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Counteracting Protein Kinase Activity in the Heart: The Multiple Roles of Protein Phosphatases

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Decades of cardiovascular research have shown that variable and flexible levels of protein phosphorylation are necessary to maintain cardiac function. A delicate balance between phosphorylated and dephosphorylated states of proteins is guaranteed by a complex interplay of protein kinases (PKs) and phosphatases. Serine/threonine phosphatases, in particular members of the protein phosphatase (PP) family govern dephosphorylation of the majority of these cardiac proteins. Recent findings have however shown that PPs do not only dephosphorylate previously phosphorylated proteins as a passive control mechanism but are capable to actively control PK activity via different direct and indirect signaling pathways. These control mechanisms can take place on (epi-)genetic, (post-)transcriptional, and (post-)translational levels. In addition PPs themselves are targets of a plethora of proteinaceous interaction partner regulating their endogenous activity, thus adding another level of complexity and feedback control toward this system. Finally, novel approaches are underway to achieve spatiotemporal pharmacologic control of PPs which in turn can be used to fine-tune misleaded PK activity in heart disease. Taken together, this review comprehensively summarizes the major aspects of PP-mediated PK regulation and discusses the subsequent consequences of deregulated PP activity for cardiovascular diseases in depth.

Keywords: PP1, PP2A, calcineurin, PIP, protein phosphatase inhibitor-1 (I-1)

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INTRODUCTION

Myriads of studies starting from the late 1930s have unequivocally shown that protein kinases (PKs) are essential for cellular homeostasis not only in the heart but in virtually any tissue of the body (Cori et al., 1939; Rapundalo, 1998; Cohen, 2002a; Harvey, 2004; Johnson, 2009; Sato et al., 2015). We have now gathered a deep understand how PKs work in the context of cardiovascular diseases (CVDs) and consequently it is anticipated that PKs may serve as the pharmacological drug target of the twenty-first century (Cohen, 2002a,b; Force et al., 2004; Belmonte and Blaxall, 2011; Roskoski, 2015). Although discovered at an equally early time point in scientific history, protein phosphatases (PPs) have received much less attention in terms of functional studies and for consideration as potential drug targets. This is somewhat astonishing as (phospho-)proteomic studies suggested that nearly one third of all protein phosphorylation events is reversible (Sefton, 2001; Olsen et al., 2006) and it seems likely that targeting phosphatases in CVDs can be similarly promising. One explanation for this observation can be certainly attributed to the difference in substrate selection between PKs and PPs (Brinkworth et al., 2003; Zhu et al., 2005; Ubersax and Ferrell, 2007;

Roy and Cyert, 2009; Slupe et al., 2011; Li et al., 2013; Peti et al., 2013; Palmeri et al., 2014). While more than 400 available PKs often recognize decent consensus sequences in their target proteins, a far smaller number of phosphatases does not rely on specific enzyme-substrate recognition but mainly interact via so called phosphatase interacting proteins (PIPs) with their respective substrate, thus decreasing druggability enormously in comparison to PKs (Cohen, 2002c; Chatterjee and Kohn, 2013; De Munter et al., 2013; Li et al., 2013). Nevertheless recent developments have revealed exciting opportunities for the application of phosphatase-regulating drugs in CVDs and will therefore be discussed at the end of this review.

Protein phosphatases can be divided into three different subgroups, namely serine-threonine, tyrosine, and dual-specific phosphatases depending on their endogenous phosphorylation substrate (Maillet et al., 2008; Auger-Messier et al., 2013; Heijman et al., 2013; Senis, 2013). Thereby more than 98% of dephosphorylation events are carried out by serine-/threonine phosphatases and within this group PP 1, 2A, and 2B (PP1, PP2A and PP2B, also called calcineurin) amount to 90% of all dephosphorylation activity in the heart (Cohen, 1989; MacDougall et al., 1991). As a consequence this review will focus on the latter PPs and their role as cellular opponents of the aforementioned PKs in the healthy and diseased heart. Nevertheless we would like to refer the reader to some recent excellent reviews and research publication showing the physiological and pathophysiological importance of phosphatases which are not discussed in this review (Pulido and Hooft van Huijsduijnen, 2008; Patterson et al., 2009; Senis, 2013; Lauriol et al., 2015). Before we will deeply dive into the functional role of PP1, PP2A, and PP2B and their potential as drug target in CVDs, we will shortly recapitulate the structure of these enzymes which are virtually all working as holo-enzymes as this is crucial for the understanding of the following chapters. **Figure 1** shows a schematic drawing of the mouse representative PP1, PP2A, and PP2B holoenzymes known so far.

In mammals, the catalytic PP1 subunit is encoded by three separate genes (PPP1CA, PPP1CB, and PPP1CC) which seem to have distinct subcellular functions as suggested by initial studies using isoform specific knock-down and knock-out studies (Aoyama et al., 2011; Liu et al., 2015). Nevertheless there is neither detailed information available about spatiotemporal control of PP1 isoforms in the healthy or diseased heart nor insights into the molecular mechanism at this time (Luss et al., 2000). Further diversification of PP1 isoforms is achieved by alternative splicing of PPP1CA ($PP1\alpha_{1-3}$) and PPP1CC ($PP1\gamma_{1/2}$). $PP1\gamma_2$ seems to be the only tissue-specific expressed PP1 (brain and testis) while all other isoforms are expressed ubiquitously (Cohen, 2002c; Ceulemans and Bollen, 2004; Korrodi-Gregorio et al., 2014). Multiple studies have suggested that there is actually no freely available PP1 within the cardiac cell but rather a competition of >150 regulatory subunits to form a holo-complex with the PP1 catalytic subunits. These regulatory subunits determine subcellular localization and substrate specificity of the different PP1 isoform catalytic subunits. A series of excellent reviews gives a comprehensive overview about this aspect of PP1 (Ceulemans and Bollen, 2004).

The composition of the PP2A holo-enzyme is even more complex. It can be either a heterodimer consisting of the catalytic (PP2A-Cα or Cβ) and the structural scaffold (PP2A-Aα or Aβ) or a trimer consisting of the catalytic, the structural scaffold and another regulatory subunit (PPP2RX; Herzig and Neumann, 2000; Heijman et al., 2013). The importance of PP2a regulation becomes obvious since altered expression and activities are closely associated with heart diseases (Lei et al., 2015). Intriguingly PP1 and PP2A seem to share a couple of substrates together (Heijman et al., 2013). If this is due to compensatory mechanisms after genetic knockdown or knockout of single PP1/PP2A genes or caused by lack of specificity of the available pharmacological inhibitors has still to be determined. For further reading about the molecular mechanisms underlying PP2A regulation in the heart we would again like to recommend another set of enlightening reviews (Janssens and Goris, 2001; DeGrande et al., 2013; Lei et al., 2015).

Finally we would like to highlight PP2B (or calcineurin) as an essential cardiac PP which links Ca^{2+} and phosphorylation-dependent signaling pathways. Initially mainly studies identified calcineurin as a regulator of Ca^{2+} mediated gene transcription during cardiac remodeling, but recently calcineurin was also shown to directly act as an essential enzyme for reversible cardiac protein phosphorylation, e.g., at the cardiac L-Type Ca^{2+}-channel (Wang et al., 2014). Heart-restricted calcineurin overexpression in mice lead to cardiac remodeling, arrhythmic events and premature, sudden cardiac death (Molkentin, 2000). Again, PP2B is mainly active as a holo-enzyme consisting of one of the three different catalytic isoforms CNAα, β or γ, the Ca^{2+}-binding subunit ($CNB_{\alpha/\beta}$) and sometimes another regulatory protein, e.g., AKAP or Cain (Lim and Molkentin, 1999; Wolska, 2009; Heineke and Ritter, 2012; Wang et al., 2014).

After clarifying the functional impact and physiological importance of PPs in the heart, we will now move on to discuss which cardiac proteins are targets of reversible protein phosphorylation by PKs and PPs and how this may relate to CVD pathology.

