoplastid PDE to be cloned and characterized. Based on inhibitor studies, TbrPDEB1 was believed to be an essential protein for the proliferation of the African trypanosomiasis parasite and regulation of cyclic nucleotide levels (Zoraghi and Seebeck, 2002). However, the parasite expresses two closely related PDEB alleles, TbrPDEB1 and TbrPDEB2, which can compensate for each other; knockdown by RNAi of both alleles together leads to severe cell cycle defects and cell death, both *in vitro* and *in vivo* (Oberholzer et al., 2007). Interestingly, the two isoforms have somewhat different cellular localizations. Whereas, TbrPDEB1 is located only in the flagellum, TbrPDEB2 additionally localizes to the cytoplasm (Oberholzer et al., 2007). As knockdown of TbrPDEB2 alone does not affect cellular viability it must be the loss of flagellar PDE activity that is critical.

More recently, a tetrahydrophthalazinone compound named CpdA, a highly potent inhibitor of both TbrPDEB isoforms, was shown to display similarly potent activity against the parasites *in vitro*. The inhibitor was discovered from a screen of more than 400,000 compounds and displayed an IC_{50} value below 10 nM against TbrPDEB1, with similar activity on TbrPDEB2, and a

mid-nanomolar effect on trypanosome viability (de Koning et al., 2012). Independent pharmacological validation of the TbrPDEB isoforms was also reported by Bland et al. (2011) using the hPDE4 inhibitor piclamilast and a number of analogs. As CpdA was also known to be a potent inhibitor of human PDE4 (Van der Mey et al., 2001a,b), it is clear that the TbrPDEB family is pharmacologically closest to this human PDE. In contrast, human PDE5 inhibitors including sildenafil and tadalafil analogs displayed only weak inhibition of TbrPDEB1 (Ochiana et al., 2012; Wang et al., 2012a). Validation of the pharmacological importance of TbrPDEB1 was further confirmed when homology modeling and docking studies were used to guide fragments of Catechol Pyrazolinones into the parasite pocket (P-pocket) of TbrPDEB1 resulting in a new series of compounds with nanomolar EC_{50} values against the enzyme while also displaying promising trypanocidal activity and stimulating cellular cAMP levels (Orrling et al., 2012). The presence of the P-pocket in the otherwise highly conserved cAMP binding site was first reported for *L. major* PDEB1 (Wang et al., 2007) but present in all members of the kinetoplastid PDEB family examined to date, including TbrPDEB1 (Jansen et al., 2013), as well as TcrPDEC (Wang et al., 2012b). This is obviously very important since it allows for the development of kinetoplastid-specific PDE inhibitors with minimal or no cross reactivity with mammalian PDEs, which lack this pocket (**Figure 2**).

In *T. cruzi*, TcrPDEB1 was located in membrane fractions of the parasite and confocal microscopy showed it to be strongly associated with the flagellum (D'Angelo et al., 2004). The very high level of homology between kinetoplastid PDEB genes, and the conserved duplication into a B1 and B2 allele in tandem, appear to indicate that these genes play a crucial regulatory function in the cells. All kinetoplastid PDEB family members contain two N-terminal cAMP-binding GAF regulatory domains and a C-terminal catalytic domain (Laxman et al., 2005; Diaz-Benjumea et al., 2006; Johner et al., 2006; Shakur et al., 2011), but none of the other kinetoplastid PDE families do (**Figure 3**). Of these PDE families, the Phosphodiesterase D (PDEDs) have as yet barely been explored beyond the mere presence of the homologous genes in the respective genomes.

As at least some of the trypanosomatid PDEs have been shown to be essential regulatory enzymes, there is now much interest in these enzymes as drug targets and substantial efforts are ongoing, ranging from high-throughput screening, to structure-based design, compound repurposing and fragment-based inhibitor design (de Koning et al., 2012; Amata et al., 2014, 2015; Blaazer et al., 2015). As discussed extensively by Seebeck et al. (2011), this strategy has many advantages to drug development for the highly NTDs caused by kinetoplastid parasites, exactly because the target is highly conserved with closely related human homologs. First of all the interest in human PDEs by Big Pharma has resulted in large compound libraries of potential inhibitors. Furthermore, the potential side-effects and toxicity issues of inhibiting any of the human PDEs have been well investigated, as have the stability and pharmacokinetic properties of most of the inhibitor scaffolds. Most crucially, it has proven to be relatively straightforward to engage with the pharmaceutical industry on inhibitors for kinetoplastid PDEs, as they already have similar programs for other diseases. This strategy has allowed the rapid identification of potent inhibitors of TbrPDEB and other kinetoplastid PDEs but needs to rely on relatively small differences in the binding pocket, notably the P-pocket (**Figure 2**) of the enzyme, to achieve selectivity over human PDEs.

