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PACAP Modulates Acetylcholine-Elicited Contractions at Nicotinic Neuromuscular Contacts of the Land Snail

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Abstract In this study, we investigate the potentiating effect of PACAP27 on cholinergic neuromuscular transmission in the recently discovered flexor muscles of the land snail, *Helix pomatia*. Using immunohistochemistry, we show that PACAP and PAC1 receptors are present in nerve fibers innervating the flexor muscles but not in the muscle itself. We also observed that PACAP27 exerts both pre- and postsynaptic effects on the cholinergic synapse and performed tests using a broad spectrum of chemicals in order to explore the possible intracellular pathways through which PACAP mediates its stimulatory effect. Our pharmacological data demonstrate that PACAP27 presynaptically enhances the release of acetylcholine by activating the adenylate cyclase-cAMP-PKA pathway. Postsynaptically, PACAP27 was found to enhance muscle contractility by PKC-mediated signaling pathway resulting in an increased Ca^{2+} release from intracellular stores. These findings suggest that regulation of Ca^{2+} release may contribute to the stimulatory effect of PACAP. Our data are the first demonstration of the potentiating effect of PACAP27 at the molluscan excitatory neuromuscular contact.

Keywords Snail · Muscle contraction · Neuromuscular contact · Cholinergic transmission · PACAP

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Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) belongs to the vasoactive intestinal peptide (VIP)-secretin-growth hormone superfamily of regulatory peptides (Arimura 1992). PACAP shows a remarkable amino acid sequence similarity at the N-terminal domain across higher and lower vertebrate species, and even invertebrate species such as worms, insects, and molluscs (Kiss and Pirger 2013). Such a high degree of sequence homology represents conservation during evolution and indicates that PACAP and its receptor molecules could be involved in the modulation of several basic physiological functions (Cardoso et al. 2007; Sherwood et al. 2000).

PACAP and its receptors are widely distributed in the central nervous system (CNS) and in the periphery of vertebrates and invertebrates. In vertebrates, two types of receptors have been characterized according to their relative affinities for PACAP and VIP: PAC1R and VPAC1/VPAC2 (Cardoso et al. 2007; Vaudry et al. 2009). The presence of both types of receptors is demonstrated in the snail CNS, making molluscs suitable models for studying the cellular mechanisms of PACAP (Hernádi et al. 2008; Kaufmann et al. 1995; Pirger et al. 2010).

The wide distribution of PACAP and its receptors implies that the peptide exerts pleiotropic physiological role to modulate ion-channels and synaptic function, by binding to specific cell-surface receptors and triggering an array of

intracellular signaling pathways (Vaudry et al. 2009). Co-existence and/or co-release of PACAP and classical transmitters have already been demonstrated in different parts of the vertebrate CNS and also at the periphery (Fahrenkrug and Hannibal 2004). For example, it has been established that PACAP increases presynaptic acetylcholine (ACh) release at neuronal nicotinic synapses in cultured ciliary ganglion neurons by activation of PACAP/PAC1R via adenylate-cyclase (AC)-dependent signaling that leads to the stimulation of NO production (Pugh et al. 2010). PACAP also modulates synaptic activity in several neuronal regions by increasing release of ACh from septal cholinergic fibers or in the CA1 region (Masuo et al. 1993; Roberto and Brunelli 2000). In the guinea pig mesenteric ganglion, PACAP enhances the level of nicotinic cholinergic transmission presynaptically and acts directly on postsynaptic neurons to increase membrane excitability (Ermilov et al. 2004). The excitatory action of PACAP has also been observed in the internal anal sphincter smooth muscle of the opossum, where it is thought to activate PACAP receptors of postganglionic nerve terminals making neuromuscular contacts (Rattan and Chakder 1997). In Drosophila, PACAP generates two temporally distinct responses on larval body wall musculature. First, the peptide generates an early inward current, thus depolarizes the membrane and the late response is an enhancement of voltage-dependent K^+ current (Zhong and Pena 1995).

PACAP also modulates the effects of transmitters at inhibitory smooth muscle neuromuscular junctions, where it elicits relaxation by increasing AC activity or by controlling smooth muscle mechanical activity. It acts directly on muscle cells via PACAP preferring receptors or indirectly by stimulating NO synthesis (Imoto et al. 1998; Lecci et al. 2002; Takeuchi et al. 2004; Zizzo et al. 2004). An inhibitory action of PACAP has been observed in the gastrointestinal tract of different vertebrates and has been attributed to the activation of apamine sensitive, Ca^{2+}-dependent K^+-channels (Kishi et al. 1996; Schworer et al. 1992). PACAP induces concentration-dependent and substantial relaxations of carotid arteries of young rats (Vamos et al. 2014). Furthermore, the peptide evokes cerebellar artery dilation by activation of ATP-sensitive and large-conductance Ca^{2+}-activated K^+ channels (Koide et al. 2014). In all cases, mentioned above the channel stimulation was likely due to the activation of adenylyl cyclase and an increase of cAMP. Preliminary experiments have demonstrated that PACAP markedly potentiates contractions of the recently described flexor muscles (FM) of the upper tentacles of the land snail evoked by synaptically released or by exogenously applied ACh (Hernádi and Teyke 2012; Kiss et al. 2013; Krajcs et al. 2012). Therefore, the main objective of the present study was to explore the mechanism underlying the potentiation of muscle contraction by PACAP.