PROTEIN PHOSPHATASES COUNTERACT PROTEIN KINASE MEDIATED PROTEIN PHOSPHORYLATION IN THE HEART

One of the hallmarks of CVDs is altered phosphorylation of cardiac proteins (Rapundalo, 1998; Luo and Anderson, 2013). Thereby most of the knowledge stems from studies of isolated cardiac myocytes, while studies about the substrates of reversible phosphorylation in cardiac fibroblasts, endothelial and smooth muscle cells are rather limited. **Table 1** gives a comprehensive and compartment-sorted overview about PP1, PP2A, and PP2B substrates in cardiac cells. In this chapter we highlight some important examples of phosphatase-dependent substrate regulation in cardiac cells and visualize them additionally in **Figure 2**.

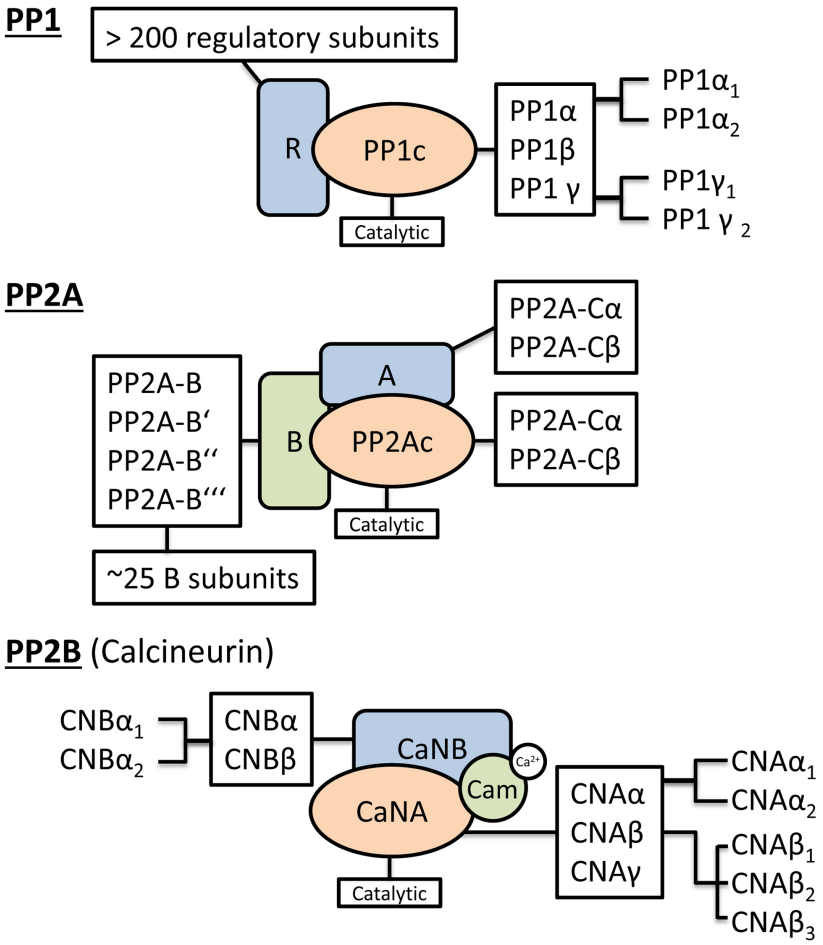

FIGURE 1 | Holoenzyme composition of protein phosphatases. The schematic drawing of the holoenzyme composition of PP1, PP2A and PP2B (Calcineurin) indicates the combinatorial complexity of the different catalytic and regulatory protein phosphatase subunits. A detailed description of the underlying nomenclature and the nature of the regulatory subunits can be found in Heijman et al. (2013).

The L-Type Calcium Channel, Na^+/K^+-ATPase and Other Ion Channels—Reversible Protein Phosphorylation at the Plasma Membrane

Reversible phosphorylation has been shown for a huge subset of different ion channels and their respective subunits. While the kinases required for phosphorylation have often been identified, the exact nature and contribution of phosphatases remained enigmatic due to the aforementioned reasons. The best studied examples of reversible protein phosphorylation at the plasma membrane are therefore the L-Type calcium channel ($Ca_V1.2$) and the Na^+/K^+-ATPase dependent substrates in cardiac cells. Phosphorylation at the α-Subunit of $Ca_V1.2$ takes place on multiple residues including Ser1512 and Ser1570 (CaMKII) as well as Ser1866 and Ser1928 (PKA; Chen et al., 2002; Yang et al., 2005; Lee et al., 2006; Lemke et al., 2008; Blaich et al., 2010; Minobe et al., 2014). There is still controversy which of these phosphorylation sites is the most important one for regulation of the $Ca_V1.2$ function, but most studies hint at an essential role of Ser1928 (Yang et al., 2005; Hulme et al., 2006; Lemke et al., 2008; Xu et al., 2010). It seems as if PP1, PP2A, and even PP2B can bind to this region and regulate dephosphorylation levels in concert (Davare et al., 2000; Hall et al., 2006; Tandan et al., 2009; Shi et al., 2012). It was reported that PP1 and PP2A act directly on the phosphorylation level of $Ca_V1.2$ and PP2B may have rather indirect effect by blocking PP1 and PP2A binding sites on the one hand and controlling $Ca_V1.2$ expression level via the CREB signaling pathway on the other hand (Tandan et al., 2009; Wang et al., 2014). Spatiotemporal control of PP2A activity at the $Ca_V1.2$ alpha-subunit is known to be mediated via the B56 (all isoforms) or PR59 subunits, while the mechanism of regulation of PP1 is still unknown (Hall et al., 2006; Xu et al., 2010). In general, PP2A-mediated dephosphorylation leads to downregulated $Ca_V1.2$ activity (Heijman et al., 2013). Finally pharmacological inhibition studies suggested that aforementioned PPs may play a role for dephosphorylation of basal as well as adrenoceptor induced $Ca_V1.2$ phosphorylation, which renders them different from PKA and CaMKII, that were shown to be either more important for basal (CaMKII) or adrenoceptor induced (PKA) $Ca_V1.2$

TABLE 1 | Targets for reversible cardiac protein phosphorylation.

