



# PACAP-38 and PACAP(6–38) Degranulate Rat Meningeal Mast Cells *via* the Orphan MrgB<sub>3</sub>-Receptor

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**Received:** 27 December 2018

**Accepted:** 08 March 2019

**Published:** 28 March 2019

### Citation:

Pedersen SH, la Cour SH, Calloe K, Hauser F, Olesen J, Klaerke DA and Jansen-Olesen I (2019) PACAP-38 and PACAP(6–38) Degranulate Rat Meningeal Mast Cells *via* the Orphan MrgB<sub>3</sub>-Receptor. *Front. Cell. Neurosci.* 13:114. doi: 10.3389/fncel.2019.00114

Infusion of pituitary adenylate cyclase activating peptide-38 (PACAP-38) provokes migraine attacks in migraineurs and headache in non-migraineurs. Adverse events like long-lasting flushing and heat sensation can be terminated with oral antihistamine treatment, indicating the involvement of mast cell activation after PACAP-infusion. Degranulation of rat peritoneal mast cells was provoked by several isoforms of PACAP *via* previously unknown receptor pharmacology. The effect might thus be mediated either *via* specific splice variants of the PAC<sub>1</sub>-receptor or *via* an unknown receptor for PACAP-38. In the present study, we characterize degranulation of rat meningeal mast cells in response to PACAP-receptor ligands. Furthermore, we investigate if PACAP-38-induced mast cell degranulation is mediated *via* PAC<sub>1</sub>-receptor splice variants and/or *via* the orphan Mas-related G-protein coupled member B3 (MrgB<sub>3</sub>)-receptor. To address this, the pharmacological effect of different PACAP isoforms on meningeal mast cell degranulation was investigated in the hemisected skull model after toluidine blue staining followed by microscopic quantification. Presence of mRNA encoding PAC<sub>1</sub>-receptor splice variants and the MrgB<sub>3</sub>-receptor in rat mast cells was investigated by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) analysis. The effect of PACAP isoforms on PAC<sub>1</sub>- and MrgB<sub>3</sub>-receptor-expressing *Xenopus laevis* oocytes were performed by two-electrode voltage-clamp (TEVC) electrophysiology. PACAP-38 is a more potent mast cell degranulating agent than Pituitary Adenylate Cyclase Activating Peptide-27 (PACAP-27) in the meninges. Presence of mRNA encoding the PAC<sub>1</sub>-receptor and its different splice variants could not be detected in peritoneal mast cells by RT-PCR, whereas the orphan MrgB<sub>3</sub>-receptor, recently suggested to be a mediator of basic secretagogues-induced mast cell degranulation, was widely present. In PAC<sub>1</sub>-receptor-expressing *Xenopus laevis* oocytes both PACAP-38, PACAP-27 and the specific PAC<sub>1</sub>-receptor agonist maxadilan were equipotent, however, only PACAP-38

**Abbreviations:** BSA, Bovine Serum Albumin; MrgB3, Mas-related G-protein coupled receptor member B3; MrgX2, Mas-related G-protein coupled receptor member X2; PACAP-27, Pituitary Adenylate Cyclase Activating Peptide-27; PACAP-38, Pituitary Adenylate Cyclase Activating Peptide-38; PACAP(6–38), Pituitary Adenylate Cyclase Activating Peptide(6–38); PLC, Phospholipase C; RT-PCR, Reverse Transcriptase-Polymerase Chain Reaction; TEVC, Two-Electrode Voltage Clamp; VIP, Vasoactive Intestinal Peptide.

showed a significant degranulatory effect on mast cells. We confirmed Pituitary Adenylate Cyclase Activating Peptide(6–38) [PACAP(6–38)] to be a PAC<sub>1</sub>-receptor antagonist, and we demonstrated that it is a potent mast cell degranulator and have an agonistic effect on MrgB<sub>3</sub>-receptors expressed in oocytes. The present study provides evidence that PACAP-induced mast cell degranulation in rat is mediated through a putative new PACAP-receptor with the order of potency being: PACAP-38 = PACAP(6–38) >> PACAP-27 = maxadilan. The results suggest that the observed responses are mediated *via* the orphan MrgB<sub>3</sub>-receptor.

**Keywords:** migraine, pituitary adenylate cyclase activating peptide, dura mater, *Xenopus laevis* oocytes, mast cell, Mas-related G-protein coupled receptor member B3, PAC<sub>1</sub>-receptor, two-electrode voltage clamp

## INTRODUCTION

Pituitary adenylate cyclase-activating peptide-38 (PACAP-38) is a 38-amino acid neuropeptide located in both sensory and parasympathetic perivascular nerve fibers (Møller et al., 1993; Mulder et al., 1994). A C-terminal truncated 27-amino acid (PACAP-27) version is endogenously present as well but is less abundant (Miyata et al., 1990; Arimura et al., 1991; Ogi et al., 1993). A 20-min intravenous infusion of PACAP-38 provokes migraine attacks in migraine patients as well as headache in non-migraineurs (Schytz et al., 2009). At present, three PACAP-receptors have been identified: PAC<sub>1</sub>, VPAC<sub>1</sub> and VPAC<sub>2</sub>. The neurotransmitter vasoactive intestinal peptide (VIP) shares high amino acid sequence homology with PACAP and its affinity to VPAC<sub>1</sub> and VPAC<sub>2</sub> equals that of PACAP (Spengler et al., 1993; Pantaloni et al., 1996) whereas binding to the PAC<sub>1</sub>-receptor is 1,000 times lower (Miyata et al., 1989, 1990; Harmar et al., 1998). Interestingly, VIP only induces a mild headache and no migraine-like attacks in migraineurs (Rahmann et al., 2008), which leads to the suggestion that PACAP and the PAC<sub>1</sub>-receptor are key targets for future migraine treatment.

Infusion of PACAP-38 caused not only migraine attacks but also heat sensation and long-lasting flushing (Schytz et al., 2009). This is in line with PACAP-38 being a mast cell degranulator and mast cells have been suggested to play a role in migraine pathogenesis (Moskowitz, 1993; Levy et al., 2006, 2007). Degranulation of mast cells can be induced either by an allergen-IgE-dependent mechanism or *via* an IgE-independent mechanism. The latter mechanism can be activated by a group of molecules known as basic secretagogues. These molecules only share one physicochemical nature, their cationic property (Ferry et al., 2002). Several of these molecules are endogenous peptides and high concentrations are required for initiation of mast cell degranulation, an effect that involves pertussis toxin-sensitive G-proteins coupled to phospholipase C (PLC) activation (Ferry et al., 2002).

Inspired by clinical findings, we have previously characterized the degranulating effect of various PACAP-analogues on isolated rat peritoneal mast cells. Based on the expectation that degranulation is mediated through the PAC<sub>1</sub>-receptor, we found an unpredicted order of potency (Baun et al., 2012). In peritoneal mast cells, the PAC<sub>1</sub>-receptor antagonist Pituitary Adenylate

Cyclase Activating Peptide(6–38) [PACAP(6–38)] caused mast cell degranulation that was as potent as PACAP-38 (Robberecht et al., 1992; Baun et al., 2012). Furthermore, the PAC<sub>1</sub>-receptor agonist maxadilan was ineffective (Baun et al., 2012).