The Role of cAMP Signaling in *T. brucei*

The presence of cAMP in trypanosomes, and its variation during the course of infection, were recognized early on (Strickler and Patton, 1975). However, the completion of various kinetoplastid genome projects has revealed that cAMP signaling in the kinetoplastids is starkly different from the pathways so extensively studied in higher eukaryotes. Some important differences include the fact that kinetoplastid genomes do not code for G-protein-coupled receptors, or for heterotrimeric G proteins or PK G. Furthermore, the ACs are structurally very different from their mammalian counterparts, although the basic catalytic mechanism seems to be conserved between them, and may have assumed the role of receptors. Finally, apart from apparent PKA subunits in *T. cruzi* (Huang et al., 2006) and *L. donovani* (Bhattacharya et al., 2012), no homologous genes for cAMP effectors were identified in these genomes. The regulatory subunit of *T. brucei* PKA does not appear to bind cAMP, but instead binds cGMP (Shalaby et al., 2001), although, as discussed above, there is no convincing evidence that cGMP is produced by these parasites. Thus, it is likely that PKA is not activated by cyclic nucleotides in *T. brucei*.

The need for parasite survival implicates the need for a developmental response to adapt to different environments encountered within the mammalian host and throughout the arthropod vector. This is especially so during preparation for transmission, where specialized developmental forms are often generated to promote survival when ingested or propagated by a biting insect (Baker, 2010; MacGregor et al., 2012). The result is a dynamic balance of transmissible and proliferative stages within a host, ensuring that the population can maximize its longevity within the host but also optimize its capacity for spread to new hosts (Mony et al., 2014). The morphotypes that characterize a certain genus are cell shape, dimensions and the positions of the kinetoplast-flagellar pocket relative to the nucleus (Svobodova et al., 2007). These complex morphological and biochemical changes during cell differentiation in trypanosomatids are environmentally driven through different ligands and/or stimulatory molecules present in these environments, most of which are yet to be identified (Parsons and Ruben, 2000).

The report of AC activity in *T. b. gambiense* in 1974 (Walter et al., 1974) implicated the possible role of cAMP signaling in the cell biology and virulence of kinetoplastids, triggering the study of cAMP levels in the different life cycle stages of *Trypanosoma lewisi* (Strickler and Patton, 1975), *T. b. brucei* (Mancini and Patton, 1981), and *L. donovani* (Walter et al., 1978). Trypanosomes living in the bloodstream proliferate as morphologically "slender" forms that evade host immunity by antigenic variation, generating characteristic waves of infection. As each wave of parasitaemia ascends, slender