Protein	Reported kinase activity	Phosphorylation site	Reported phosphatase activity	Reference
Aurora B	n.d.	n.d.	PP1	Murnion et al. (2001)
$Ca_V1.2$ α	CaMKII	Ser1512, Ser1570	PP1 PP2A PP2B	Davare et al. (2000), Kamp and Hell (2000), Chen et al. (2002), Yang et al. (2005, 2007), Hall et al. (2006), Hulme et al. (2006), Lee et al. (2006), Sun et al. (2006), Lemke et al. (2008), Tandan et al. (2009), Blaich et al. (2010), Xu et al. (2010), Brandmayr et al. (2012), Shi et al. (2012), Minobe et al. (2014)
	PKA	Ser1574, Ser1866, Ser1928	PP1 PP2A	
PKC	Ser1928	PP1 (major) PP2A (minor)		
PKG	Ser1928			
Akt/PKB	n.d.	n.d.		
$Ca_V1.2$ β	PKC	Ser496	PP1 PP2A	Gerhardstein et al. (1999), Grueter et al. (2006), Yang et al. (2007), Abiria and Colbran (2010)
	CaMKII	Thr498		
PKA	Ser458, Ser478, Ser479			
PKG	Ser496			
cMyBP-C	PKA	Ser282	PP1	Mohamed et al. (1998), Florea et al. (2012), Heijman et al. (2013)
	PKC	Ser273 (HF) Ser302 (HF)	PP2A	
Cold shock domain protein A CSDA	Bcr-Abl kinase PI3K/Akt Ribosomal S6 kinase (RSK)	Ser134	PP1	Sears et al. (2010), Chiang et al. (2015)
Connexin 43	PKC	Ser368	PP1 PP2A	Ai and Pogwizd (2005), Ai et al. (2011)
	PKA	Ser262		
CREB	PKA PKC Casein kinase II CaMKII P90 (RSK)	Ser133	PP1 PP2A	Montminy et al. (1990), Miller et al. (1998), Li et al. (2006)
eIF2α	n.d.	n.d.	PP1	Ceulemans and Bollen (2004)
FAK	n.d.	n.d.	PP1α *in vitro*	Yamakita et al. (1999)
Glycogen synthase kinase	PKA	Ser67 Ser48	PP1 PP2A	Woodgett and Cohen (1984), Walker et al. (2000)
HDAC4	CKIP PKA CaMKII	Ser246 Ser467 Ser632	PP2A	Zhang et al. (2002), Backs et al. (2006)
Histone3	CaMKII	Thr3 Ser28 Ser10	PP1	Ceulemans and Bollen (2004), Awad et al. (2013)
I-1	PKA	Thr35	PP2A PP2B	El-Armouche et al. (2006a), Wittkopper et al. (2010a)
	PKCα	Ser67		
Na^+/K^+ ATPase	PKC	Ser18	PP2A	Palmer et al. (1991), Feschenko and Sweadner (1997)
$Na_V1.5$	CaMKII	Ser571 Thr594 Ser516	n.d.	Murray et al. (1997), Zhou et al. (2000), Baba et al. (2004), Wagner et al. (2006), Hund et al. (2010), Ashpole et al. (2012), Toischer et al. (2013)
	PKA	Ser525 Ser528		
	PKC	n.d.		
NCX1	PKA (*in vitro*), PKC	Thr731 (*in vitro*)	PP1 PP2A PP2B	Palmer et al. (1991), Schulze et al. (2003), Wei et al. (2003, 2007), Shigekawa et al. (2007), Zhang and Hancox (2009), Wanichawan et al. (2011)
Neurabin	P70S6 kinase	n.d.	PP1	McAvoy et al. (1999), Sakisaka et al. (1999), Oliver et al. (2002)
	PKA	Ser461		

(Continued)

TABLE 1 | Continued

Protein	Reported kinase activity	Phosphorylation site	Reported phosphatase activity	Reference
NFATs	PKA GSK3β DYRK1/2 CK1 MEKK1 JNK P38MAPK	multiple	PP2B	Beals et al. (1997), Chow et al. (1997, 2000), Zhu et al. (1998), Chow and Davis (2000), Gomez del Arco et al. (2000), Sheridan et al. (2002), Yang et al. (2002), Okamura et al. (2004), Gwack et al. (2006)
PDE4D3	PKA	Ser13 Ser54	PP1 PP2A	Egloff et al. (1997), Carlisle Michel et al. (2004), Dodge-Kafka et al. (2006), De Arcangelis et al. (2008)
	ERK5	Ser579		
PDE5A	n.d.	n.d.	PP1	Chiang et al. (2015)
Phospholemman	PKA PKC	Ser68 Ser63 Ser68	PP1	Palmer et al. (1991), El-Armouche et al. (2011)
PLB	PKA	Ser16	PP1 PP2A	MacDougall et al. (1991), Luo et al. (1994), Jackson and Colyer (1996), Chu and Kranias (2002)
	CaMKII	Ser17		
PMCA	PKA PKC	n.d.	PP1 PP2A	Zylinska et al. (1998), Zylinska and Soszynski (2000)
P70S6 Kinase	n.d.	n.d.	PP1 PP2A	Bettoun et al. (2002)
Retinoblastoma protein (Rb)	CDK	Thr320	PP1	Liu et al. (1999), Ceulemans and Bollen (2004)
RyR2	PKA	Ser2808 Ser2030	PP1 PP2A PP2B	Marx et al. (2000), Pare et al. (2005),Xiao et al. (2005, 2006), Meng et al. (2007), Liu et al. (2011, 2014), Zhang et al. (2012)
	CaMKII	Ser2814		
SERCA2a	CaMKII	Ser38 (Ser167, Ser531 → *in vitro*)	n.d.	Brandl et al. (1986), Narayanan and Xu (1997)
SF2/ASF	PKA	n.d.	PP1	Gu et al. (2011), Huang et al. (2014)
Tn-inhibitor (TnI)	PKA	Ser23 Ser24	PP1 PP2A	Cole and Perry (1975)
T-type calcium channel (Cav3.2)	PKA	Ser1107 Thr2214 Ser1144	n.d.	Chemin et al. (2007), Hu et al. (2009)
Vitamin D receptor	P70S6 PI-3 kinase B/Akt	n.d.	PP1 PP2A	Bettoun et al. (2002)
Yotiao/KCNQ1	PKA	Ser27	PP1	Westphal et al. (1999), Marx et al. (2002), Terrenoire et al. (2009)

This table lists currently known substrates, which PK-mediated phosphorylation is counteracted by PP1, PP2A, or/and PP2B.

phosphorylation (Brandmayr et al., 2012). As tight control of $Ca_V1.2$ activity during EC-coupling is essential and deregulated $Ca_V1.2$ function has been shown in a multitude of different CVDs, e.g., hypertension, cardiac arrhythmia and heart failure, specific targeting of PP-$Ca_V1.2$ interactions might be a versatile drug targeting in the future (Splawski et al., 2004; Tang et al., 2008; Domes et al., 2011; Hong et al., 2012; Tajada et al., 2013).

Another important target for reversible dephosphorylation at the plasma membrane is the Na^+/K^+-ATPase, which can be either indirectly regulated via PP1-mediated dephosphorylation of the Na^+K^+-ATPase subunit phospholemman at position Ser68 or by direct recruitment of PP2A via its subunit PP2A-B56 and subsequent dephosphorylation at position Ser18 (Neumann et al., 1999; El-Armouche et al., 2006a; Bhasin et al., 2007). Interestingly binding of PP2A to Na^+K^+-ATPase diminishes binding of arrestin, thus increasing membrane localization of the pump and thereby actively modulating function of the Na^+K^+-ATPase (Kimura et al., 2011). The above mentioned studies also provided an important breakthrough in better understanding the role of reversible Na^+K^+-ATPase phosphorylation for CVD development. The described mechanism is markedly perturbed in failing hearts favoring phospholemman dephosphorylation and Na^+K^+-ATPase deactivation and thus may contribute to maladaptive hypertrophy and arrhythmogenesis via chronically increased intracellular Na^+ and Ca^{2+} concentrations (Neumann et al., 1999; Schwinger et al., 2003; Cheung et al., 2010; El-Armouche et al., 2011).