Several PAC<sub>1</sub>-receptor splice variants have been cloned and characterized by ligand binding and signal transduction (Spengler et al., 1993; Pantaloni et al., 1996; Pisegna and Wank, 1996; Dautzenberg et al., 1999; Lutz et al., 2006). In 2006, Tatemoto et al. (2006) found the Mas-related G-protein coupled receptor member X<sub>2</sub> (MrgX<sub>2</sub>) to be present in human mast cells. Mast cell degranulation induced by basic secretagogues appeared in the same concentrations as responses found in MrgX<sub>2</sub>-expressing cells. The rat counterpart of MrgX<sub>2</sub> was found to be the Mas-related G-protein coupled receptor member B<sub>3</sub> (MrgB<sub>3</sub>; Tatemoto et al., 2006). In the present study, we hypothesized that PACAP mediated degranulation by rat peritoneal and dural mast cells were either caused by a splice variant of the PAC<sub>1</sub>-receptor or *via* MrgB<sub>3</sub>-receptors.

## MATERIALS AND METHODS

### Animals

A total of 115 male Sprague-Dawley rats weighing 320–440 g (Taconic Europe, Ejby, Denmark) were used in this study. The rats were group-housed under a 12-h light/dark cycle and allowed *ad libitum* access to a standard rodent diet and water. All rats were euthanized by inhalation of a CO<sub>2</sub>/O<sub>2</sub>-mixture followed by CO<sub>2</sub> asphyxiation. Experimental procedures were approved by the National Danish Animal Experiments Inspectorate (License number 2014-15-0201-00256) and carried out in accordance with Danish legislation.

### Mast Cell Degranulation in Hemi-skull Preparations

Mast cell degranulation was performed as previously described (Pedersen et al., 2015). In brief, skulls were cut mid-sagittal and the brain halves were removed, leaving the dura mater undisturbed. This was followed by immediate addition of either 350 μl 0.1, 1 or 10 μM PACAP-38 (custom synthesis by Caslo Laboratory ApS, Lyngby, Denmark) or vehicle (saline) in phosphate buffered saline (PBS). After 30 s incubation, the reactions were terminated, and the skulls were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS);

**TABLE 1** | Primers designed for detection of the housekeeping gene  $\beta$ -actin, the N-terminal part and the hip-hop variants of the PAC<sub>1</sub>-receptor and the MrgB<sub>3</sub>-receptor.

Primer	Forward (5' → 3')	Reverse (5' → 3')	Product size
$\beta$ -actin	tca aca ccc cag cca tgt acg	cag gaa gga agg ctg gaa gag	422 bp
N-terminal	tct gac tgc atc ttc aag aag	acc gac agg tag taa taa tcc	392 bp
Hip + Hop	ctt gta cag aag ctg cag tcc cca gac atg	cag gtg ctt gaa gtc cat agt gaa gta acg gtt cac ctt	471 bp / 387 bp
MrgB <sub>3</sub>	ccc ctg gaa tgt tct ttt gtg tag	aca gtg aaa aat gca gga act tog	259 bp

Each set of forward and reverse primer was designed in Primer3 (Broad Institute) against the *Adcyap1r1* gene (NM\_001270582.1), *RGD1560730* (XM\_006229262.3) encoding the MrgB<sub>3</sub>-receptor, and  $\beta$ -actin and tested for specificity by BLAST alignment tool (NCBI). The sizes of the amplified sequences are shown as base pair (bp).

Glostrup Hospital Pharmacy, Denmark). The dura mater was dissected from the skull, whole mounted on slides, and mast cells were visualized by staining with 0.1% acidified toluidine blue (Sigma Aldrich, Germany). Tissues were dehydrated in graded alcohols prior to cover slip mounting. The level of mast cell degranulation was evaluated by 400 $\times$  magnification (Nikon Eclipse Ni microscope) by a researcher blinded to the treatment and was counted in 10 consecutive fields along the stem part of the middle meningeal artery. Mast cells were considered degranulated if an extensive dispersion of more than 10 extruded granules were localized outside the cell or if an extensive loss of staining gave the cell a “ghostly” look.

## Peritoneal Mast Cell Isolation and RNA-Extraction

Peritoneal mast cells were harvested from three rats by injecting 20 ml oxygenated buffer (137 mM NaCl, 2.7 mM KCl, 1 mM MgCl<sub>2</sub>, 0.5 mM CaCl<sub>2</sub>, 0.4 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM HEPES and 5.6 mM glucose, pH 7.6) in the peritoneal cavity of asphyxiated rats. The cavity was then gently massaged and subsequently opened by midline incision for the lavage to be removed by pipetting. Cells were washed three times by sedimentation at 13°C by a 7 min centrifugation at 400 g. The pellet was re-suspended in 5 ml oxygenated buffer and layered on top of a BSA-Percoll<sup>®</sup> density gradient (81%) containing 162  $\mu$ l 35% bovine serum albumin (BSA), 8.1 ml Percoll<sup>®</sup> (GE Healthcare, Buckinghamshire, UK), 580  $\mu$ l distilled water and 1.16 ml salt solution (1.54 M NaCl, 27 mM KCl, 3.8 mM CaCl<sub>2</sub>). Cell types were separated by centrifugation at 225 g for 25 min at 13°C. The density gradient was discarded, and the pellet was again washed three times. The purity of mast cells was determined by histological characterization of the percentwise mast cell fraction. Only samples with a purity >90% were used for further analysis. Peritoneal mast cell RNA was extracted using the Isolation of Small and Large RNA Kit (Macherey-Nagel, Germany) in combination with TRIzol<sup>®</sup> (Qiagen) according to manufacturer's recommendations.

## Reverse Transcriptase-Polymerase Chain Reaction

cDNA was synthesized from 500 ng peritoneal mast cell RNA using the iScript cDNA Synthesis kit (BioRad) according to instructions. PAC<sub>1</sub>-receptor splice variants were identified in peritoneal mast cells and spinal cord using HotStarTaq<sup>®</sup> DNA polymerase (QIAGEN) with 10  $\mu$ M primer. The MrgB<sub>3</sub>-receptor was only tested in mast cell RNA. Primers

against the *Adcyap1r1* gene (NM\_001270582.1, encoding the PAC<sub>1</sub>-receptor), *RGD1560730* (XM\_006229262.3, encoding the MrgB<sub>3</sub>-receptor), and  $\beta$ -actin were designed in Primer3 (Broad Institute) and tested for specificity by BLAST alignment tool (NCBI) and ordered from DNA Technology, Aarhus, Denmark (Table 1). PAC<sub>1</sub>-primers were specifically designed to span the extracellular N-terminal (exon 3 to exon 8), exon 14 (known as “hip”) and exon 15 (known as “hop”).  $\beta$ -actin was included as a positive control. The amplification protocol was as follows: initial heat activation at 95°C for 15 min, followed by 45 cycles with denaturation at 95°C for 1 min, annealing at variable temperatures depends on the primer set (50°C for  $\beta$ -actin and N-terminal, 56°C for MrgB<sub>3</sub> and 66°C for Hip + Hop) for 1 min, and extension at 72°C for 1 min; final extension at 72°C for 10 min. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) products were visualized by agarose gel electrophoresis.