Materials and Methods

Preparation and Contraction Measurement

Adult specimens of the pulmonate snail, *Helix pomatia*, were collected in the surrounding area. FMs of the posterior tentacles were isolated, and their isotonic contraction was recorded as described previously (Krajcs et al. 2012). Briefly, one end of a single muscle was fixed into a slot cut out of Plexiglass, and the other end was free. Changes in muscle length were measured in control and test solutions using the ocular micrometer of a stereomicroscope. Contractions were expressed as a percentage shortening of the muscle compared to its resting length. Chemicals were dissolved in snail physiological solution containing (in mM): 80 NaCl, 4 KCl, 10 $CaCl_2$, 5 $MgCl_2$, and 10 Tris–HCl (pH = 7.4), and were applied onto the muscle via a rapid gravity perfusion system. The geometry of the perfusion chamber enabled a fast exchange of solutions (2–3 s). All experiments used preparations containing the olfactory nerve connecting the cerebral ganglion (CG) with the FM. The CG and part of the olfactory nerve were placed in a pit of the recording chamber separated from the muscle by a Vaseline gap. For the electrical stimulation of innervated muscles, a pair of silver-hooked electrodes was placed under the nerve. Electrical pulses of 10 ms and 5–10 V, 1–1.5 Hz were applied to the olfactory nerve, and contractions of the M3 FM were recorded (Hernádi and Teyke 2013).

Immunohistochemistry

The posterior tentacles with the three recently described tentacle FMs (Hernádi and Teyke 2012) were dissected and fixed in 4 % paraformaldehyde (buffered with 0.1 M phosphate buffer, pH 7.4) for 6 h at 4 °C. The FMs were separated from the tentacles and used for immunohistochemistry (IHC) as whole mount preparations. The preparations were incubated for 24 h at room temperature with either the anti-PAC1R antibody, or the anti-PACAP antibody. Anti-PAC1R antibodies purchased from two different producers were tested. The epitopes of the two PACAP receptor antibodies were different. Anti-PACAP and anti-PAC1R from Sigma were diluted to 1:500 in PBS-TX-BSA. After a short wash in PBS-TX, the immunoreaction was visualized using the One-Step Polymer-HRP IHC Detection System (anti rabbit diluted 1:4 in PBS-TX-BSA, BioGenex, USA). Anti-PAC1R antibody from Abcam was diluted to 1:100 in PBS-TX-BSA. The immunoreaction was visualized using NL-493 conjugated anti-rabbit IgG, diluted to 1:200 (R&D Systems, Biomedica Kft, Budapest, Hungary). No immunoreactivity was observed using preabsorption and method controls. The specificity of anti-PAC1R antibody (Sigma) had already been described (Hernádi et al. 2008).

Following a short wash in PBS-TX, the samples were mounted in PBS-glycerol (2:1) and viewed under a light microscope or a fluorescence microscope equipped with the appropriate filters.

Chemicals

Acetylcholine chloride (ACh), forskolin, N^6,2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt (DB-cAMP), chelerythrine chloride (ChelCl), ruthenium red (RR), cadmium chloride ($CdCl_2$), potassium chloride (KCl), and anti-pituitary adenylate cyclase-activating polypeptide type 1 receptor (PAC1R) antibody (rabbit polyclonal, P8872) were purchased from Sigma (Budapest, Hungary). Another anti-PAC1R antibody was purchased from Abcam (rabbit polyclonal, ab95278, Cambridge, UK). Anti-PACAP antibody (rabbit polyclonal) was purchased from Abcam (ab174982). H-7 dihydrochloride (H-7) was purchased from Biomedica (Budapest, Hungary). Decamethylene-bis-dimethylammonium bromide (DMDA), a nicotinic AChR antagonist, was purchased from Fluka AG (Buchs, Switzerland). M65 was purchased from Bachem (Bubendorf, Switzerland). PACAP38, PACAP27, and PACAP6-27 were synthetized at the Department of Medical Chemistry, University of Szeged (Szeged, Hungary).

Statistical Analysis

Data are presented as mean ± S.E.M. Statistical significance was assessed in all experiments using a paired sample *t* test. Statistical significance was accepted when $p < 0.05$ or $p < 0.01$.

Results

Effect of PACAP on Nerve-Evoked Contractions (Presynaptic Mechanism)

FM contractions evoked by stimulating the olfactory nerve were mimicked by external ACh application. In the presence of nicotinic acetylcholine receptor antagonists, contractions were powerfully attenuated, suggesting that ACh was released from nerve endings (Kiss et al. 2014). Direct application of PACAP38 or PACAP27 at 10^{-7}–10^{-4} M did not cause a change in the resting tone, or in the phasic contraction of the muscle (data are not shown). However, when muscles were pretreated for 15 min with 10^{-7} M PACAP27, nerve evoked contractions were significantly potentiated (Fig. 1a). No such effect was observed when muscles were pretreated with PACAP38. The potentiating effect of PACAP27 was statistically significant when it was added to the muscle compartment of the organ chamber (67 % potentiation on average), and also observed when added to the ganglionic compartment (27 %, although not significant). This observation underpins the hypothesis that PACAP27 exerts potentiating effect both pre- and postsynaptically. The potentiation was long-lasting, and full recovery was obtained washing with control physiological solution for 15–20 min. The potentiating effect of PACAP27 on nerve evoked contractions was effectively antagonized by truncated peptide PACAP6-27 (an antagonist of PACAP receptor), suggesting that PACAP was released upon nerve stimulation and that a specific PACAP-sensitive receptor was involved (Fig. 1b). These observations imply that there is a presynaptic interaction between the neuropeptide and neurotransmitter, suggesting that PACAP27 is released simultaneously with ACh when the olfactory nerve is stimulated.