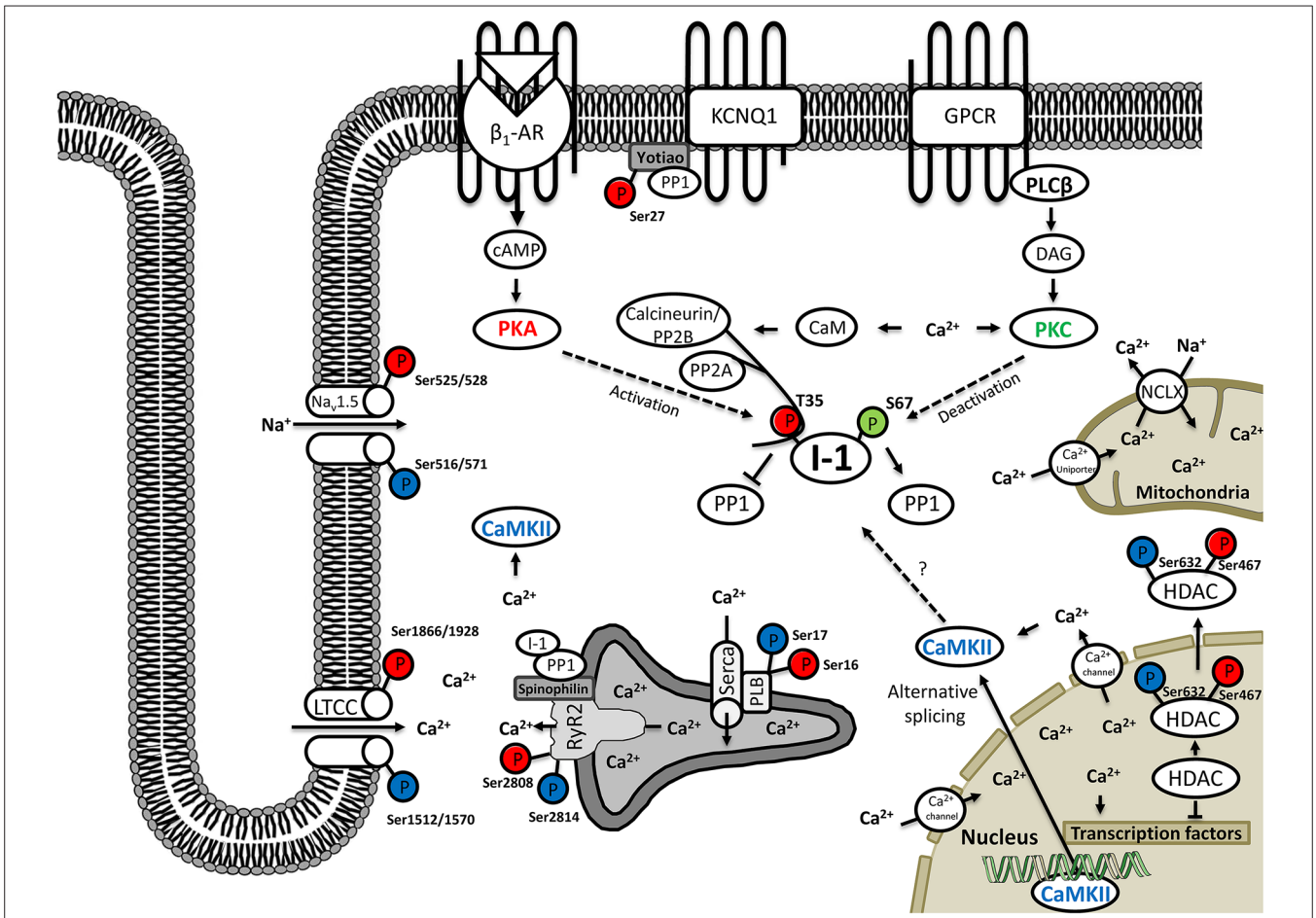

FIGURE 2 | Protein phosphatase inhibitor-1 (I-1) fulfills a nodal role during reversible protein phosphorylation in cardiomyocytes. On the primary level I-1 is mainly regulated by the complex interplay of protein kinases: PKA (red color), PKC (green color) and indirectly also CaMKII (blue color) vs. protein phosphatases 2A and 2B. While PKA (and CaMKII) have activating function, PKC, PP2A, and PP2B are limiting I-1 activity. On the secondary level, CaMKII and PP2B and thus subsequently also I-1, are regulated by intracellular calcium levels, which are mediated by a complex flux balance from internal Ca^{2+} stores of the sarcoplasmic reticulum, the mitochondria and the nucleus as well as Ca^{2+} influx from extracellular space. In turn I-1 is mainly acting on protein phosphatase 1 which dephosphorylates a multitude of functional proteins of the cardiomyocyte (see **Table 1**) and can act on different cellular functions including contraction, ion flux/currents, transcription and/or splicing, accordingly.

Finally there are some reports indicating reversible phosphorylation of Sodium and Potassium Channels, e.g., $Na_V1.5$ and KCNQ1, that are essential for membrane excitation (Murray et al., 1997; Terrenoire et al., 2009; Marionneau et al., 2012; Toischer et al., 2013). In case of $Na_V1.5$ many different phosphorylation sites have been described, with a priority of PKA-mediated phosphorylation in peak I_{Na} and CamKII-mediated phosphorylation during late I_{Na} (Baba et al., 2004; Bers and Herren, 2012; Marionneau et al., 2012). To our knowledge there is just one report about a protein tyrosine phosphatase (PTP1) working as a counteracting phosphatase after PKA or CamKII-mediated $Na_V1.5$ phosphorylation, so additional studies to identify putative PP-mediated $Na_V1.5$ dephosphorylation might be helpful (Jespersen et al., 2006). Even less is known with respect to reversible KCNQ1 phosphorylation. Initial studies show that PKA can mediated phosphorylation of KCNQ1 at position Ser27 at its C-terminal tail, which may by counteracted by PP1 and regulated by the accessory protein Yotiao (Marx et al., 2002; Nicolas et al., 2008). However, more studies are needed to understand the functional implication of reversible phosphorylation in this macromolecular protein complex at the plasma membrane are needed.

Phospholamban and the Ryanodine Receptor—Reversible Protein Phosphorylation at the Sarcoplasmic Reticulum

Disturbed Ca^{2+}-handling is coming along with most CVDs and numerous studies using genetically modified or pharmacologically treated animals have revealed the importance of tight spatiotemporal control of the two sarcoplasmic reticulum (SR) residing proteins ryanodine receptor type-2 (RyR2) and phospholamban (PLB) in this context. Consequently, tremendous

efforts have been undertaken to identify the exact contribution of kinases and phosphatases toward deregulated phosphorylation of the latter proteins. While the large intracellular C-terminus of RyR2 contains multiple putative phosphorylation sites, only three of them have been extensively studied until today. Although still under debate, it seems as if Ser2808 and Ser2030 are the major PKA and Ser2814 is the major CaMKII phosphorylated site (Marx et al., 2000; Marks et al., 2002; El-Armouche and Eschenhagen, 2009). Dephosphorylation can be carried out by either PP1, which is targeted by spinophilin and/or PP2A which is targeted by its subunit PR130 to the multiprotein RyR2 holo-complex (Marx et al., 2001; Reiken et al., 2003; Chiang et al., 2014). There are no reports about calcineurin mediated RyR2 dephosphorylation but it has been suggested that deregulated RyR2 activity can modulate calcineurin expression in turn, thus serving as a potential hub for feedback inhibition of reversible cardiac protein phosphorylation (Zou et al., 2011).

In contrast PP1 is the major dephosphorylating enzyme of PLB, which critically modulates the activity of the SR Ca^{2+}-ATPase (SERCA2a). A small contribution has been also shown for PP2A, but this relates to less than 30% of the dephosphorylation events at positions Ser16 (PKA-controlled) and Thr17 (CaMKII-controlled; MacDougall et al., 1991; Jackson and Colyer, 1996; Chu and Kranias, 2002; Aye et al., 2012). Increased targeting of PP1 to PLB is achieved by R_{GL} or HSP20, which leads to reduced SERCA2a Ca^{2+} uptake and thus attenuated β-AR stimulation-induced lusitropy (Berrebi-Bertrand et al., 1998).

Troponin-I and cMyBP-C—Reversible Protein Phosphorylation at the Myofilaments

The most important consequences of the elevated systolic Ca^{2+}-level is the activation of the contractile apparatus, which is again dependent on the amount of phosphorylation of the associated contractile proteins, e.g., the cardiac myosin-binding protein-C (cMyBP-C) or the Inhibitor of the Troponin complex, Troponin-I (Barefield and Sadayappan, 2010). For the latter, PP1 seems to preferentially dephosphorylate Ser23 and Ser24 on TnI, while the exact substrate of PP2A is yet undetermined (Jideama et al., 2006). Nevertheless it could be detected that targeting of PP2A is B56α-dependent and β-AR stimulation seems to be able to dissociate this localized interaction (Yin et al., 2010).

Phosphoproteomic studies revealed a number of putative phosphorylation sites also for cMyBP-C (Solaro and Kobayashi, 2011). Interestingly reduced phosphorylation at position Ser282 coincides with increased PP1 and PP2A activity in patients with atrial fibrillation (El-Armouche et al., 2006b). Similar results were received in end-stage heart failure, however here phosphorylation was also diminished at positions Ser273 and Ser302 (El-Armouche et al., 2007b; Sumandea and Steinberg, 2011).