## In vitro Transcription

DNA of the coding region of *Rattus Norvegicus* *Adcyap1r1* variant 5 (NM\_001270582.1) encoding the null-splice variant (short N-terminal and neither exon 14 or 15) of the PAC<sub>1</sub>-receptor (Vector: EX-Rn10199-M03) was ordered from GeneCopoeia. DNA encoding the *RGD1560730* gene (XM\_006229262.3, GenScript), was cloned into the pXOOM vector as previously described (Jespersen et al., 2002). DNA was purified using Plasmid DNA Purification NucleoBond Xtra Midi-kit (Macherey-Nagel). The inserts were fully sequenced (MWG Operon) to confirm the expected sequence (data not shown). Extracted plasmids were linearized down-stream the poly(A) segment using the *XhoI* restriction enzyme (New England Biolabs, Ipswich, MA, USA). RNA was *in vitro* transcribed by synthetization from the T7 RNA polymerase promoter using the mMessenger mMachine kit (Ambion) according to manufacturer's protocol. Messenger RNA was purified using the MEGAclean kit (Ambion). Transcribed RNA integrity was assessed by agarose gel electrophoresis.

## Expression in *Xenopus laevis* Oocytes and Two-Electrode Voltage Clamp

Stage V and VI defolliculated *Xenopus laevis* oocytes were purchased from EcoCyte Bioscience (Dortmund, Germany) and kept in Kulori medium (90 mM NaCl, 4 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, and 5 mM Hepes, pH 7.4). Oocytes were micro-injected with 50 nl mRNA solution containing 30 ng RNA per oocyte and incubated at 19°C. Currents were

**TABLE 2** | The number of degranulated meningeal mast cells in % of total number of mast cells after 30 s incubation with either vehicle, PACAP-38 or PACAP-27 in concentrations ranging from 0.1 to 10  $\mu$ M.

Conc. ( $\mu$ M)	Number of exp.	Vehicle to PACAP-38 (% degranulation)	PACAP-38 (% degranulation)	Vehicle to PACAP-27 (% degranulation)	PACAP-27 (% degranulation)
0.1	5	8.5 $\pm$ 3.0	12.4 $\pm$ 3.8	4.7 $\pm$ 1.9	7.1 $\pm$ 3.2
1.0	6	7.2 $\pm$ 1.3	17.9 $\pm$ 3.7	7.2 $\pm$ 2.0	16.9 $\pm$ 5.5
10.0	6	6.2 $\pm$ 1.7	48.4 $\pm$ 6.2****	5.9 $\pm$ 1.3	12.1 $\pm$ 3.8

Values are presented as means  $\pm$  SEM. Statistical analysis (Two-way ANOVA followed by Sidak's test) was performed to find significant differences in mast cell degranulation induced by each substance in each concentration. Significance is given as \*\*\*\* $p < 0.0001$ .

measured after 2–5 days using a conventional two-electrode-voltage clamp (TEVC). The oocytes were placed in a 200  $\mu$ l chamber and continuously exposed to a flow of Kulori medium with or without ligands (1 ml/min) while impaled with both a current and a voltage electrode filled with 3 M KCl and connected to an Oocyte Clamp Amplifier [Warner Instruments Corp. (OC-725 B) and a PC-interface (Digidata1440A, Molecular Devices)]. Current amplitude in absence or presence of ligands were analyzed using pClamp 10.2 software (Molecular Devices). All experiments were performed with oocyte membrane voltages constantly clamped to  $-70$  mV and the temperature was kept between 19 and 22°C. In activation experiments, non-responding as well as low-responding (<10 nA) oocytes were excluded from the dataset. Ligands PACAP-38, PACAP-27, PACAP(6–38) were custom synthesized by Caslo (Lyngby, Denmark) while maxadilan was purchased from Bachem (Bubendorf, Switzerland).

Due to receptor desensitization, it was not possible to repeat measurements on individual oocytes, so only one dose could be tested per egg. Different batches of oocytes showed different expression levels and all figures are based on several batches of oocytes.

## Statistical Analysis

Concentration-response curves for both oocytes and dural mast cells were analyzed for overall effects of PACAP-38 and -27 by two-way analyses of variance (ANOVA) followed by Sidak's test for multiple pairwise comparisons. Effects of PAC<sub>1</sub>-receptor ligands on mast cell degranulation or on receptor-expressing oocytes were analyzed with a one-way ANOVA followed by Tukey's multiple comparisons test. The effect of PACAP-38 and PACAP(6–38) on MrgB<sub>3</sub>-expressing oocytes were analyzed by an unpaired two-tailed *t*-test. Differences between groups were considered significant when  $p < 0.05$  and data are presented as mean with standard error of the mean ( $\pm$ SEM). GraphPad Prism 7 (GraphPad Prism Software, San Diego, CA, USA) was used for statistical analysis.

## RESULTS

### Effects of PACAP-38 and PACAP-27 on Dura Mast Cell Degranulation

PACAP-provoked mast cell degranulation was characterized by stimulating the dura mater with PACAP-38, PACAP-27 or saline in concentrations ranging from 0.1 to 10  $\mu$ M ( $n = 5$ –6; **Table 2**). PACAP-38 stimulation resulted in an

eight-fold increase and highly significant mast cell degranulation at the 10  $\mu$ M concentration ( $p < 0.0001$ ). However, even at the highest tested concentration, PACAP-27 did not induce mast cell degranulation that was significantly different from saline treatment.

### Splice Variants of PAC<sub>1</sub>-Receptor in Rat Peritoneal Mast Cells

Because mast cell degranulation induced by increasing concentrations of the different PACAP-isoforms did not follow the known order of potencies for PAC<sub>1</sub>-, VPAC<sub>1</sub>- or VPAC<sub>2</sub>-receptors, we investigated the presence of PAC<sub>1</sub>-receptor mRNA and possible splice variants by using RT-PCR (**Figure 1**). As a positive control, we included rat spinal cord tissue in which the PAC<sub>1</sub>-receptor previously has been localized (Dickinson et al., 1999). We performed RT-PCR analysis targeted to several areas involved in splice variation of the PAC<sub>1</sub>-receptor (N-terminal, exon 14 and exon 15) and found the PAC<sub>1</sub>-receptor to be absent in mast cells and present in spinal cord (**Figure 1**). The lack of PAC<sub>1</sub>-receptor mRNA expression in rat peritoneal mast cells indicates mast cell degranulation to be mediated *via* a non-PAC<sub>1</sub>-receptor.