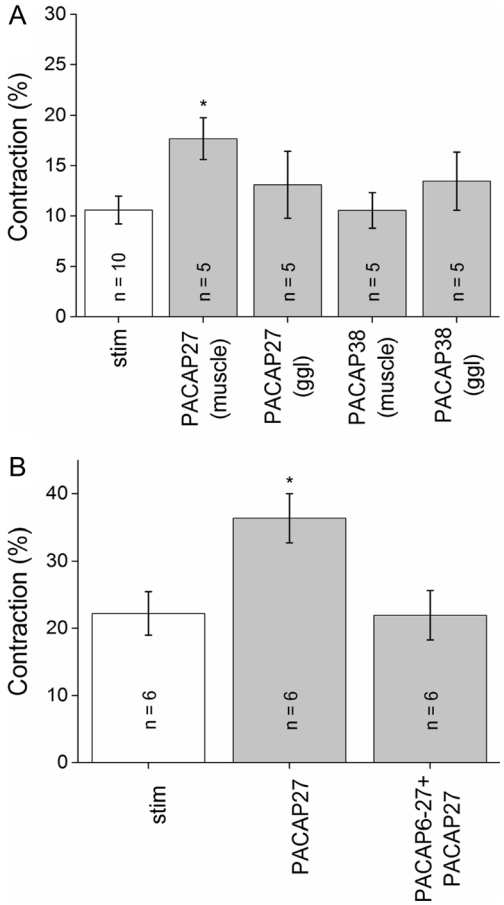

Fig. 1 PACAP27 but not PACAP38 enhances muscle contractions. **a** Pretreatment with 10^{-7} M PACAP27, applied to the muscle compartment, increased by 67 % the amplitude of muscle contractions evoked by electrical stimulation (stim) of the olfactory nerve. When PACAP27 was applied to the ganglionic compartment (ggl) contractions increased by 24 %. Application of PACAP38 over the muscle had no effect, while application to the ggl augmented the contractions by 27 %. **b** The PACAP receptor antagonist PACAP6-27 (10^{-6} M) effectively antagonized the stimulatory effect of 10^{-7} M PACAP. *Asterisks* indicate a significant difference from the control value at $^*p < 0.05$, *n* = number of muscles. All pretreatments lasted for 15 min

To examine the potential modulatory role of PACAP for nicotinic neuromuscular transmission, its localization in the olfactory nerve, which innervates all FMs, was next established using PACAP and PAC1R antibody (Ab) labeling. Consistent with a modulatory role, PACAP27 Ab immunolabeled fibers were observed in the olfactory nerve (Fig. 2). Intense PACAP-LI was present in axon bundles running along the longitudinal axes of each of the FMs (Fig. 2a); the labeled axons also projected thin perpendicular side branches onto the muscle fibers (Fig. 2b, c).

Applying PAC1R Ab revealed PAC1R-like immunoresponse in axons within thick fiber trunks running longitudinally in the muscles (Fig. 3a). Fine varicose side branches of PAC1R-LI containing axons, running both longitudinally and transversally, were seen both on the surface and deep within the muscles (Fig. 3b). The labeled axons densely innervated FMs and terminated on muscle fibers, displaying an endplate like appearance (Fig. 3c). Similar appearance of PAC1R-labeled axons could be observed in cross-section of the muscle using another PACAP receptor antibody (Fig. 3d). The anti-PAC1R antibodies revealed that these receptors were confined exclusively to nerves innervating the FM but muscles supporting presynaptic action of PACAP27. Further evidence for presynaptic modulation by PACAP was obtained applying AChR blocker DMDA.

DMDA was found to reduce nerve-elicited contractions by 40–50 % both in control- and PACAP27-pretreated muscles (Fig. 4a). These data provide supporting evidence first that ACh was released from nerve terminals during stimulation and second that the stimulating effect of PACAP27 was due to the increased transmitter release. The blocking effect in both cases was statistically significant.

Since PACAP27 acts via PAC1R, a selective high-affinity G-protein coupled receptor, the role of the intracellular signaling cascade was investigated. Specific activators and inhibitors of AC were applied during olfactory nerve-evoked FM contractions and compared with those obtained in muscles pretreated with 10^{-7} M PACAP27. Forskolin, an activator of AC, and DB-cAMP, a permeable analog of cAMP, both enhanced muscle contractions (Fig. 4b). These data suggest that PACAP27 enhances ACh release via presynaptic activation of cholinergic nerve terminals in the FM. Next, the possible involvement of protein kinases was evaluated using ChelCl and H-7. ChelCl, a specific PKC inhibitor, did not change the PACAP27 induced potentiation of the nerve evoked contractions (Fig. 4b). In contrast, H-7, a PKA, PKC, PKG, and myosin light-chain kinase inhibitor significantly attenuated these contractions (Fig. 4b).