I-1 and PDE4D3—Reversible Protein Phosphorylation in the Cytoplasm

An excellent showcase for the description of a typical substrate for reversible phosphorylation of a cardiac protein represents the PP1-specific inhibitor of protein phosphatase-I (I-1). I-1 fulfills the function as a central signaling hub in feedback control of phosphatase activity and is thus topic of a number of reviews itself (Nicolaou et al., 2009; Wittkopper et al., 2010a, 2011). I-1 activity depends on PKA-dependent Thr35 phosphorylation, which seems to be mediated via AKAP18 (Endo et al., 1996; Singh et al., 2011). During β-AR stimulation, PKA-controlled I-1 activation and subsequent PP1 inhibition form a positive feed-back loop amplifying the phosphorylation of substrates like PLB, RyR2, and phospholemman (Carr et al., 2002; El-Armouche et al., 2003). Dephosphorylation of I-1 at position Thr35 is carried out by PP2A and PP2B, thus creating a crosstalk between the different phosphatases as well as between PKA and Ca^{2+}-related second-messenger systems (via PP2B mediated dephosphorylation of I-1; El-Armouche et al., 2006a). Moreover, I-1 is also phosphorylated by PKCα on Ser67, this time resulting in reduced I-1 activity and decreased PLB phosphorylation. Conversely, the Ser67Ala substitution, preventing inactivation, increases PLB phosphorylation (Huang and Paudel, 2000; Braz et al., 2004). **Figure 1** gives a schematic overview about the nodal role of I-1 during reversible cardiac protein phosphorylation.

Much less is known about reversible phosphorylation of phosphodiesterases, another important players of the adrenergic response. PP2A could be shown as a negative regulator of PDE4D3 activity in a complex with mAKAP, RyR2 and PKA (Carlisle Michel et al., 2004; Dodge-Kafka et al., 2006; De Arcangelis et al., 2008). In this case PP2A activity is itself controlled by PP2B (Dodge-Kafka et al., 2010). Further studies will be needed to elucidate the contribution of reversible phosphorylation toward function of cardiac PDEs, as malfunction of this enzymes can be beneficial or detrimental in multiple CVDs.

Histone 3 and HDAC4—Reversible Protein Phosphorylation in the Nucleus

Several studies have implicated localization and activity of phosphatases in different compartment of the nucleus including chromatin bound fractions (Bollen and Beullens, 2002; Moorhead et al., 2007). There is extensive knowledge about the counteracting role of PPs against PKs during different phases of the cell cycle in non-cardiac cells (Ceulemans and Bollen, 2004; Novak et al., 2010; Jeong and Yang, 2013). As adult cardiac myocytes are mainly non-dividing cells, phosphatase targets may be different and up to this point there are only sparse reports about nuclear phosphatase targets in this regard (von Holtey et al., 1996; Heineke and Ritter, 2012). An exception hereof is histone deacetylase 4 (HDAC4), which phosphorylation status was well studied. While CKIP and CaMKII seem to be the main kinases phosphorylating Ser246, and Ser632, PP2A was identified as a major phosphatase to control HDAC4 phosphorylation status and subsequent subcellular localization, thus modulating HDAC4 activity (Paroni et al., 2008; Ling et al., 2012; Liu and Schneider, 2013; Kreusser and Backs, 2014). Knockdown experiments of PP1 did not reveal a significant contribution toward HDAC4 dephosphorylation. This might however be different for another important phosphatase substrate of the nucleus, which is histone 3 (H3). Phosphorylation of H3 occurs at multiple positions, including Ser10 and Ser28 (Awad et al., 2013; Korrodi-Gregorio

et al., 2014). PP1 was reported to significantly contribute toward dephosphorylation of at least Ser10 in non-cardiac cells rendering it a potential target also in cardiac myocytes (Awad et al., 2013). In summary PPs may act as important and rapid modifiers of the epigenetic code and predispose DNA and protein sequences for subsequent methylation—a concept that is also known as the methyl-phospho-switch hypothesis (Chen et al., 2008; Sawicka and Seiser, 2014).

Nuclear Factors of Activated T-Cells (NFATs)—Regulation of Transcription by Ca^{2+}-dependent Dephosphorylation

Nuclear factors of activated T-cells (NFATs) are a family of transcription factors in which the nuclear localization signals (NLS) are masked by multiple phosphorylations in their serine-rich domains. Upon activation of PP2B by Ca^{2+}/calmodulin, they are dephosphorylated, leading to their nuclear translocation, initiation of transcription and induction of cardiac hypertrophy as well as electrical remodeling (Heineke and Molkentin, 2006; Heineke and Ritter, 2012). A potential role in cardiac hypertrophy has been shown for NFATc2, NFATc3, and NFATc4. A number of PKs have been inflicted in cardiac NFAT phosphorylation, among them PKA, GSK3β, DYRK1/2, CK1, MEKK1, JNK and P38MAPK, and some isoform specificity is present. Importantly, PKA and DYRK1/2 can act as priming kinases, enabling other kinases to access the NFAT phosphorylation sites (Pan et al., 2013). The importance of PP2B/NFAT signaling in cardiac hypertrophy was first recognized in 1998, when Molkentin et al. (1998) demonstrated that overexpression of activated PP2B of NFATc4 lead to cardiac hypertrophy. Later it became clear that NFATc3 as well as c4, but not c2 are necessary for cardiac hypertrophy in multiple models (Wilkins et al., 2004; Bourajjaj et al., 2008). Moreover, PP2B/NFAT signaling plays an important role in shaping physiological cardiac repolarization (Rossow et al., 2006) as well as in the generation of an arrhythmogenic substrate in heart failure (Rossow et al., 2004). Since cardiomyocytes dramatically increase their intracellular Ca^{2+} concentration during each heartbeat, an important problem to be solved is the question whether the "contractile Ca^{2+}" released by LTCC and RyR2 or a different pool of Ca^{2} regulated by LTCC and/or canonical transient receptor potential channels (TRPC1, 3 or 6) activate PP2B in CVD (Rossow et al., 2004).

Connexin 43 and SAP97—Reversible Protein Phosphorylation at Cell–Cell Contacts

Cardiac electrical excitation is mainly conducted from cell to cell by connexins. In heart especially connexins 43 and 45 fulfill an important function for conduction and increased phosphatase activity of either PP1 or PP2A was shown to induce conduction slowing possibly due to influence on protein stability and nearby channel activation (Jeyaraman et al., 2003; Ai and Pogwizd, 2005; Ai et al., 2011). Decreased connexin expression and phosphorylation was recently identified as a novel inducer of arrhythmic events (Delmar and Makita, 2012; Remo et al., 2012). Another important protein involved in such cell-cell contacts is synapse-associated protein 97 (SAP97). Numerous studies in brain have suggested CaMKII dependent phosphorylation and PP2B-mediated dephosphorylation of this scaffolding protein (Nikandrova et al., 2010). While SAP97 is also expressed in heart and initial studies in mice have revealed potential impact on potassium channel function and thereby action potential duration, there is a lack of knowledge regarding reversible SAP97 phosphorylation in the heart.

Glycogen Synthase and Phosphorylase—Reversible Protein Phosphorylation During Cardiac Metabolism

Although being the first PP substrates studied in terms of regulation and mechanism, the knowledge about functional impact of phosphatases on proteins of the glycogen metabolism has been underestimated for cardiac function a long time. However, recent milestone publications have led to a revival of the study of metabolic pathways and their contribution toward CVDs (Kolwicz et al., 2013; Chouchani et al., 2014; Mirtschink et al., 2015). The most important substrates of PP1 and PP2A are glycogen synthase, glycogen phosphorylase and to a lesser extent, phosphorylase kinase (Ceulemans and Bollen, 2004). PP1 and PP2A-mediated dephosphorylation of the rate-limiting enzymes of glycogen metabolism results in the storage of glycogen, in accordance with the proposed function of PP1 as an energy conserving enzyme (Ceulemans and Bollen, 2004). Future studies will be necessary to understand the exact contribution of the different phosphatases in cardiac cells in this process and to translate these findings for the understanding of pathophysiological processes. In this regard it will also be essential to check reversible phosphorylation in other metabolic pathways, e.g., lipid, amino acid, and nucleotide metabolism.