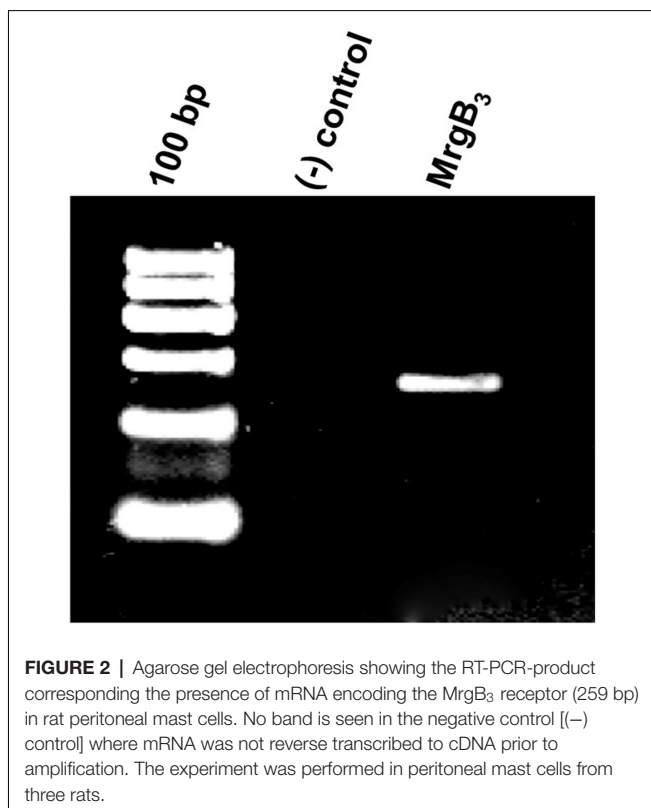
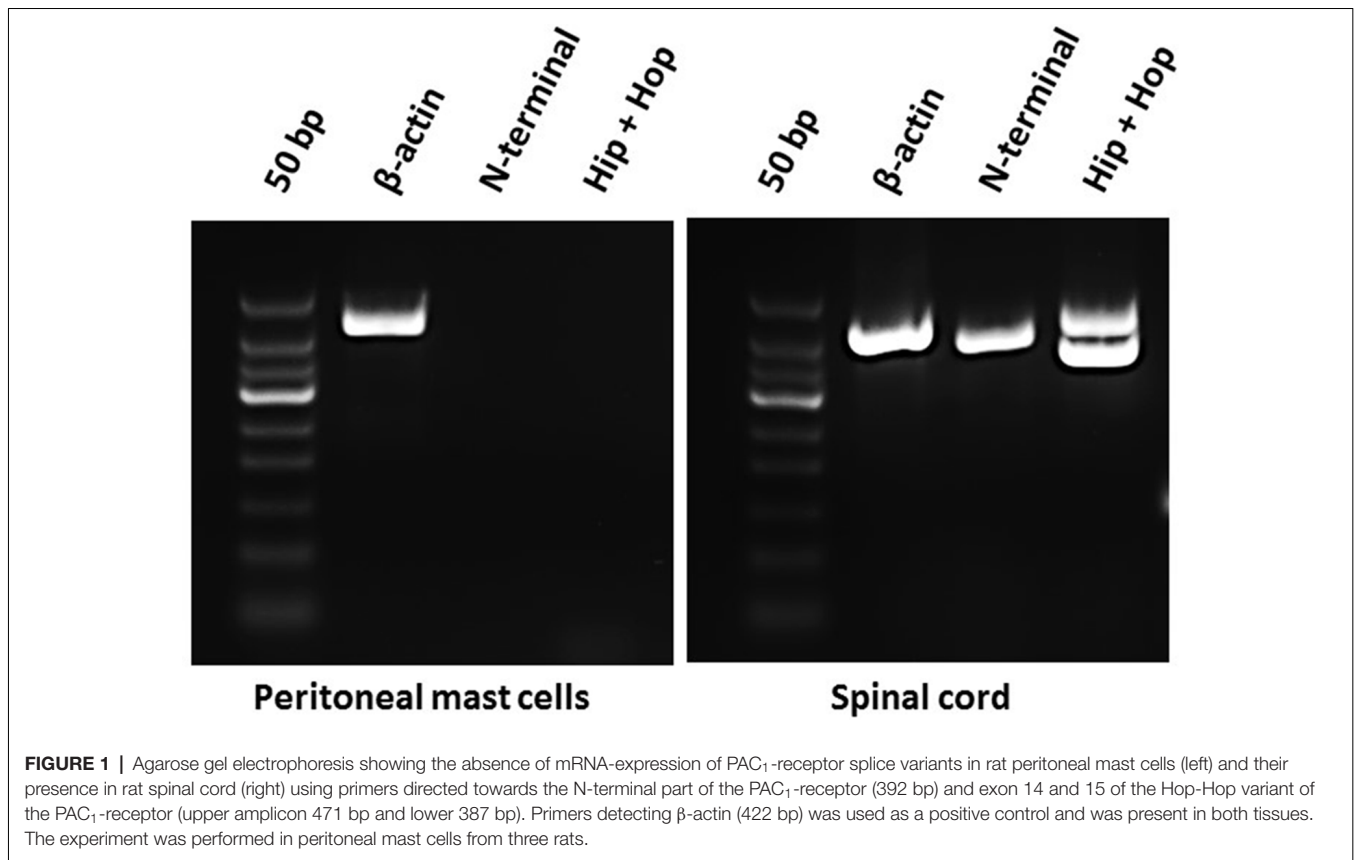
### mRNA Expression of MrgB<sub>3</sub>-Receptor in Rat Peritoneal Mast Cells

The PAC<sub>1</sub>-receptor was not expressed in rat peritoneal mast cells. Thus, we investigated a possible expression of the MrgB<sub>3</sub>-receptor as previously shown (Tatemoto et al., 2006). Using primers directed towards MrgB<sub>3</sub>-receptor mRNA, we found it to be expressed in rat peritoneal mast cells (**Figure 2**). Therefore, we decided to study the effect of PACAP isoforms on MrgB<sub>3</sub>-receptors and to compare the pharmacological profile on the PAC<sub>1</sub>-receptor using TEVC in the *Xenopus laevis* oocyte expression system.

### Effects of PACAP-38 and -27 on PAC<sub>1</sub>- and MrgB<sub>3</sub>-Receptors Expressed in *Xenopus laevis* Oocytes

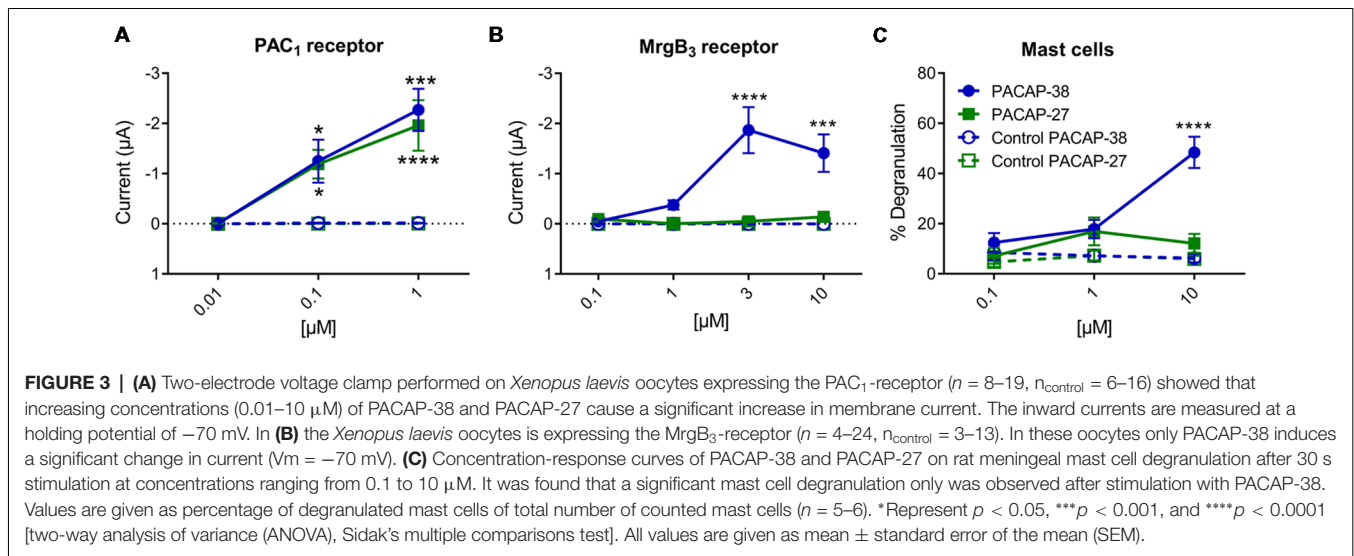
Effects of PACAP-38 and PACAP-27 on PAC<sub>1</sub>- and MrgB<sub>3</sub>-receptors expressed in *Xenopus laevis* oocytes were investigated by TEVC. Upon addition of PACAP-38 and/or PACAP-27 at concentrations ranging from 0.01 to 1  $\mu$ M to PAC<sub>1</sub>-receptor-expressing *Xenopus laevis* oocytes, a rapid concentration-dependent inward current was observed (**Figure 3A**) consistent with activation of an endogenous Cl<sup>-</sup> current following receptor activation. These findings confirmed the aforementioned studies