Effect of PACAP on ACh-Elicited Contraction (Postsynaptic Mechanism)

To assess whether postsynaptic mechanisms were also involved in the contraction-enhancing effect of PACAP27, ACh (10^{-5} M) was next directly applied to the denervated muscle. The relatively high concentration of ACh ($EC_{50} = 6 \times 10^{-6}$ M) assured the prevalence of a postsynaptic site of action (Krajcs et al. 2014). Pretreating the muscle with 10^{-7} M PACAP27 for 15 min induced a significant enhancement of the ACh evoked contraction (Fig. 5). This enhancement was successfully attenuated in the presence of 10^{-6} M PACAP6-27 or M65, suggesting that specific PACAP receptors were involved. However, PAC1R receptors could not be identified in the muscles using immunohistochemistry (see

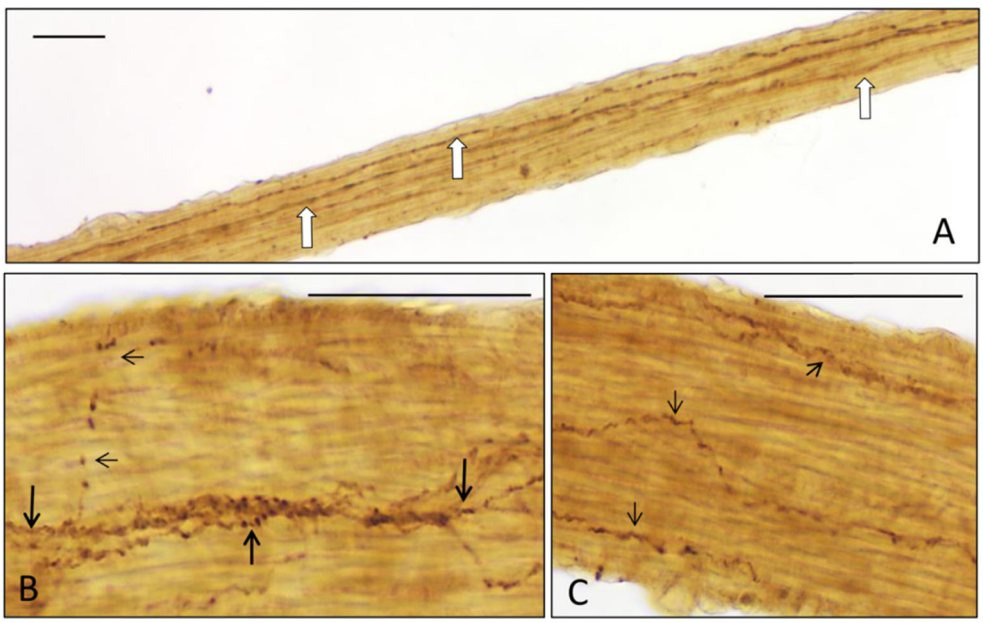

Fig. 2 PACAP27 immunolabeled elements in the flexor muscles. **a** In the M1 flexor muscle PACAP27-LI axons are shown in nerve trunks (*large arrows*) running longitudinally in the muscle. **b** In the nerve fiber trunks, numerous labeled axons can be observed (*thin black arrows*). The axons may send off transversally running varicose axonal side branches (*arrow heads*). **c** Fine varicose axon branches running longitudinally on the surface of the muscle can also be seen (*arrow heads*). *Bars* represent 50 μm