While this chapter indicated the widespread target portfolio of PPs in the heart, the molecular regulation of PPs themselves is at least equally important for cardiac physiology and manifestation of CVDs. Therefore we are going to review the multiple checkpoints of PP regulation in the heart in the light of PP-dependent PK activity regulation.

REGULATING THE REGULATORS: MOLECULAR CONTROL OF PROTEIN PHOSPHATASE ACTIVITY IN THE HEALTHY AND DISEASED HEART

PPs themselves can be regulated at multiple checkpoints. We will first discuss and highlight some of this regulative mechanisms and subsequently summarize the consequences of deregulated phosphatase activity in CVDs.

The perhaps most obvious but still not really well studied mechanism of transcriptional control is the expression of different isoforms of the catalytic subunits of PP1, PP2A, and PP2B (Cohen, 2002c; Korrodi-Gregorio et al., 2014). Especially for PP1, isoform analysis in non-cardiac cells has shown difference in subcellular distribution of catalytic isoforms and response toward cellular stressors like hypoxia and/or apoptosis (Wang

et al., 2001; Comerford et al., 2006; Iacobazzi et al., 2015). The most obvious but however not really well studied mechanism of transcriptional control is the expression of different isoforms of the catalytic subunits of PP1, PP2A, and PP2B (Herzig and Neumann, 2000; Felkin et al., 2011; DeGrande et al., 2013; Korrodi-Gregorio et al., 2014). Especially for PP1, isoform analysis in non-cardiac cells has shown difference in subcellular distribution of catalytic isoforms and response toward cellular stressors like hypoxia and/or apoptosis (Ceulemans and Bollen, 2004; Trinkle-Mulcahy et al., 2006; Djouder et al., 2007; Iacobazzi et al., 2015). Mutants of different PP1 isoforms in *Drosophila* or mice give rise to apparently different phenotypes (Raghavan et al., 2000; Kirchner et al., 2007). For cardiac myocytes there is yet very limited evidence that isoform-specific distribution of PP catalytic subunits underlies functional implication during CVDs, thus awaiting further examination in suitable cellular or animal models. So far preliminary reports suggest predominant expression of PP1α in myofibrillar, PP1β in longitudinal SR and PP1γ in the junctional SR fraction under basal conditions (Aoyama et al., 2011). However, the small number of PP1c isoforms, their near 90% amino-acid sequence identity and their broad and similar substrate specificities *in vitro* support the current hypothesis that it is predominantly the regulatory subunits with which PP1c interacts that controls the specificity and enormous diversity of PP1 function. To our knowledge there is only one report about isoform specific distribution of PP2A or PP2B catalytic subunits in cardiac cells until today enforcing the importance of regulatory subunits for substrate specificity and subcellular distribution also for the two latter phosphatases (DeGrande et al., 2013). In addition there is some limited knowledge about the characteristic of the calcineurin promoter, while we are not aware of any reports about PP1 or PP2A promoter analysis in cardiac cells leaving a huge unexplored research field of PP activity regulation (Oka et al., 2005). Finally some sparse studies hint at epigenetic regulation mechanisms of, e.g., PP2A expression control but again specific and detailed mechanistic studies in cardiac cells are missing (Keen et al., 2004). Thus although deregulated expression of PPs have been reported during a variety of different CVDs, and the underlying mechanistic nature has not been studied in detail yet.

An interesting opportunity of post-transcriptional PP activity control is conducted via regulated expression of microRNAs (miRs). However, most of the known examples in the heart have revealed control of PP1, PP2A, and PP2B regulatory subunit rather than catalytic subunit expression by miRs so far. Prominent examples are, e.g., miR-1 and miR-133 control of RyR2/PP2A mediated arrhythmogenesis, miR-34-a control of PPP1R10 during cardiac aging and contraction, as well as miR-212/132 control of calcineurin activity during cardiac hypertrophy (Belevych et al., 2011; Ucar et al., 2012; Boon et al., 2013). It is anticipated that miR-mediated control of PP catalytic and regulatory subunits may serve as a novel attractive and highly specific pharmacological target among CVDs.

The most intensive studied way of PP activity control is, however, post-translational PP activity control, mediated either by a regulatory or an inhibitory subunit. Recent studies have suggested that the number of PIPs is bigger than the number of PP, leading to a situation of competition between the different regulatory and inhibitory subunits for the catalytic subunits of PPs (Ceulemans and Bollen, 2004). Genetic studies in transgenic mice with knock-out or overexpression of PP regulatory subunits have enforced our understanding about this process (Heijman et al., 2013). While there are, e.g., already more than 150 known regulatory subunits for PP1, we have decided to focus on two complex examples of PP activity regulation that may serve a prototype for PP regulation and inhibition, namely I-1 and spinophilin. For a comprehensive overview we would like to recommend some excellent reviews dedicated to posttranslational PP activity control (Cohen, 2002c; Redden and Dodge-Kafka, 2011).

Protein Phosphatase Inhibitor-1 (I-1)

One of the best studied examples of PP-inhibitory subunits in the heart is the I-1. Originally identified in rabbit skeletal muscle, it's expression has now been verified in nearly every tissue with highest expression in skeletal muscle, adipocytes, kidney and liver and brain (Huang and Glinsmann, 1976; Hemmings et al., 1992; Aleem et al., 2001). Interestingly I-1 can only be found in vertebrates and may therefore represent rather recent addition of the fine-tuning interplay between PKA and PP1 from an evolutionary view (Ceulemans and Bollen, 2004). I-1 is one of the few thermostable proteins and cannot be precipitated by 1% trichloroacetic acid (Aitken and Cohen, 1982; El-Armouche et al., 2004; Wittkopper et al., 2011). Although being relatively small and without any transmembrane domains, the crystal structure of I-1 could not be elucidated until recently (Huang et al., 2010). As the exact activation and inhibition mechanism of I-1 has been already been described before in this review we will know focus on the consequence of deregulated I-1 expression on PP activity and *vice versa* for different CVDs.

The first example comes from failing human myocardium, which showed hyperactive PP1 (Neumann et al., 1997; Huang et al., 1999; Schwoerer et al., 2008). It was tempting to speculate that I-1 might be dysregulated in heart failure and in fact I-1 mRNA and total I-1 protein amount were reduced by 60%, and its PKA-dependent phosphorylation level even by 80% (El-Armouche et al., 2003, 2004). The reason of decreased I-1 phosphorylation possibly reflects desensitization of β-AR signaling with decreased cAMP levels and PKA activation in the failing myocardium. Additionally an increase in calcineurin activity, especially in settings of elevated diastolic Ca^{2+} levels, may contribute to the decrease in I-1 phosphorylation described above (Lim and Molkentin, 1999). Finally I-1 protein expression was also markedly downregulated in an experimental dog model of heart failure arguing for a central role of I-1 mediated PP1 activity control during heart failure (Gupta et al., 2003; El-Armouche et al., 2007a). All these data suggest that I-1 downregulation may be part of the desensitization process taking place among the irregular β-adrenergic signal transduction pathway in failing hearts. A hypothesis that could in part be validated by studies using rats with chronic isoprenaline treatment showing decreased I-1 expression and activation levels as well (El-Armouche et al., 2007a). Knock-out of I-1 in mice in two different studies