of PACAP-38 and PACAP-27 being equipotent on the PAC<sub>1</sub>-receptor (Shivers et al., 1991; Pisegna and Wank, 1993). At 0.1 μM, PACAP-38 and PACAP-27-induced currents of  $-0.93 \pm 3 \mu\text{A}$  ( $n = 19$ ) and  $-1.04 \pm 3 \mu\text{A}$  ( $n = 15$ ), respectively. Stimulation with 1 μM of PACAP-38 and PACAP-27 resulted in currents of  $-2.27 \pm 4 \mu\text{A}$  ( $n = 15$ ) and  $-1.96 \pm 5 \mu\text{A}$  ( $n = 13$ ), respectively.

In MrgB<sub>3</sub>-receptor-expressing oocytes, PACAP-38, but not PACAP-27, induced a rapid concentration-dependent inward current in the concentration range of 1–10 μM ( $n = 3$ –24). The maximum current induced by PACAP-38 was  $-1.87 \pm 5 \mu\text{A}$  ( $n = 12$ ) at 3 μM (Figure 3B). The maximum response to PACAP-27 was found at 10 μM resulting in a current of  $-0.14 \pm 0.4 \mu\text{A}$  ( $n = 10$ ), which was not significantly different from baseline. These rapid responses were not seen in un-injected oocytes. In concentrations between 0.1 and 3 μM no effect was observed in un-injected oocytes, indicating that *Xenopus laevis* oocytes do not endogenously express PAC<sub>1</sub>- or MrgB<sub>3</sub>-receptors. However, concentrations at 10 μM PACAP-38 (15 out of 19 oocytes) but not PACAP-27 occasionally caused a delayed long-lasting response in un-injected oocytes, which was distinct from the above described fast responses. Thus, 10 μM PACAP-38 was not included in the experiments. Taken together, expression of the PAC<sub>1</sub>-receptor and the MrgB<sub>3</sub>-receptor in *Xenopus laevis* oocytes shows that PAC<sub>1</sub> is activated by PACAP-27 as well as PACAP-38, whereas the MrgB<sub>3</sub>-receptor is activated by PACAP-38 only. Thus, the activation profile



found for MrgB<sub>3</sub> resembles the effects of ligands observed for mast cell degranulation (Figure 3C). Taken together, mast cell degranulation induced by PACAP-38 and PACAP-27 in the meninges resembled mostly the current changes evoked in MrgB<sub>3</sub>-receptor transfected oocytes.

## Pharmacological Characterization of MrgB<sub>3</sub>- and PAC<sub>1</sub>-Receptor-Expressing Oocytes as Compared to Mast Cell Degranulation