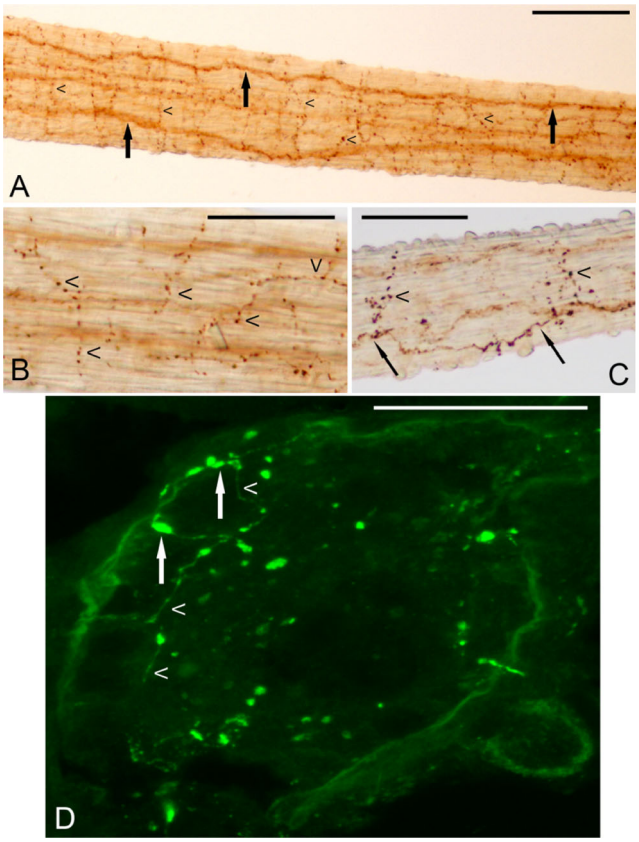

Fig. 3 PAC1-R immunolabeled elements in the flexor muscles. **a** PAC1R-LI fiber trunks (*thick arrows*) run longitudinally in the muscle. Labeled axons in the trunks give off varicose side branches (*arrow heads*) which densely innervate the muscles. **b** Varicose side branches (*arrow heads*) run both longitudinally and transversally on the surface of the muscle. **c** In the deeper layer of the muscle, the labeled axons (*thin arrow*) give off transversal varicose side branches (*arrow heads*) which terminate on muscle fibers and display an endplate-like appearance. **d** Cross-section of the muscle showing longitudinally (*thick arrows*) and transversally (*arrow heads*) running, PAC1R-containing axons. *Bars* represent 50 μm (**a**–**c**) or 25 μm (**d**)

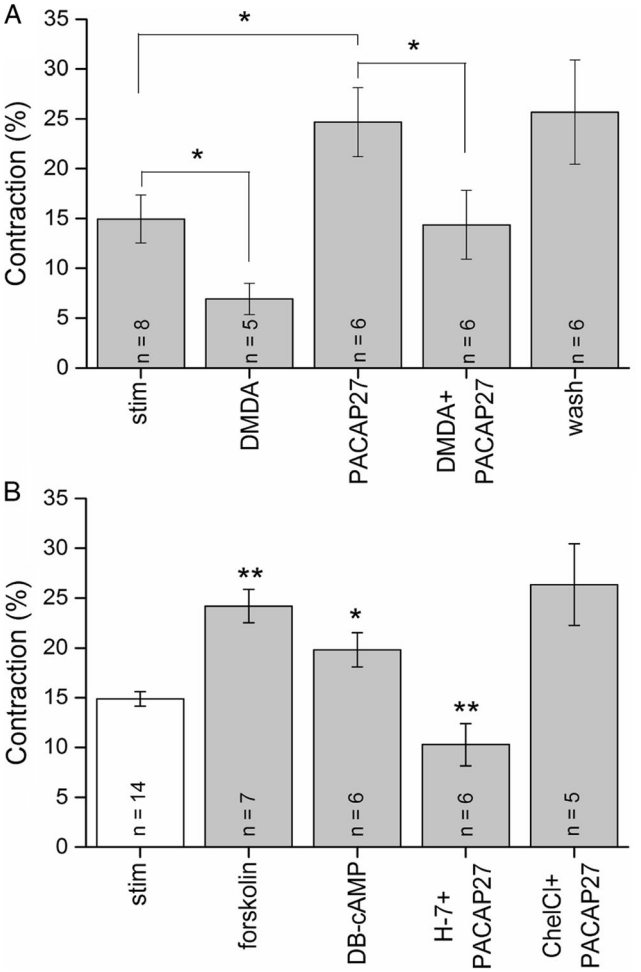

Fig. 4 **a** Pretreating the muscle with 10^{-5} M DMDA significantly reduced the muscle contractions evoked by electrical nerve stimulation. 10^{-5} M DMDA completely blocked the potentiating effect of 10^{-7} M PACAP27. **b** PACAP27 acts via second messenger signaling pathway. Similarly to PACAP27, pretreatment of the muscle with forskolin (10^{-5} M) and DB-cAMP (10^{-6} M) for 15 min increased the nerve stimulation-evoked muscle contractions by 63 and 34 %, respectively. Joint application of PACAP27 (10^{-7} M) and H-7 (10^{-5} M) decreased the contractions by 30 %, while pretreatment of PACAP27 (10^{-7} M) and chelerythrine chloride (ChelCl, 10^{-6} M) at the same time augmented the contractions by 77 %. *Asterisks* indicate a significant difference from the control value at $*p < 0.05$, $**p < 0.01$, n = number of muscles. All pretreatments lasted for 15 min

Fig. 3). The potentiating effect of PACAP27 on ACh-evoked contractions was blocked in the presence of a PKC and PKA inhibitor, indicating that the potentiating effect of PACAP27 is mediated by a cAMP/PKA- and PKC-dependent mechanism (Fig. 5). Forskolin did not have statistically significant effect on the ACh-elicited contraction suggesting that PACAP postsynaptically used different intracellular pathway from that observed presynaptically. PACAP27 therefore appears to exert its potentiating effect on the FM contraction by acting on different receptors or directly on the muscle, either by increasing the extracellular Ca^{2+}-influx through voltage-dependent Ca^{2+} channels (VDCC) or by increasing Ca^{2+} release from intracellular stores.