led to either none or a subtle decrease in basal contractile parameters and a significantly blunted β-adrenergic response (Carr et al., 2002; El-Armouche et al., 2008). Isolated atria from I-1 deleted mice showed normal isometric force under basal and maximally stimulated conditions but a rightward shift of the concentration-response curve of isoprenaline (Carr et al., 2002). Importantly, neither the β-AR density nor the PP1 protein levels differed from wild type littermates, indicating no obvious major compensatory changes in the I-1 KO hearts. Finally, germline deletion of I-1 did not result in apparent heart disease and did not negatively affect life expectancy and/or heart structure in aged mice arguing against a causative role of diminished I-1 expression for heart failure progression (Carr et al., 2002; El-Armouche et al., 2008; Heijman et al., 2013). Further studies are needed to better understand the functional implication of I-1 deletion during experimental models of heart failure and to elucidate if pharmacological inhibition of I-1 might be beneficial or detrimental during initiation and progression of heart failure. In contrast extreme overexpression of full-length I-1 (200-fold) led to spontaneous cardiac hypertrophy and cardiac dysfunction accompanied by compensatory PP1 expression already at young age of 3 months (El-Armouche et al., 2008). Instead conditional cardiomyocyte-specific and moderate overexpression of a constitutively active I-1 (I-1c) show a hypercontractile response comparable to PLB knock out mice but exaggerated contractile dysfunction upon catecholamine application in young mice and spontaneous development of dilated cardiomyopathy in adult mice (Wittkopper et al., 2010b, 2011). A comparative PP1 upregulation could however not be detected in this mouse model. Another study did not show any overt cardiac phenotypes in young mice after moderate I-1 overexpression and therefore postulated moderate I-1 overactivation/overexpression as a potential pharmacological approach (Pathak et al., 2005). Indeed subsequent studies using models of pressure overload and chronic isoprenaline infusion-induced heart failure, suggested that I-1 overexpression is associated with a preserved cardiac function, an attenuated development of cardiac hypertrophy and a lower degree of fibrosis and apoptosis (Pathak et al., 2005; Chen et al., 2010; Ishikawa et al., 2014).

A second example of I-1 controlled PP1 activity in a pathological condition has been described for the occurrence and persistence of atrial fibrillation (El-Armouche et al., 2006b; Chiang et al., 2015). Similar to the situation in failing myocardium some proteins which are important for Ca^{2+} handling, such as the L-type Ca^{2+} channel or cMyBP showed increased dephosphorylation hinting at hyperactive PPs (El-Armouche et al., 2006b; Dobrev and Nattel, 2008; Nattel et al., 2008). Nevertheless some other Ca^{2+}-handling proteins like the RyR2 (at Ser2814) or PLB (at Thr17) were in turn hyper-phosphorylated arguing for rather subcellular than general upregulation of PP activity (Neef et al., 2010). The exact mechanism for this observation is however still unknown and deserves further investigation. Another contrast to the situation in failing myocardium were the level of I-1 as well as S67-phospho I-1, which were unchanged between AF and non-AF patients (Bhasin et al., 2007). Even more surprisingly, PKA-dependent phosphorylation of I-1 at Thr35 was roughly 10-fold higher in cAF patients, which is thought to be completely suppressive for SR-bound PP1 activity, thus leading to increased phosphorylation of PLB (Ser16) and RyR2 (Ser2814; El-Armouche et al., 2006b; Chelu et al., 2009; Greiser et al., 2009). Another contributor toward RyR2 phosphorylation in cAF patients may be overactivated CaMKII which overcomes the globally enhanced PP1 activity causing greater steady-state RyR2 phosphorylation (Chelu et al., 2009).

As a final example for I-1 controlled PP activity during CVDs we have picked ventricular arrhythmias, which may be responsible for almost half of death cases in patients with heart failure (Packer, 1985; Roberts-Thomson et al., 2011). In general ventricular arrhythmias are initiated and maintained by either focal (ectopic) or re-entry mechanisms, which have been linked to increased diastolic SR Ca^{2+} leak from RyR2 and subsequent activation of the Na^{+}/Ca^{2+}-exchanger (NCX) at the cellular level (Weiss et al., 2000; Jalife et al., 2003; Prystowsky et al., 2012; Qu and Weiss, 2015). Increased and/or prolonged NCX activity may in turn lead to delayed after-depolarizations underlying arrhythmogenesis (Vermeulen et al., 1994; Schillinger et al., 2003; Sipido et al., 2007). Although still under debate if hyperactivated PKA or CaMKII play the major role for increased RyR2 activation and subsequent SR Ca^{2+} leak, it is common sense that β-adrenoceptor stimulation is the general trigger for SR Ca^{2+} leak in both atrial and ventricular arrhythmias (Eisner et al., 2009; Eschenhagen, 2010; Dobrev et al., 2011). In contrast much less is known about the physiological and pathological role of RyR2 dephosphorylation by phosphatases. Different studies have shown that both, PP1 and PP2A are coupled to the RyR2 macromolecular complex (Marx et al., 2000). Consequently, the dual PP1/PP2A inhibitor okadaic acid increases the RyR2 open probability and phosphorylated RyR2 subunits have been shown to be dephosphorylated using recombinant PP1 (Sonnleitner et al., 1997).

Interestingly, I-1 deleted mice were partly protected from structural (hypertrophy, fibrosis, and dilatation) and functional effects (loss of inotropic response to dobutamine) of chronic β-adrenoceptor stimulation via isoprenaline infusions with minipumps (Wittkopper et al., 2011). In addition, injections of increasing doses of isoprenaline with continued ECG monitoring showed marked protection from fatal catecholamine induced arrhythmias in I-1 knock-out mice (El-Armouche et al., 2008). Notably, the protection from β-adrenergic-mediated cardiotoxicity in I-1 knock-out mice was not associated with changes in heart rate regulation. Further studies using, e.g., isoform-specific PP1 and/or PP2A overexpressing mice or cardiac myocytes may help to strengthen these preliminary results.

Spinophilin

Another fascinating example of phosphatase activity regulation with potential implication in CVD can be found within the interaction of spinophilin and PP1. First evidence that spinophilin can act as a regulatory PP1 subunits stems from *in vitro* co-immunoprecipitation studies using overexpressed GST-RyR2 fusion proteins (Marx et al., 2001). While *in vitro* co-incubation of spinophilin and PP1 with the model substrate phosphorylase A led to decreased dephosphorylation activity, *in*

vivo experiments showed that spinophilin acts as a scaffolding protein to target PP1 toward its endogenous substrates, thus rather facilitating dephosphorylation activity than decreasing it (Feng et al., 2000; Oliver et al., 2002). Elegant studies using different PP1/spinophilin or other PP1/PIP crystal complexes helped to understand the underlying mechanistic principles of these interactions (Ragusa et al., 2010, 2011).

It was shown that the C-terminal domain (aa 417–494) of spinophilin is necessary and sufficient for complete interaction with PP1 (Hsieh-Wilson et al., 1999). However, crystallization studies revealed that the PP1-binding domain of spinophilin is highly unstructured and has contact with multiple residues on PP1. In fact, the spinophilin binding domain entirely folds only upon binding to PP1 (Ragusa et al., 2010). Interestingly, spinophilin does not only bind via the well-recognized RVXF binding motif, but also by multiple interaction with the C-terminal groove of PP1. Most importantly, despite all the above described PP1/spinophilin interaction sites, spinophilin does not affect the PP1 active site, nor two further putative substrate binding sites, enabling full PP1 activity when bound to spinophilin and leaving the chance for further PP1/PIP interactions. As a consequence really unique PP1 holoenzymes can exist, which may allow for very specific counteracting and fine-tuning of phosphorylation events conducted by the plethora of specific kinases within the cell. It would be of major interest to solve the structure of PP1/I-1 complexes as well, to better understand the nature of the so far mainly phenomenologically described interaction/inhibition.