### Effect of PAC<sub>1</sub>-Receptor Agonist Maxadilan on MrgB<sub>3</sub>- and PAC<sub>1</sub>-Receptors

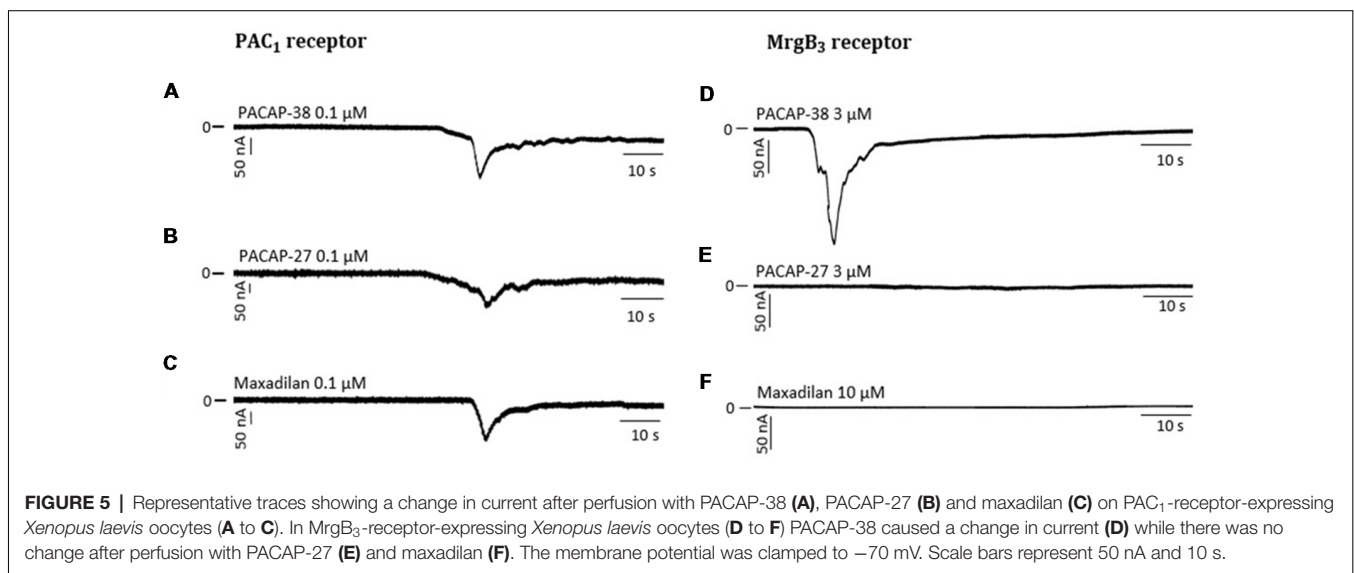
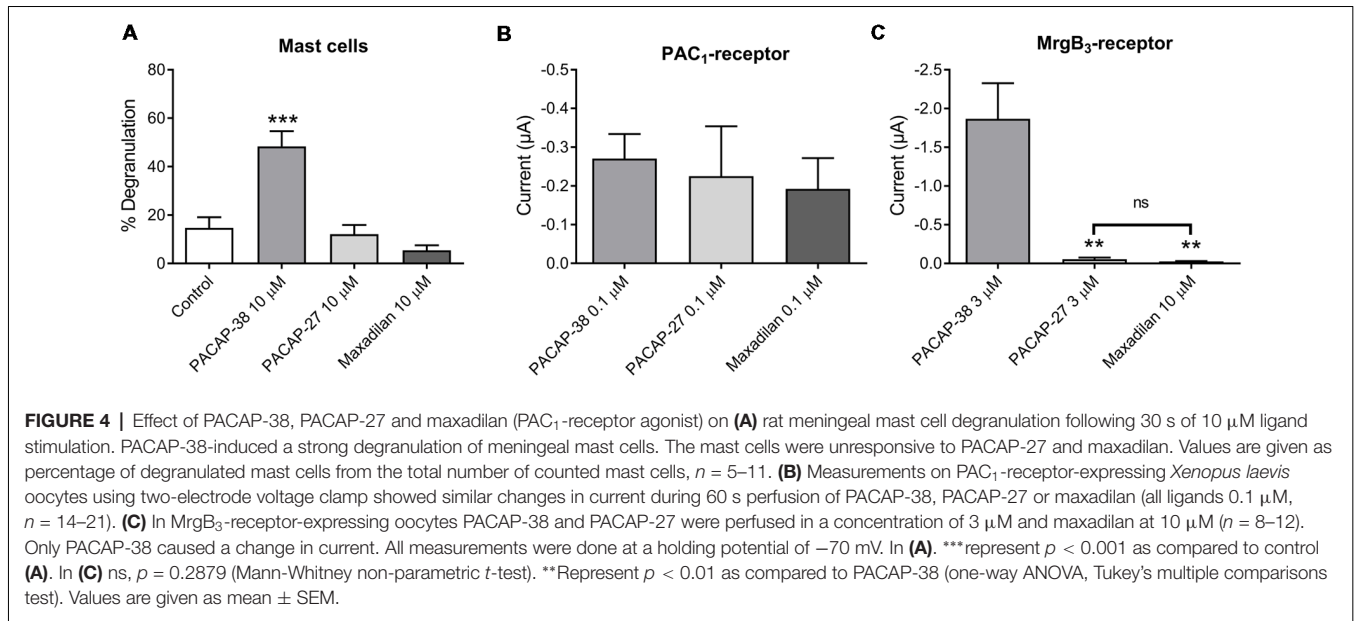
Next, we characterized mast cell degranulation in response to the specific PAC<sub>1</sub>-receptor agonist maxadilan. We found that neither PACAP-27 nor maxadilan caused mast cell degranulation ( $12 \pm 4\%$ ,  $n = 6$  and  $5 \pm 2\%$ ,  $n = 5$ , respectively) despite the high concentration (10  $\mu\text{M}$ ) tested (Figure 4A). When we compared the potencies of the same agonists in a relevant concentration (0.1  $\mu\text{M}$ ) in PAC<sub>1</sub>-expressing *Xenopus laevis* oocytes, we observed that PACAP-27 and maxadilan induced changes in currents of  $-0.26 \pm 1 \mu\text{A}$  ( $n = 14$ ) and  $-0.21 \pm 0.8 \mu\text{A}$  ( $n = 15$ ), respectively. These responses were not significantly different ( $p = 0.8669$ ) from currents observed after application of PACAP-38 ( $-0.27 \pm 0.6 \mu\text{A}$ ,  $n = 21$ ; Figures 4B, 5A–C). In MrgB<sub>3</sub>-expressing oocytes, 3  $\mu\text{M}$  PACAP-38 caused currents of  $-1.87 \pm 5 \mu\text{A}$  ( $n = 12$ ) that was significantly stronger than the effect induced by either 3  $\mu\text{M}$  PACAP-27 ( $-0.06 \pm 0.2 \mu\text{A}$ ,  $n = 8$ ) or up to 10  $\mu\text{M}$  maxadilan ( $-0.03 \pm 0 \mu\text{A}$ ,  $n = 9$ ; Figures 4C, 5D–F). Currents were not observed when PACAP-38, PACAP-27 or maxadilan were added to un-injected oocytes in the same concentrations (*data not shown*). Thus, this series of experiments conclude that maxadilan, PACAP-27 and PACAP-38 activates the PAC<sub>1</sub>-receptor with apparently equal potencies, whereas maxadilan does not activate MrgB<sub>3</sub>-receptors.

### Effect of PAC<sub>1</sub>-Receptor Antagonist PACAP(6–38) on MrgB<sub>3</sub>- and PAC<sub>1</sub>-Receptors

Pharmacological characterization of PACAP-mediated mast cell degranulation was studied using the PAC<sub>1</sub>-receptor antagonist PACAP(6–38). As previously shown in peritoneal mast cells this antagonist showed agonistic properties in meningeal mast cells by inducing a significant ( $p < 0.0001$ ) and almost complete degranulation ( $93 \pm 2\%$ ,  $n = 7$ ) when administered in a concentration of 10  $\mu\text{M}$  (Figure 6A). This response was very similar to degranulation induced by PACAP-38 ( $96 \pm 3\%$ ,  $n = 5$ ). In PAC<sub>1</sub>-receptor-expressing oocytes PACAP(6–38) in a concentration of 0.1  $\mu\text{M}$  had, as expected, no effect ( $-5 \pm 6$  nA,  $n = 9$ ). However, the effect of PACAP-38 ( $-0.18 \pm 0.5 \mu\text{A}$ ,  $n = 11$ ) was significantly antagonized when PACAP(6–38) was administered together with PACAP-38 in 0.1  $\mu\text{M}$  ( $-0.05 \pm 0.02 \mu\text{A}$ ,  $n = 10$ ; Figure 6B). In MrgB<sub>3</sub>-receptor-expressing oocytes, 3  $\mu\text{M}$  PACAP(6–38) induced a change in the current of  $-2.17 \pm 3 \mu\text{A}$  ( $n = 27$ ), which was not significantly different from the response induced by PACAP-38 ( $-1.62 \pm 4 \mu\text{A}$ ,  $n = 21$ ; Figure 6C).

## DISCUSSION

PACAP-38, but not the related peptide VIP, induces migraine headache in migraineurs suggesting the specific PACAP-receptor, PAC<sub>1</sub>, as a potential target for migraine treatment (Rahmann et al., 2008; Schytz et al., 2009). Furthermore, all participants in the clinical provocation studies experienced long-lasting flushing, especially on the face and trunk, which could be terminated by antihistamine treatment, suggesting the involvement of mast cell degranulation (Schytz et al., 2009). In a previous series of experiments performed on rat peritoneal mast cells, we found that PACAP-38, but not PACAP-27 and VIP, caused degranulation. In addition, we showed that the selective PAC<sub>1</sub>-receptor agonist maxadilan had no effect on mast cell degranulation. It was also found that



the selective PAC<sub>1</sub>-receptor antagonist, PACAP(6–38), induced a pronounced mast cell degranulation (Baun et al., 2012). Based on these observations, we suggested that the PACAP-provoked meningeal mast cell degranulation is mediated through another receptor than the PAC<sub>1</sub>-receptor (Baun et al., 2012). Our results suggest that PACAP-induced degranulation of rat peritoneal and meningeal mast cells is mediated *via* the orphan MrgB<sub>3</sub>-receptor.