To test the power of PACAP to induce a rise in cytosolic Ca^{2+} concentration, high K^{+}-evoked contractions were compared in control and in PACAP27-pretreated muscles (Fig. 6a). In PACAP27-pretreated muscles, KCl-evoked contractions were not potentiated; in fact, they decreased on average by ~32.4 %. In the presence of 100 μM Cd^{2+}, ~69 % of KCl-evoked contractions were decreased, suggesting the involvement of Ca^{2+} influx via VDCCs in the KCl-induced contraction (Fig. 6a). Next, the effect of PACAP27 on ACh-evoked contractions in Ca^{2+}-free solution was assessed. The mean amplitude of the ACh-evoked contractions of muscles placed in Ca^{2+}-free extracellular solution decreased by ~60 % compared to the control contractions, supporting the view that an inward Ca^{2+} movement from the extracellular space was involved (Fig. 6b). When FM placed in Ca^{2+}-free solution was challenged with PACAP27 for 15 min, the ACh-evoked

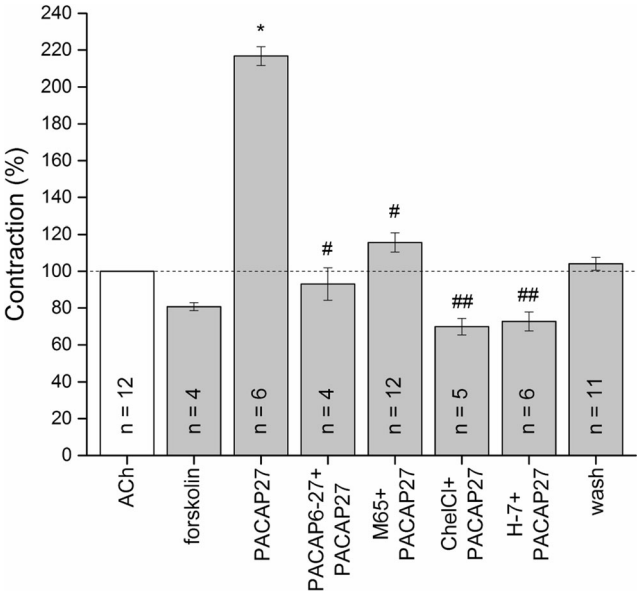

Fig. 5 Effect of PACAP27 on the externally applied ACh-evoked muscle contractions. Forskolin (10^{-5} M) pretreatment did not increase the 10^{-5} M ACh-evoked contractions. 10^{-7} M PACAP27 pretreatment augmented ACh-elicited contractions by 104 %. However, when PACAP27 was applied together with10^{-6} M PACAP6-27, the increasing effect of PACAP was completely blocked. Applying M65 (10^{-6} M) together with PACAP27, the effect of PACAP was almost completely blocked. Similarly, chelerythrine chloride (ChelCl, 10^{-6} M) and H-7 (10^{-5} M) inhibited the increasing effect of PACAP by ~67 and 63 %, respectively. *Dashed line* shows ACh contractions marked as 100 %. *Asterisks* indicate a significant difference from the control value at $*p < 0.05$. # indicates significant difference from the PACAP-augmented contractions at $p < 0.05$, ## indicates significant difference from the PACAP-augmented contractions at $p < 0.01$, n = number of muscles. All pretreatments lasted for 15 min

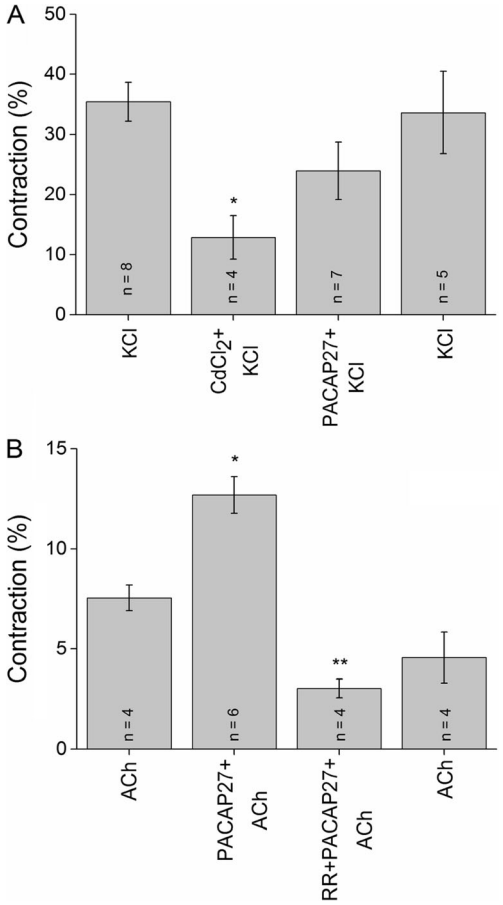

Fig. 6 Effect of PACAP27 on cytosolic Ca^{2+} level. **a** 40 mM KCl evoked muscle contractions by 35 %. After pretreating the muscle with 100 μM $CdCl_2$, the KCl-evoked contractions were decreased by 64 %. Applying 10^{-7} M PACAP27 reduced the KCl-elicited contractions by 32 %. The recovery was complete washing the preparations with physiological saline. **b** Effect of PACAP27 on ACh-evoked contractions in Ca^{2+}-free ringer solution. 10^{-5} M ACh evoked muscle contractions increased by ~70 % following 10^{-7} M PACAP27 pretreatment. The potentiating effect of PACAP was decreased by 60 % pretreating the muscle with 40 μM ruthenium red (RR) compared to the PACAP27+ACh-elicited contractions. *Asterisks* indicate a significant difference from the control value at $*p < 0.05$, $**p < 0.01$, n = number of muscles. All pretreatments lasted for 15 min

contraction increased by ~70 %. The ratio of the contraction recorded in PACAP-treated and non-treated muscle in control and Ca-free solution was nearly equal, 2 and 1.7, respectively. The ACh-evoked contraction recorded in Ca^{2+}-free solution was further decreased in the presence of RR, which is a blocker of Ca^{2+}-release from mitochondria and ryanodine/caffeine-sensitive intracellular stores (Fig. 6b). Collectively, these data revealed that the underlying mechanism of PACAP27-induced potentiation of ACh-evoked contractions involved the increase of cytosolic free Ca^{2+} from intracellular stores.

