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The neuropeptide proctolin potentiates contractions and reduces cGMP concentration *via* a PKC-dependent pathway

Berit Philipp, Nicole Rogalla and Sabine Kreissl*
Department of Biology, University of Konstanz, 78457 Konstanz, Germany
*Author for correspondence (e-mail: s.kreissl@uni-konstanz.de)

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Summary

As in many other arthropods, the neuropeptide proctolin enhances contractures of muscles in the crustacean isopod *Idotea emarginata*. The enhancement of high K^+-induced contractures by proctolin (1 μmol l^{-1}) was mimicked upon application of the protein kinase C (PKC) activator phorbol-12-myristate 1-acetate (PMA) and was inhibited by the PKC inhibitor bisindolylmaleimide (BIM-1). The potentiation was not inhibited by H89, a protein kinase A (PKA) inhibitor. Proctolin did not change the intracellular concentration of 3′,5′-cyclic adenosine monophosphate (cAMP) whereas it significantly reduced the intracellular concentration of 3′,5′-cyclic guanosine monophosphate (cGMP). The reduction of cGMP was not observed in the presence of the PKC inhibitor BIM-1. 8-Bromo-cGMP, a membrane-permeable cGMP analogue, reduced the potentiating effect of proctolin on muscle contracture. We thus conclude that proctolin in the studied crustacean muscle fibres induces an activation of PKC, which leads to a reduction of the cGMP concentration and, consequently, to the potentiation of muscle contracture.

Key words: *Idotea emarginata*, neuropeptide, modulation, cAMP, cGMP, PKC, PKA.

Introduction

Neuropeptides are endogenous signalling molecules with complex effects on sensory integration and motor behavior. The myotropic pentapeptide proctolin (RYLPT) was first identified in the hindgut of the cockroach *Periplaneta americana* (Brown, 1967; Brown and Starrat, 1975) and was subsequently localised in nervous systems of other arthropods (Bishop et al., 1984; Brüstle et al., 2001; Kingan and Titmus, 1983; Lange et al., 1986; Schwarz et al., 1984; Sullivan, 1979; Witten and O'Shea, 1985). In insects and crustaceans, it is released from neurohaemal organs into the hemolymph (Lange, 2002; Kingan and Titmus, 1983, Schwarz et al., 1984), or it is contained within identified neurones being released in the central nervous system and at neuromuscular synapses occasionally acting as a cotransmitter (Bartos et al., 1984; Bishop et al., 1987; Brüstle et al., 2001; O'Shea, 1985; Orchard et al., 1989; Siwicki et al., 1987). Both in insects and crustaceans, proctolin induces stimulatory responses of skeletal and visceral muscles (Evans and Myers, 1986; Lange, 2002; O'Shea, 1985; Orchard et al., 1989) and potentiates nerve-evoked contractions of skeletal muscles (Adams and O'Shea, 1983; Bartos et al., 1994; Bishop et al., 1987; Facciponte et al., 1996; Mercier and Wilkens, 1985). The pentapeptide exerts its potentiating action through pre- and postsynaptic mechanisms. Presynaptically, proctolin enhances transmitter release (Belanger and Orchard, 1993; Jorge-Rivera et al., 1998; Rathmayer et al., 2002a) whereas the postsynaptic effects of proctolin on several arthropod muscles are manyfold. It directly evokes contractures in some insects (Adams and O'Shea, 1983; Baines and Downer, 1992; Bartos et al., 1994; Bauer, 1991; Lange et al., 1986; Wegener and Nässel, 2000) and crustaceans (Schwarz et al., 1980), as well as in *Limulus polyphemus* (Watson and Hoshi, 1985). Proctolin potentiates contractions of skeletal muscles elicited by depolarization in current clamp (Erxleben et al., 1995), and in high K^+ salines (Bishop et al., 1987; Cook and Holman, 1980). It enhances muscle contractions caused through mobilization of Ca^{2+} release from intracellular Ca^{2+} stores by caffeine (Brüstle et al., 2001; Wegener and Nässel, 2000). Additionally, proctolin induces the phosphorylation of a 30 kDa thin filament protein (Brüstle et al., 2001). Thus, proctolin exerts its potentiating action by regulating proteins localised at the sarcolemma and by modulating the contractile machinery.