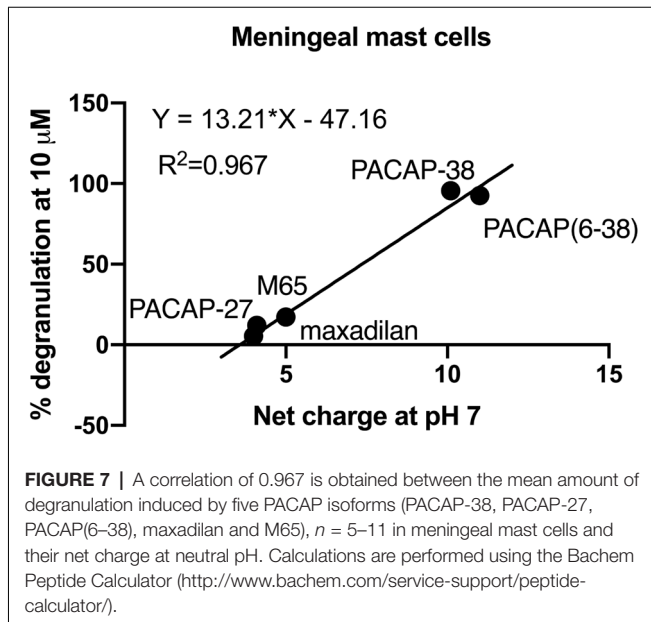
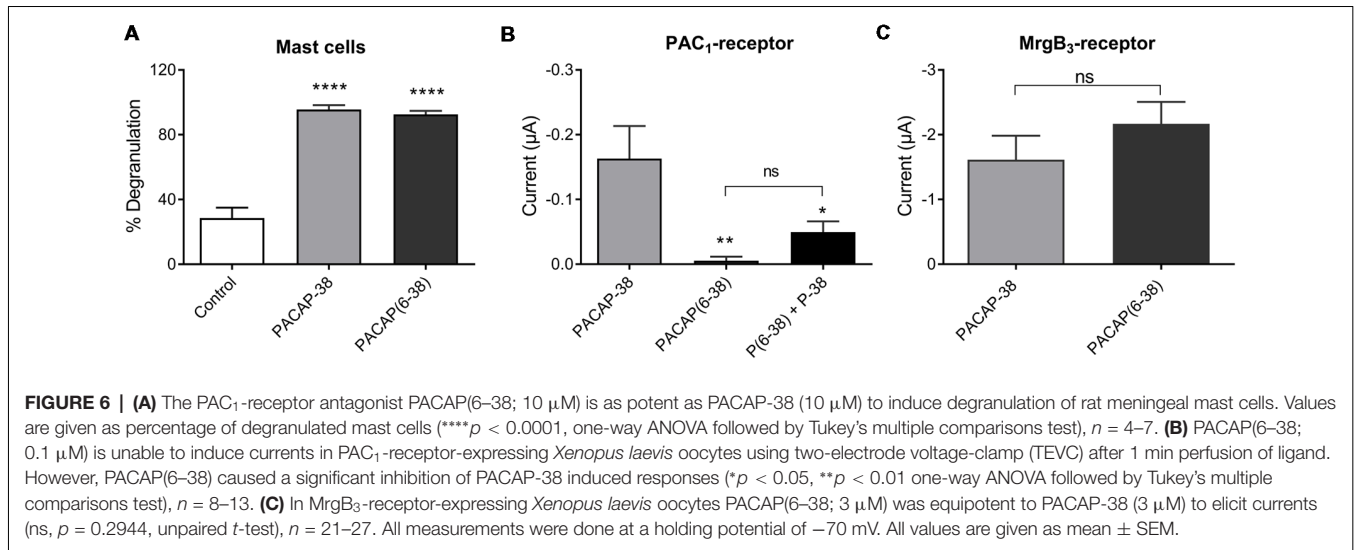
## Pharmacology of PACAP on Mast Cell Degranulation

The weak degranulating effect of VIP and PACAP-27 compared to the strong degranulation effect of PACAP-38 in rat mast cells is inconsistent with the previously reported equipotent profiles of PACAP-38, PACAP-27, and VIP on

VPAC<sub>1</sub>- and VPAC<sub>2</sub>-receptors (Harmar et al., 2012). In migraineurs, VIP does not provoke migraine headache, which suggests VPAC<sub>1</sub>- and VPAC<sub>2</sub>-receptors to be of minor importance in comparison to the PAC<sub>1</sub>-receptor (Rahmann et al., 2008). Furthermore, the PAC<sub>1</sub>-receptor antagonist PACAP(6–38) has not been shown to have an affinity to VPAC<sub>1</sub>- and VPAC<sub>2</sub>-receptors (Harmar et al., 2012). Taken together, this leads us to rule out the possible involvement of VPAC<sub>1</sub>- and VPAC<sub>2</sub>-receptors in PACAP-mediated mast cell degranulation.

## Expression of PAC<sub>1</sub>-Receptor Splice Variants

Several different splice variants of the PAC<sub>1</sub>-receptor have been identified in rats. Splice sites in the extracellular N-terminal



domain and the third intracellular loop account for fine tuning of ligand affinity and signal transduction through adenylyl cyclase or PLC activation (Deutsch and Sun, 1992; Spengler et al., 1993). The presence of a 21-amino acid domain in the extracellular N-terminal domain (PAC<sub>1</sub>-full, short) impairs PACAP-27 binding (Pantaloni et al., 1996). Hip and hop (exon 14 and 15, respectively) insertions into the third intracellular loop are suggested to modulate G-protein coupling and favor PACAP-38 induced PLC activation *via* Gq-proteins as compared to PACAP-27 (Spengler et al., 1993; Blechman and Levkowitz, 2013). This could explain the difference found in PACAP-38 and PACAP-27 provoked mast cell degranulation. We, therefore, designed primers directed towards the N-terminal part and towards exon 14 and 15. However, using the RT-PCR analysis we were unable to identify PAC<sub>1</sub>-receptor expression in

the mast cell transcriptome. To further confirm the validity of the primers, we made parallel RT-PCR experiments on mRNA from spinal cord showing the presence of both the N-terminal part and the hip-hop variants of the PAC<sub>1</sub>-receptor. Based on these findings, we suggest PACAP-38-provoked mast cell degranulation to act *via* a target distinct from PAC<sub>1</sub>-receptors.

### PACAP as a Basic Secretagogue

Degranulation of mast cells induced *via* the IgE-independent pathway is mediated by a variety of compounds collectively designated as basic secretagogues. This is a mechanism highly conserved among mammals and birds, which appoints it to be ancient and fundamental (Halpern and Wood, 1950; Taneike et al., 1988). In general, basic secretagogues are positively charged, although hydrophobic structured compounds causing rapid mast cell degranulation [within ~10–20 s through PLC stimulation, which is sensitive to Gi-protein inhibition, e.g., pertussis toxin (Ferry et al., 2002; Tatamoto et al., 2006)]. PACAP-38 induced an almost total mast cell degranulation within the first 10–20 s after application. The degranulation was impaired by the PLC-inhibitor U-73122, whereas adenylyl cyclase inhibitor SQ22536 was ineffective (Baun et al., 2012), indicating PLC activation as the responsible transduction pathway for PACAP-induced mast cell degranulation.