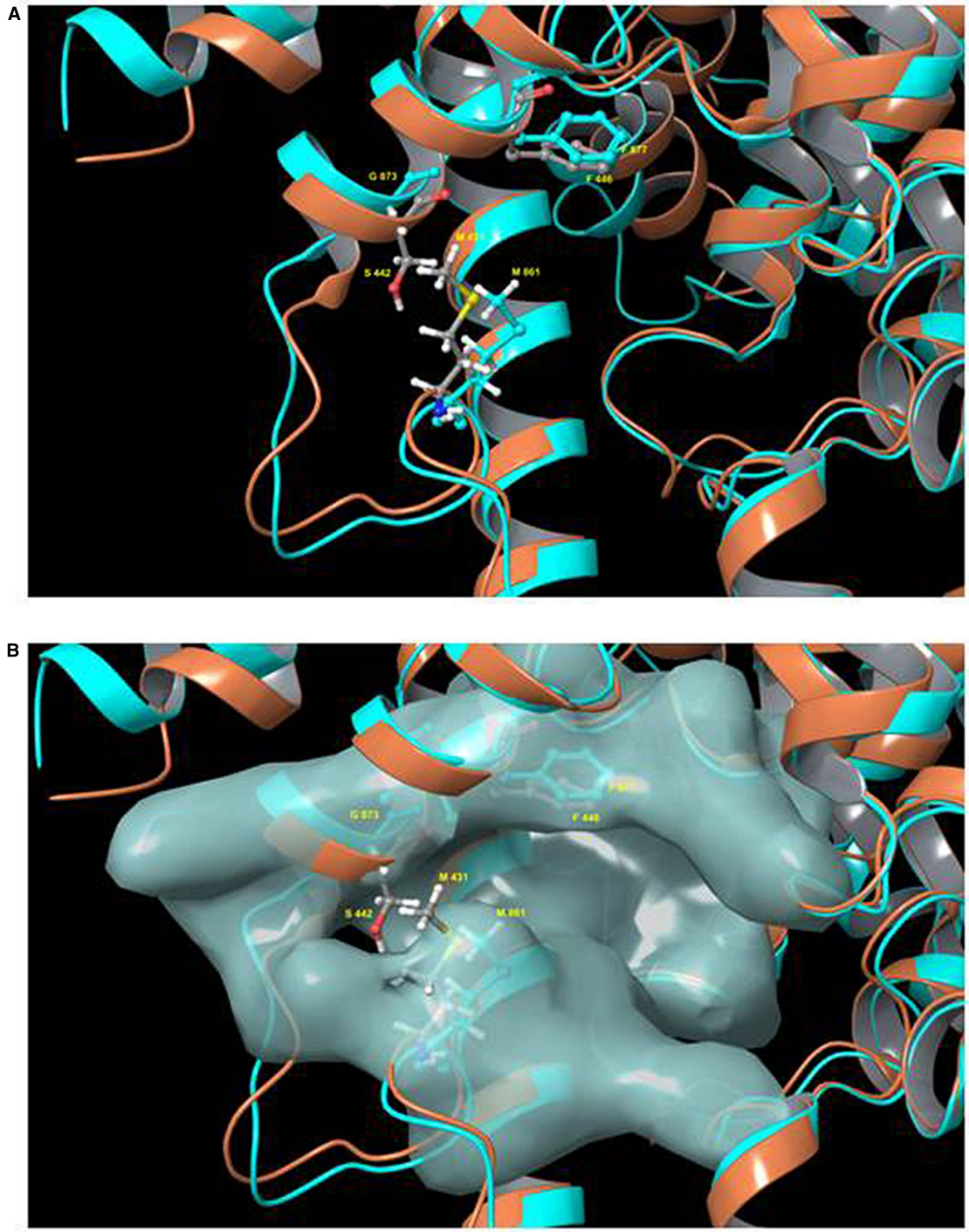

FIGURE 2 | Model of the binding pocket of TbrPDEB1 and hPDE4. Model of the superimposed binding pockets of TbrPDEB1 (turquoise ribbons and carbon atoms) and hPDE4B (orange ribbons, gray carbon atoms). The figure depicts chain A from the published 4I15 PDEB1 structure and chain B of hPDEB structure 1XM4 (alignment RMSD 1.847 Angstrom). **(A)** Ribbon model of the cAMP binding pocket. **(B)** Same view but with the molecular surface for TbrPDEB1 residues shown. Side chains for the conserved hydrophobic clamp phenylalanine residue in TbrPDEB1 (Phe877, turquoise) and hPDE4B (Phe446, gray carbons) are shown to illustrate the orientation of the P pocket relative to this canonical binding site feature. Side chains for the pair of amino acid residues at the entrance to the P pocket in TbrPDEB1 and hPDE4B are also shown—Met861 and Gly873 in TbrPDEB1 (turquoise), and Met431 and Ser442 in hPDE4B (colored by element—carbon gray, hydrogen white, nitrogen blue, oxygen red, sulfur yellow). For TbrPDEB1 the P-pocket is clearly visible in Frame B, directly adjacent to the main ligand binding site and delineated by M861 and G873, where in hPDE4B this space is filled entirely by M431 and S442. The models were constructed by Dr. R. K. Campbell of the Marine Biology Laboratory, Woods Hole, MA, USA, using Maestro software release 2015-2 (Schrödinger, Portland, OR, USA).