Multiple cellular targets may be influenced by proctolin through its binding to G-protein coupled receptors and subsequent activation of intracellular signalling pathways (Baines et al., 1996; Egerod et al., 2003; Johnson et al., 2003). In a crayfish muscle, a 3′,5′-cyclic adenosine monophosphate (cAMP) analogue mimics the proctolin-induced increase in Ca^{2+} channel activity (Bishop et al., 1991). Agonists of the intracellular cAMP signalling pathway also mimic the

proctolin-induced increase of voltage-dependent Ca^{2+} channel activity and the inhibiting effect of proctolin on a non-voltage sensitive K^+-channel in extensor muscle fibres of the isopod *Idotea emarginata* (Erxleben et al., 1995; Rathmayer et al., 2002b). The protein kinase inhibitor H7 suppresses the Ca^{2+} currents potentiated by proctolin in this preparation (Rathmayer et al., 2002b). The enhancement of the myogenic rhythm by proctolin in a specialized bundle of slow fibres of the locust's extensor tibiae muscle is mimicked by experimentally elevating intracellular cAMP concentration (Evans, 1984). Only in these fibers was a proctolin-sensitive adenylate cyclase observed (Swales and Evans, 1988). However, in various arthropod muscles, no proctolin-induced increase in cAMP concentration can be detected when cAMP is measured directly (Baines and Downer, 1992; Evans and Myers, 1986; Goy et al., 1984; Groome and Watson, 1989; Mazzocco-Manneval et al., 1998).

Besides cAMP, phosphoinositides are discussed as mediators of the proctolin-induced effects. In several arthropod muscles, proctolin increases the inositol 1,4,5-trisphosphate ($InsP_3$) concentration (Baines et al., 1990; Hinton and Osborne, 1995; Lange, 1988; Mazzocco-Manneval et al., 1998). As a conjoint effect of $InsP_3$ production, proctolin may activate protein kinase C (PKC) and subsequently decrease cyclic GMP (cGMP) (Jaiswal, 1992), because phorbol esters mimic the potentiating effect of proctolin on contractions as well as its inhibiting effect on K^+ conductance (Baines and Downer, 1992; Lange and Nykamp, 1996; Walther et al., 1998; Wegener and Nässel, 2000).

The different reports on the transduction mechanisms underlying the myostimulatory effects of proctolin prompted us to further investigate the postsynaptic intracellular mechanisms of this neuropeptide in fibres of the extensor muscle of the marine isopod *Idotea emarginata*, where proctolin is present in efferent neurones supplying the dorsal extensor muscles and the pericardial organ (Brüstle et al., 2001). We determined intracellular concentrations of the cyclic nucleotides cAMP and cGMP and studied the role of protein kinase A (PKA) and PKC signalling pathways in mediating the proctolin-induced increase of contractures. We show that in *Idotea* muscle fibres proctolin does not induce the cAMP–PKA signalling pathway. Proctolin rather induces a decrease in intracellular cGMP concentration *via* a PKC-dependent inhibition of guanylate cyclase.

Materials and methods

Animals

All experiments were performed on adult males of the marine isopod *Idotea emarginata* (Fabricius 1793). Specimens were obtained from the Marine Station at Helgoland (Germany) and were reared at the Animals Facility of the University of Konstanz. Animals were kept in tanks with circulating artificial seawater under a 14 h:10 h light:dark cycle at 16°C.

Physiological solutions and chemicals

Artificial seawater (ASW) used as saline had a composition of (in mmol l^{-1}): 490 NaCl, 8 KCl, 10 $CaCl_2$, 48 $MgCl_2$, 30 D(+) glucose and 20 Hepes buffer at pH 7.4. When high K^+ (30 mmol l^{-1}) was used to induce muscle contractures, Na^+ was substituted with equimolar K^+.

The following stock solutions were prepared and diluted in saline prior to experiments: proctolin, 1 mmol l^{-1} in distilled water; octopamine, 1 mmol l^{-1} in distilled water; bisindolylmaleimide-1 (BIM-1), 10 μmol l^{-1} in distilled water; 8-bromo-cGMP, 10 mmol l^{-1} in distilled water; H89 dihydrochloride (H89), 1 mmol l^{-1} in dimethyl sulfoxide (DMSO); 3-isobutyl-1-methylxanthin (IBMX), 200 mmol l^{-1} in DMSO; phorbol-12-myristate-13-acetate (PMA), 1 mmol l^{-1} in DMSO. All stock solutions were stored at –20°C, except for H89 and BIM-1 (+4°C).

During contracture measurements final DMSO concentrations were kept constant throughout any experiment and did not exceed 0.1%. DMSO at a concentration of 0.2% had no effect on properties or responses of extensor muscle fibres in control experiments (Weiss et al., 2001). During cyclic nucleotide measurements with IBMX the DMSO concentration was 0.5%. Proctolin and octopamine were obtained from Sigma (Deisenhofen, Germany) and BIM-1, 8-bromo-cGMP, H89, IBMX and PMA from Calbiochem-Novabiochem (La Jolla, USA).