Interestingly, basic secretagogues seem to activate connective type mast cells independent of their putative receptor but only when applied in high concentrations (Ferry et al., 2002). Plotting the net charge of PACAP related molecules at neutral pH towards the level of degranulation induced by these PACAP analogues at 10 µM (**Figure 7**), we found a linear relationship with an *R*<sup>2</sup> value close to 1. Thus, a high net charge of the molecules correlates with a high mast cell degranulating effect of the PACAP analogues tested, and several factors indicate that PACAP may act as a basic secretagogue to cause mast cell degranulation despite the absence of PAC<sub>1</sub>-receptors.



## Expression of MrgB<sub>3</sub>-Receptors

The exact mechanism of basic secretagogue-mediated mast cell degranulation remains unclear. The ability of basic secretagogues to act as direct activators of purified G-proteins as well as numerous failed attempts to identify an endogenously expressed receptor could suggest a receptor-independent mechanism of action (Mousli et al., 1990, 1994; Seebeck et al., 1998). However, this would require the ability of peptides, despite their positive charge, to diffuse across the membrane in order to reach intracellular G-proteins, and as this has not been shown, the hypothesis seems unlikely (Tatemoto et al., 2006). The identification of a basic secretagogue receptor has for a long time been sought but without success (Ferry et al., 2002). In 2006, Tatemoto et al. (2006) studied members of the Mrg family. These are G-protein coupled receptors and expressed in a subset of nociceptive sensory neurons, thus making them interesting targets (Dong et al., 2001; Lembo et al., 2002). Expression of MrgX<sub>2</sub>-receptors in humans and MrgB<sub>3</sub>-receptors in rats were shown to be present in connective type mast cells and with affinity to various peptides like PACAP(6–27), mast cell depleting peptide and [D-Trp<sup>7,9,10</sup>]-substance P could link these receptors to PACAP-mediated mast cell degranulation (Tatemoto et al., 2006). In a recent study, MrgX<sub>2</sub>-receptor (human) and MrgB<sub>2</sub>-receptor (which is the mouse orthologue of the human MrgX<sub>2</sub>-receptor and the rat MrgB<sub>3</sub>-receptor) was convincingly demonstrated to be mast cell-specific and responsible for inflammatory activation by basic secretagogues (McNeil et al., 2015). By RT-PCR, we found the MrgB<sub>3</sub>-receptor to be present in rat peritoneal mast cells and decided to study the effect of selected PACAP analogues on *Xenopus laevis* oocytes expressing MrgB<sub>3</sub>-receptors and to compare the responses to the effects obtained by the same PACAP analogues on PAC<sub>1</sub>-receptor-expressing oocytes.

## Experiments on MrgB<sub>3</sub>-Receptor and PAC<sub>1</sub>-Receptor-Expressing Oocytes as Compared to Mast Cell Degranulation

In rat MrgB<sub>3</sub>-receptor-expressing oocytes, we found that PACAP-38, but not PACAP-27 and maxadilan induces currents. The effect mediated by the different PACAP agonists in the oocytes had the same characteristics as those previously found to induce a significant degranulation of rat meningeal mast cells. The findings were also in line with our previous results from peritoneal mast cells (Baun et al., 2012). Importantly, the concentrations required for effects were in the 1–10 μM range. In PAC<sub>1</sub>-receptor-expressing oocytes, PACAP-38, PACAP-27 and maxadilan were equipotent and responses were found to be significant at 10 times lower concentrations.

In line with our results from peritoneal mast cells (Baun et al., 2012), we found the PAC<sub>1</sub>-receptor antagonist PACAP(6–38) both to be a potent degranulator of rat meningeal mast cells and to induce a significant current in MrgB<sub>3</sub>-receptor-expressing oocytes. Contrarily, PACAP(6–38) showed the predicted antagonistic effect on PACAP-38-induced currents in PAC<sub>1</sub>-receptor-expressing oocytes (Harmar et al., 2012). Taken together, our studies suggest mast cell degranulation

to be mediated *via* MrgB<sub>3</sub>-receptors and not *via* the PAC<sub>1</sub>-receptor. However, the conclusion is limited by the fact that currently no selective antibodies or antagonists directed towards the MrgB<sub>3</sub>-receptor are available to provide the final pharmacological evidence.

Interestingly, a similar finding of PAC<sub>1</sub>-receptor pharmacology and functional observations in the rat trigemino-vascular system was reported. In these studies, PACAP-38, but neither VIP, PACAP-27 nor maxadilan, mediated the release of the sensory vasodilator peptide, calcitonin gene-related peptide (CGRP) from the trigeminal nucleus caudalis. Furthermore, the response to PACAP-38 seemed not to be mediated *via* PAC<sub>1</sub>-receptors due to lack of inhibition by the PAC<sub>1</sub>-receptor antagonist M65 (Jansen-Olesen et al., 2014). Future studies will have to rule out if MrgB<sub>3</sub>-receptors are involved in PACAP-38-induced CGRP release in trigeminal nucleus caudalis.

## CONCLUSION

In the present study, we found PACAP agonists and antagonists to have the same pharmacological effect in meningeal mast cells as previously found in peritoneal mast cells. By RT-PCR, we showed that there was no PAC<sub>1</sub>-receptor transcription in peritoneal mast cells thus excluding the possibility of the degranulating effect to be mediated *via* PAC<sub>1</sub>-receptor splice variants. However, we found mRNA encoding the rat MrgB<sub>3</sub>-receptor to be expressed in mast cells. This receptor was previously suggested to mediate mast cell degranulation in rat after application of basic secretagogues. This finding led us to investigate the effect of different PACAP analogues on *Xenopus laevis* oocytes expressing either PAC<sub>1</sub>- or MrgB<sub>3</sub>-receptors. The expressed MrgB<sub>3</sub>-receptor but not the PAC<sub>1</sub>-receptor share the same order of potency for PACAP analogues as found in rat peritoneal and meningeal mast cells. Thus, we hereby suggest the MrgB<sub>3</sub>-receptor to be a mediator for PACAP-induced mast cell degranulation.

## DATA AVAILABILITY

All datasets generated for this study are included in the manuscript.

## AUTHOR CONTRIBUTIONS

IJ-O, SP and DK designed the research. KC and FH contributed with valuable intellectual input for improvement of the study. SP and SC performed the experiments. IJ-O, SP, CK, DK and SC performed data analysis. IJ-O and SP drafted the manuscript. JO, DK, KC, FH and SC read and corrected the manuscript. JO, IJ-O, SP and DK received financial support for the study. All authors read and approved the final manuscript.

## FUNDING

This study was financially supported by the Lundbeck Foundation (R77-A6952), Candy's Foundation, The Danish

Research Council (11–107831), the Novo Nordisk Foundation, Frimodt-Heineke Foundation, Else and Mogens Wedell-Wedellsborg Foundation, and the A.P. Moller Foundation for the Advancement of Medical Science.

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## ACKNOWLEDGMENTS

The authors would like to thank Vibeke Grøsfjeld Christensen for excellent laboratory assistance.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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