Discussion

In this study, the effect of PACAP on the contractions evoked by stimulation of the olfactory nerve or by exogenous ACh application was studied in the FM of the upper tentacle of the snail. PACAP27 caused an increase in the amplitude of muscle contractions in both cases. The goal of this study was to investigate the signaling pathway that couples PACAP to a potentiation of both synaptically and exogenously applied ACh-elicited muscle contraction, and to unravel the site of PACAP action.

The data shown here provide pharmacological, physiological, and immunohistochemical evidence in support of the hypothesis that PACAP27, but not PACAP38, increases nerve evoked contractions by pre- and postsynaptic mechanisms working together at the gastropod neuromuscular junction. In order to determine whether the effect of PACAP was elicited through pre- and/or postsynaptic mechanisms, the localization of PACAP and its receptors was assessed by IHC. It was observed that PACAP27-LI and PAC1R-LI immunoreactive nerve fibers were present throughout the muscles. Based on distribution pattern of labelled elements, we suggested that

at least part of PACAP-containing fibers were localized presynaptically to the ACh-containing nerve endings. The presence of PACAP-LI and PAC1R immunoreactive nerve fibers in the FM, verified here, confirms therefore that the peptide acted presynaptically to enhance nerve evoked contractions and that the effect of PACAP27 is mediated by specific PAC1Rs. However, the ability of PACAP27 to enhance nerve-evoked contractions may be explained by different mechanisms, namely, (a) by presynaptic mechanisms when PACAP27 binds to specific, presynaptically localized PAC1Rs leading to the increased release of neurotransmitter, (b) by postsynaptic mechanisms, whereby PACAP27 increases the contractility or facilitates excitation-contraction coupling of the muscle in response, or (c) both, when presynaptic and postsynaptic mechanisms work together.

Previously, it has been described that contractions of the FM evoked by stimulation of the olfactory nerve are mimicked by external ACh, suggesting that ACh is an excitatory transmitter at the neuromuscular contacts. On binding to the postsynaptic, slowly desensitizing αBgTx-sensitive α7-like AChRs, ACh evokes a concentration-dependent contraction of the FM with $EC_{50} = 6 \times 10^{-6}$ M (Kiss et al. 2014; Krajcs et al. 2014).

PACAP27 had no significant effect on the resting tone of the FM. The data presented here suggest, however, that PACAP27 is potentially able to increase synaptically elicited contraction presumably by increasing the amount of ACh released at the neuromuscular contact.

The ability of PACAP to modulate ACh-containing nerve terminals throughout the nervous system of vertebrates is well known (Liu et al. 2000; Margiotta and Pardi 1995; Pugh et al. 2010). Evidence indicates that PACAP stimulates ACh release from presynaptic cholinergic terminals in rat hippocampus and that this action is highly Ca^{2+}-dependent (Masuo et al. 1993). Less is known about the effect of PACAP at the excitatory neuromuscular junction in either vertebrates or invertebrates (Parkman et al. 1997; Yoshitomi et al. 2002). PACAP has been shown to be distributed in the myenteric neurons throughout the gastro-intestinal tract, where it exerts both inhibitory and excitatory effects depending on the sites and mechanisms of action (Rattan and Chakder 1997). The inhibitory effects may be direct while the excitatory may be indirect, and appear to be mediated via the activation of specific PAC1Rs (Katsoulis et al. 1993).

Here, investigation into the intracellular mechanisms of the presynaptic PACAP27 effect revealed that the increase in FM contraction elicited by nerve stimulation was attenuated by the protein kinase A inhibitor H-7. In contrast, ChelCl (PKC inhibitor) had no effect. PACAP also increased cAMP accumulation by stimulating adenylate cyclase (AC) activity, because both forskolin and a membrane permeable cAMP analog increased synaptically evoked contractions. These observations