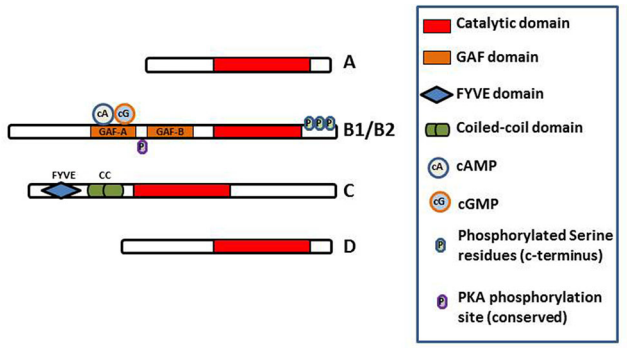

FIGURE 3 | Diagram of the domain structure of the kinetoplastid PDEs. The conserved catalytic domain is the main functional domain of the PDEs and binds cAMP. The GAF-A domains of PDEB1 and B2 bind cAMP and cGMP and regulate the function of the catalytic domain (Laxman et al., 2005). FYVE finger has been shown to bind two Zn^{2+} ions. Coiled-coil domains are important in stabilizing protein structure and thus for protein function. Phosphorylation of the indicated serine residues of PDEB1 has been observed in the *T. brucei* phosphoproteome (Nett et al., 2009) whilst a probably functionally conserved PKA phosphorylation site is predicted in PDEB1/B2 (Shakur et al., 2011).

forms stop proliferating and undergo transformation to stumpy forms, the parasite's adaptation for transmission to the tsetse fly vector (Vickerman, 1985). Earlier cAMP measurements showed increased cAMP levels in long slender BSFs during the cyclical wave of proliferation of these cells, and relatively low cellular cAMP concentrations when the abundance of stumpy forms increases (Mancini and Patton, 1981). The differentiation of longer slender forms to short stumpy forms has been shown to be density dependent (Vassella et al., 1997) with resemblance to quorum-sensing systems found in microbial communities (Waters and Bassler, 2005). The response to this density-dependent differentiation is triggered by a yet to be identified low molecular weight molecule called SIF, and it has been speculated that this triggers a cAMP response as the cAMP analog 8-(4-chlorophenylthio)-cAMP (8-pCPT-cAMP) was demonstrated to have the same differentiation-inducing effect as SIF. Additionally, trypanosomes incubated with a conditioned medium containing SIF displayed a twofold to threefold increase in the intracellular concentration of cAMP compared with cells grown in a non-conditioned medium (Vassella et al., 1997; Breidbach et al., 2002). Moreover, there had been a tentative link between cAMP signaling and differentiation of the BSFs to the insect procyclic forms, a process that is accompanied by the shedding of its VSG surface coat (Barry and McCulloch, 2001). However, monitoring of AC activity and VSG shedding after triggering differentiation to procyclic forms showed that AC stimulation was not responsible for the release of VSG (Rolin et al., 1993), and cAMP was not required for differentiation to occur (Strickler and Patton, 1975; Mancini and Patton, 1981). High concentrations of extracellular cAMP, 5′-AMP or adenosine did not significantly affect the proliferation of *T. brucei*, suggesting that the antiproliferative effect caused by the nucleotide analogs was mediated by an intracellular "receptor." And although 8-pCPT-cAMP did induce differentiation into stumpy-like non-proliferative forms, a hydrolysis-resistant analog did not, whereas the hydrolysis products of 8-pCPT-cAMP (i.e., the equivalent AMP and adenosine analogs) had a more potent effect than 8-pCPT-cAMP itself (Laxman et al., 2006). The clear conclusions of this study were that (1) cAMP is not the primary effector of the differentiation signal and (2) the hydrolysis products of 8-pCPT-cAMP trigger a differentiation-like transformation in *T. brucei* long-slender BSFs.

This insight was used to good effect when a genome-wide RNAi target sequencing (RITseq) approach was used to identify signaling components driving stumpy formation by exposing and selecting proliferative monomorphic cell lines unresponsive to 8-pCPT-cAMP or 8-pCPT-2-*O*-methyl-5-AMP-driven stumpy formation. This led to the identification of a cohort of genes implicated in each step of the signaling pathway, from genes involved in purine metabolism and signal transduction (kinases, phosphatases) to gene expression regulators (Mony et al., 2014). Identified genes at each step of the signaling pathway were independently validated in cells naturally capable of stumpy formation, confirming their role in density sensing *in vivo*. The putative RNA-binding protein, RBP7, was required for normal quorum sensing and promoted cell-cycle arrest and transmission competence when overexpressed. Thus, quorum sensing signaling in trypanosomes shares similarities to fundamental quiescence pathways in eukaryotic cells, its components providing targets for quorum-sensing interference-based therapeutics (Mony et al., 2014).