Further reports showed that spinophilin is able to direct PP1 to the membrane of the SR in cardiac myocytes. Cardiomyocytes from spinophilin knock out mice revealed significantly reduced length, increased Ca^{2+} amplitude as well as maximal rate of Ca^{2+} rise during systole, decreased shortening amplitude and maximal rate of shortening, while β-adrenergic stimulation remained intact (Petzhold et al., 2011). Mechanistic studies suggested that spinophilin-mediated PP1 targeting to RyR2 and Phospholamban lead to site-specific enhanced dephosphorylation, thus evoking the phenotypic consequences described in the latter study. Very recently the group around Xander Wehrens transferred this knowledge into better understanding of disease mechanisms in atrial fibrillation putatively underlying deregulated PP1/spinophilin interactions and subsequent hyperactivation of RyR2 (Chiang et al., 2014). In detail they found that knockout of sphinophilin-1 resulted in strongly reduced interaction of PP1 and RyR2, while RyR2 phosphorylation was significantly increased at serine (Ser) 2814 but unchanged at Ser2808. As a result RyR2s from spinophilin-1 KO mice showed the expected increase in RyR2 open probability in lipid bilayer experiments, increased Ca^{2+} spark frequency in isolated atrial myocytes, which could be reverted by CaMKII inhibition, and most importantly increased atrial ectopy and susceptibility to pacing-induced atrial fibrillation *in vivo*, which could be reverted by crossing in phosphorylation-dead RyR2-Ser2814A mice (Chiang et al., 2014). In summary this reports showed the first time that decreased local PP1 regulation of RyR2 contributes to RyR2 hyperactivity and promotion of atrial fibrillation susceptibility. This milestone study may set the basis for more exact examination of localized PP activity in CVDs, thus enabling novel drug targeting strategies in the future.

The last chapter of PP-dependent PK activity regulation will be opened in the upcoming section of this review. Hereby direct activity control of PKs via PP-mediated dephosphorylation will be discussed in light of a rapid molecular mechanism to achieve direct feedback control of PK activity.

DIRECT CONTROL OF PROTEIN KINASE EXPRESSION AND ACTIVITY BY PROTEIN PHOSPHATASES

It is a basic principle that PKs themselves are activated by (auto-)phosphorylation (Ubersax and Ferrell, 2007). In order to avoid excessive PK activation it is necessary that in most cases PK phosphorylation has to be reversible. Indeed several examples (see also **Table 1**) show direct PP-mediated dephosphorylation of PKs in cardiac cells. However, PP-mediated dephosphorylation of two of the most abundant and essential PKs in cardiac myocytes, namely PKA and CaMKII has only be studied in non-cardiac tissues until today (Blitzer et al., 1998; Bradshaw et al., 2003; Humphries et al., 2005; Otmakhov et al., 2015).

Dephosphorylation of PKA at position Thr-197 by PP2A was shown *in vitro* and *in vivo*. Interestingly, dephosphorylation activity strongly depends on oxidative state, which is mechanistically mediated via the neighboring cysteine 199 (Humphries et al., 2005). CaMKII is auto-activated at position Thr286 and can be dephosphorylated by PP1 and PP2B (Reese et al., 2011). In this case the dephosphorylation activity in dendritic spines is calcium dependent. It is tempting to speculate that PKA as well as CaMKII are also direct substrates of PP-mediated dephosphorylation in cardiac physiology and especially in CVDs, thus predisposing further studies to clarify this issue.

In addition, there is yet another possibility of more or less direct control of PK activity by PP mediated initiation of alternative splicing in cardiac myocytes. The best studied example in this case is the CaMKIIδ-isoform, which plays an important role during evolvement of heart failure and cardiac arrhythmias (Gray and Heller Brown, 2014). Over- or deregulated expression of single splicing variants of CaMKIIδ are sufficient to induce cardiac hypertrophy, dilated cardiomyopathy and subsequent heart failure in experimental animal models (Erickson, 2014). Two studies have shown that reversible phosphorylation of the splicing factor SF2/ASF by PKA and PP1 is able to change the splicing pattern of CaMKIIδ and subsequently trigger functional consequences on typical CaMKII substrates such as PLB (Gu et al., 2011; Huang et al., 2014).

As described before Histones, Histone Deacetylases as well as transcription and translation factors have also been identified as PP substrates (Backs et al., 2006; Heijman et al., 2013; Kreusser and Backs, 2014). All these components are necessary prerequisites for (epi-)genetic control of PK activity. Finally, as CaMKIIδ is by far not the only cardiac PK undergoing alternative splicing it will be worth to examine this novel way of PP-mediated PK regulation in more detail in the future. Regulation of alternative splicing in cardiac myocytes has emerged as a highly innovative

and novel drug target in multiple studies and would be interesting to better understand the contribution of PPs in this regard. This conclusion closes the gap toward the final chapter of this review, which discusses the current state-of-the-art knowledge regarding pharmacological PP activity control that may serve as an additional or synergistic therapeutic approach toward pharmacological compounds targeting PKs.

THERAPEUTIC PERSPECTIVE

The widespread tasks of PP1, PP2A, and PP2B in healthy and diseased described in the chapter above, predispose the PPs as novel drug targets. In order to receive similar attention as kinase inhibitors, there are however still some challenges that have to be tackled.

The most straightforward approach to block the activity of an enzyme is the design of small molecules (SMOLs) which bind to the active site of the enzyme, thus blocking access for their endogenous substrates. Due to the very high structural conservation in PP1's catalytic site with that of PP2A, it has remained difficult to specifically target the active site of either phosphatase (Terrak et al., 2004; Cho and Xu, 2007). Nevertheless, some natural toxins such as calyculin, cantharidin, tautomycin and okadaic acid did reveal subtle selectivity between PP1 and PP2A *in vitro* and have thus been employed the last decades to study the role of PP1 and PP2A in cellular signaling processes (Hescheler et al., 1988; Aggen et al., 1999; Fagerholm et al., 2010). Even better selectivity is achieved by the endogenous inhibitors of PP1 and PP2A. As an example the heat stable endogenous protein inhibitors Inhibitor-1 as well as Inhibitor-2 specifically inhibit PP1 at very low nanomolar concentrations without affecting the any other known serine/threonine phosphatase (Kwon et al., 1997). The usefulness of the latter inhibitors in functional assays or as drug leads is limited by the size, availability, stability as well as the cellular permeability of these proteins, but they could nevertheless be used as starting points for peptide-based drug design (Chatterjee and Kohn, 2013). Due to the multitude of phosphatase substrates direct and solely targeting of a single phosphatase catalytic subunit will putatively be insufficient or induce serious toxic side effects so further strategies rely on either disrupting the interaction between (a) the catalytic and respective regulatory subunit or (b) the regulatory subunit and the respective substrate. As a proof-of-concept specific modulators of PP1 (and PP2A) holoenzymes have been developed not only for the treatment of CVDs but also for the treatment of diabetes, Parkinson's disease and drug addiction (Uehata et al., 1997; Armstrong et al., 1998; McConnell and Wadzinski, 2009; Yger and Girault, 2011). Concrete examples are disruption of the PP1/GADD34 complex by salubrinal or guanabenz or I-1/I-2 overexpression in rodent/pig models of myocardial infarction (Boyce et al., 2005; Tsaytler et al., 2011; Liu et al., 2012; Neuber et al., 2014). Moreover synthetic peptides containing the PP1c-binding motif (RVxF) have been successfully used to disrupt PP1 complexes in a neuronal and cardiac context, inducing beneficial effects on synaptic transmission and PLB phosphorylation (Reither et al., 2013; Sotoud et al., 2015). Thus, clinical targeting of PPs side-by-side with PK inhibition may represent a promising opportunity to treat CVDs in the near future.

Last but not least we propose two novel strategies to further enhance the feasibility of PP targeting drugs in CVDs: 1. Engaging subcellular and isotype-specific control of PPs: a recent review has highlighted the isotype-specific interaction of PP1 with different regulatory subunits and inhibitors. Combined with compartment-specific PP1 targeting this knowledge might lead to the development of really specific PP targeting drugs. 2. Combination of PK inhibitor and Phosphatase activating drugs and *vice versa*: in order to increase potency of currently used drugs, exact knowledge about the contribution of kinases and phosphatases during reversible protein phosphorylation of a specific substrate may help to develop novel and successful combination therapies—similar to the success story of LCZ-696/Entresto (Gu et al., 2010; Solomon et al., 2012; Jessup et al., 2014; Desai et al., 2015).

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