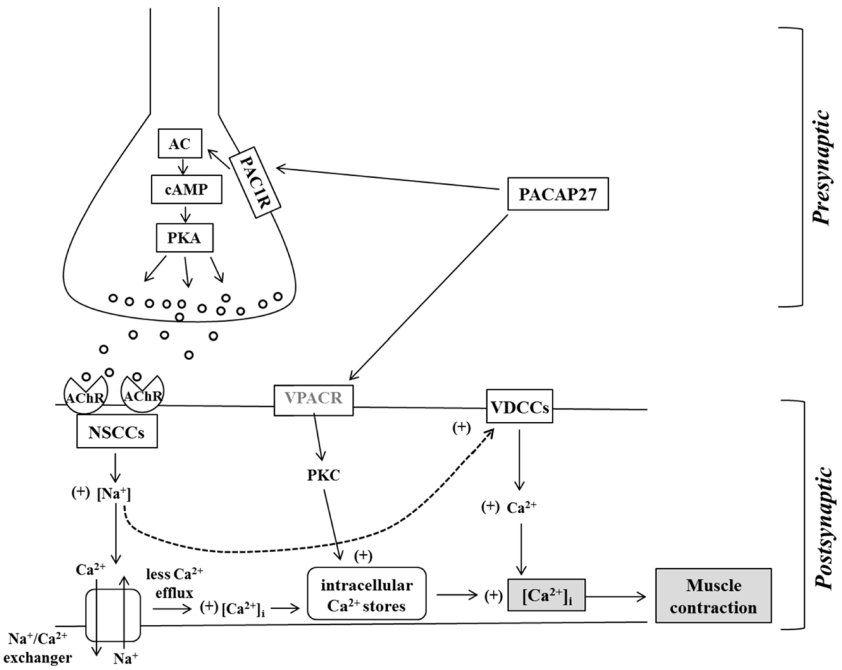

Fig. 7 Schematic drawing illustrates the possible pathways, via which PACAP develops its enhancing effect on contraction. Acting presynaptically, PACAP27 binds to PAC1Rs of the nerve endings and through the AC-cAMP-PKA pathway stimulates the ACh release. ACh activates the non-selective cation channels (NSCCs), and it results in an elevated Na^+ influx, which causes membrane depolarization, leading to the opening of voltage-dependent calcium channels (VDCCs). Elevation of $[Na^+]_i$ inhibits the Na^+/Ca^{2+} exchanger and the less efflux of Ca^{2+} contributes to the rise of $[Ca2^+]_i$. Postsynaptically, PACAP may have a direct positive effect on the intracellular Ca^{2+} stores through PKC-mediated pathway. The final effect is the elevation of the intracellular Ca^{2+} level and therefore the increase of muscle contraction

indicate that the effect of PACAP on muscle contraction represents signaling mechanisms that involve the activation of PKA presynaptically. Only PAC1Rs, which couples via AC/PKA-mediated signal transduction, were required to enhance neuromuscular transmission. Taken together, these results indicate that cyclic nucleotides and cyclic-nucleotide-dependent protein kinases are necessary components of the pathway that underlies presynaptic modulation by this peptide. The data presented here are the first to demonstrate the potentiating effect of PACAP at the neuromuscular contact in molluscs.

Pharmacological experiments suggested, however, that both a postsynaptic and presynaptic action of PACAP27 were present, because exogenously applied ACh-evoked contractions were also potentiated. Interestingly, these contractions were not affected by forskolin suggesting a different intracellular signaling pathway; however, contractions were significantly attenuated in the presence of PACAP6-27 and M65 although IHC failed to localize PAC1Rs on the muscle using the same antibodies as for nerves innervating the muscle. To explain the positive immunoresponse, we have different possibilities: (1) Eight isoforms of PAC1Rs, resulting from alternative splicing, have been characterized so far (Payet et al. 2003); therefore, we cannot exclude that the PAC1R Ab used in these experiments was unable to recognize the PACAP receptor variant localized on the muscle cells. (2) It is also possible that VPACR1 or VPACR2 is present on the FM, as it has been demonstrated in smooth muscle cells of mammalian stomach (Vaudry et al. 2009). However, this possibility was not examined by immunohistochemistry. Bearing in mind that PACAP could facilitate contractions of the FM via a number of mechanisms, an alternative explanation would be a direct effect on the muscle not requiring specific receptors. It has been shown previously that caffeine is able to elicit contraction even in Ca^{2+}-free solution, involving an activation of caffeine sensitive Ca^{2+} stores that can be blocked by RR (Krajcs et al. 2012). In this study, the enhancement of the ACh-evoked contraction by PACAP was observed both in normal and Ca^{2+}-free solution, clearly showing that voltage-dependent Ca^{2+} channels were not involved in PACAP-induced Ca^{2+} release. The ratio of PACAP27-induced potentiation in Ca^{2+}-free solution was almost the same as in a solution of normal ion-content, supporting the conclusion above. Moreover, the potentiating effect of PACAP on ACh-evoked contraction was still observed even when VGCCs were blocked by cadmium. When ryanodine/caffeine sensitive Ca^{2+}-release was inhibited by RR, the potentiating effect of PACAP27 was significantly blocked, confirming our conclusion that PACAP stimulated Ca^{2+}-release from intracellular stores through a PKC dependent pathway. Thus the possibility exists that PACAP may enhance muscle contraction not only by increased cAMP production, but also by mobilizing cytosolic Ca^{2+} concentrations. Direct measurement of the cytosolic Ca^{2+} level was not performed, however, it is known that PACAP is able to increase cytosolic Ca^{2+} concentration in a number of cell types including nerve, adrenal medulla chromaffin and pancreatic β-cells (Masuo et al. 1993; Payet et al. 2003).

The mechanisms of PACAP-induced potentiation of ACh-elicited contraction are summarized in Fig. 7. Thus, it appears that PACAP is able to utilize a variety of mechanisms by which it can regulate the excitability of contractions of the FM, including presynaptic regulation of ACh release, as well as the post-synaptic regulation of contractility by increasing the release of Ca^{2+} from intracellular stores.

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