While a direct role for cAMP in the initiation of trypanosome differentiation events has thus become more doubtful, the role and importance of cAMP in flagellar motility and signaling is increasingly being dissected, with interesting findings. For example it is commonly believed that the flagellum, as an important host-parasite interface, has essential sensory functions (Tetley and Vickerman, 1985; Rotureau et al., 2009). For example in *Chlamydomonas reinhardtii*, the triggering of zygote formation is initiated by cAMP signaling in response to flagellum adhesion in gametes (Pan and Snell, 2000). Recently, it has been shown that cAMP regulates social motility in procyclic *T. brucei*, with social motility absent when TbrPDEB1 was inhibited by CpdA or knocked down with RNAi. The reduction in PDEB activity appeared to disrupt the generation of an extracellular signal necessary for the behavior, as the social motility was completely restored in mixed TbrPDEB1 knockdown and wild-type cells (Oberholzer et al., 2015). This is similar to social motility observation in *D. discoideum* where cAMP signaling is critical for surface motility (Firtel and Meili, 2000). It is believed that the social motility exhibited by the procyclic forms is essential for their migration from the tsetse midgut to the insect's salivary gland, which allows it to complete it life cycle.

In BSFs of *T. brucei*, the most unambiguous role of cAMP is in cytokinesis, as either the knockdown of ACs (Salmon et al., 2012a), knockdown of TbrPDEB1 and B2 (Oberholzer et al., 2007) or the pharmacological inhibition of these PDEs (de Koning et al., 2012) all lead to severe defects in the cytokinesis phase of cell division, resulting in misshaped cells with multiple nuclei and kinetoplasts, that are ultimately non-viable.

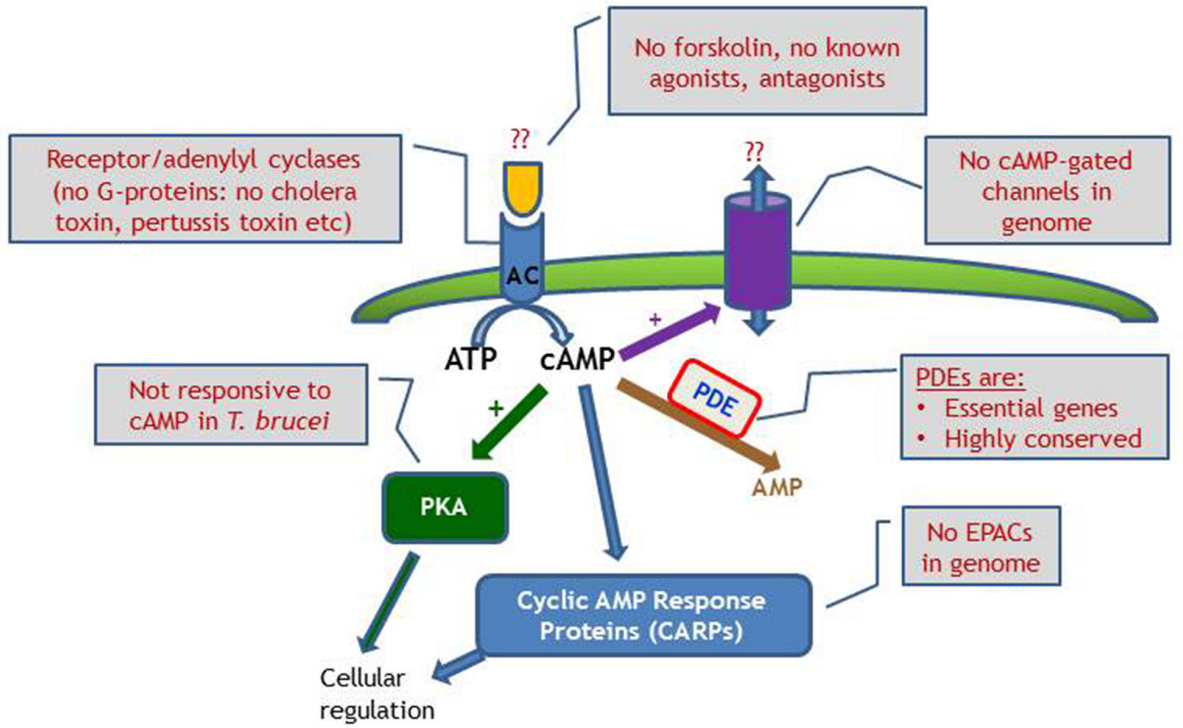

FIGURE 4 | Schematic diagram of cyclic nucleotide signaling in *T. brucei*, emphasizing the lack of investigative tools compared with the classical mammalian model, where manipulation of receptors, G-proteins and cyclases are all possible. EPAC, exchange protein directly activated by cAMP.

Novel Downstream Effectors of cAMP in Trypanosomes

Although cAMP has thus been implicated in important cellular functions and behavior of trypanosomes, the effectors that mediate this regulatory activity have been elusive. As noted above, the only potential effector protein identified, PKA, was not responsive to cAMP (Shalaby et al., 2001), and it became clear that, instead of searching for mammalian homologs, an unbiased approach to identify novel effector proteins was required. Accordingly, Gould et al. (2013) generated two CpdA-resistant lines. The first method involved exposing wild-type *T. b. brucei* trypanosomes briefly to the mutagen methyl methanesulfonate (MMS; Sigma), followed by culture in increasing but sub-lethal concentrations of CpdA. The second method employed the use of a genome-wide *T. b. brucei* RNAi library (Alsford et al., 2011, 2012; Baker et al., 2011) to select for resistance under CpdA pressure. This screen revealed four distinct genes that were knocked down, which were designated cAMP Response Proteins (CARP1–4; Gould et al., 2013). Targeted RNAi knockdown of these CARPs confirmed a significant increase in resistance to CpdA and to elevated cellular cAMP levels, confirming that they are genuine downstream effectors of cAMP signaling. One of the genes knocked down in the CpdA-resistant cultures was Tb427tmp.01.7890 (*CARP1*; Tb927.11.16210 in *T. b. brucei* reference strain TREU 927), encoding a 705-amino-acid protein containing two apparently intact and one partial cyclic AMP binding-like domain, that is conserved in synteny in each of the kinetoplastid genomes sequenced. Recently, the homolog of CARP1 in *T. cruzi* TcCLB.508523.80 has been reported to bind cyclic nucleotides, using cAMP and cGMP displacement assays (Jäger et al., 2014), further validating the role of CARP1 as a downstream cAMP signaling effector. CARP2–4 are proteins of as yet unknown functions but some of them have a probable flagellar localization, consistent with a role in mediating or regulating a cAMP signal (Gould et al., 2013).

Summary and Outlook

Differences in cAMP signaling between the mammalian system and trypanosome are well documented, such as the many and varied AC; no GPCRs or G-proteins; inactive PKA in *T. brucei*; and as yet to be identified AC triggers (**Figure 4**). However, from these differences opportunities may arise, as the downstream effects as well as the cAMP modulating receptor ligands appear to be unique to kinetoplastid parasites, and may offer promising targets for therapeutic intervention. The cAMP PDEs are already the focus of considerable drug development programs at the interface of academic research and pharmaceutical industry[1,2].

[1]http://www.openlabfoundation.org/research/projects/details12.html
[2]http://www.tipharma.com/pharmaceutical-research-projects/neglected-diseases/phosphodiesterase-inhibitors-for-neglected-parasitic-diseases-pde4npd.html

It is believed that as further studies of the downstream effectors progress, many more similarities and/or differences with mammalian regulatory pathways will come to the fore, which will provide much needed insights into these important biological processes in eukaryotic pathogens. This is even more so as studies of cAMP signaling and its associated effect on flagellar function and social motility is increasingly revealing particularly important cellular activities of the trypanosome. The importance of the flagellum to the trypanosome and how it interacts with its environment cannot be overstated. Thus, trypanosomal cAMP signaling, and the role of the flagellum therein, offer a ready and important biological system for much needed, innovative strategies for antiprotozoal drug development.

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