Contracture measurements and electrophysiological techniques

To expose the extensor muscle (for anatomical details, see Kreissl et al., 1999), the preparation was pinned with the ventral side exposed in a Sylgard-coated dish. Sternites with attached flexor muscles and the ventral nerve cord were removed. For all contracture experiments, the individually identifiable short extensor muscle fibre 2 of pleon segment 2 was used. The intersegmental membrane between pereion segment 7 and pleon segment 1 was cut to yield an isolated preparation consisting of the dorsal half of pleon segment 2, pleon segment 1 and half of the pleotelson. The long fibres 5 and 6, spanning two segments, were then removed. The cleaned preparation contained only the short fibres 1–4, but fibres 3 and 4 were cut to prevent them from contributing to force generation. This left only fibre 2 and the thin fibre 1 intact. The final preparation was transferred into a small bath (300 μl volume) lined with Sylgard. The pleon segment 1 was fixed with a fine pin. In order to record muscle tension, a small metal pin connected to a force transducer was attached to the posterior end of the preparation (see below). Finally, the preparation was stretched to *in situ* resting length of the muscle fibres. This tension was taken as zero tension in the contraction measurements. The bath was continuously perfused with cooled (18°C) ASW at a flow rate of 0.5–2.5 ml min^{-1}. Contractures were induced by elevating extracellular K^+ concentration (high K^+ contractures, 30 mmol l^{-1}) for 5 min either without or with simultaneous application of drugs. Changes of solutions were performed by means of a switching port in the perfusion system.

Muscle tension was measured isometrically using a KG3 force transducer (Scientific Instruments Güth, Heidelberg, Germany). The tension results were digitised at 10 kHz and low-pass filtered between 0.5–3 kHz. Intracellular and tension recordings were performed using an AxoClamp 2B amplifier (Axon Instruments). Data acquisition was controlled with the help of pClamp 8.0 software (Axon Instruments). Conventional intracellular electrodes were filled with 3 mol l^{-1} KCl and had d.c. resistances between 5 and 8 MΩ. Records were digitised at 10 kHz and filtered at 3 kHz during acquisition. Data analysis was performed using Clampfit 8.2 (Axon Instruments) and Excel 2000 (Microsoft Corporation).

Preparation of dorsal extensor fibres for cyclic nucleotide measurements

Before dissection, animals were chilled, then their head and legs removed. The animals were pinned with the ventral side up in a Sylgard-coated dish. The pereion sternites, with attached flexor muscles, and the ventral nerve cord were cut away. The gut, the tubes of the digestive gland, the gonads with the vasa deferentia and the heart were removed. In order to match conditions in cyclic nucleotide and tension measurements and to prevent artefacts due to dissection, the preparation was equilibrated for 10 min in fresh ASW, except when stated otherwise. The preparation was then transferred to a different dish with ASW at 18°C, containing either neuromodulators or enzyme inhibitors (experimental conditions), or no modulators and no enzyme inhibitors (control conditions). The final concentrations of the substances were: proctolin 1 μmol l^{-1}, 1 nmol l^{-1}; octopamine 10 μmol l^{-1}; IBMX 0.5 mmol l^{-1}; BIM-1 100 nmol l^{-1}. The incubation times were 0.5 min, 1 min, 3 min or 15 min. When effects of the enzyme inhibitors and the neuromodulators on cyclic nucleotide concentrations were tested together, the substances were applied simultaneously except when stated otherwise. The incubation was stopped with ice-cold perchloracid (0.1 mol l^{-1}, in ASW). Subsequently, the long fibres 5 were dissected, immediately frozen and stored in liquid nitrogen.

cAMP and cGMP measurements

The cAMP and cGMP concentration measurements were performed with a cAMP or a cGMP enzymeimmunoassay (*Biotrak* cellular communication assay, Amersham Pharmacia Biotech, Buckinghamshire, England). Fibres of the controls and tests were pooled in separate groups. For cyclic nucleotide measurements, 20 isolated long fibres 5 were pooled in 100 μl ice-cold 0.1 mol l^{-1} perchloric acid and subjected to sonication for 30 min at 4°C. After centrifugation for 20 min at 2000 ***g*** (4°C), the supernatant was taken off and the pellet analysed for protein content (Bradford, 1976). In order to neutralise the samples, 9 μl 1 mol l^{-1} KOH and 10 μl of 1 mol l^{-1} sodiumphosphate buffer (pH 5.8) were added to 85 μl of the supernatant. The samples were kept overnight at 4°C and were then centrifuged for 20 min at 2000 ***g*** and 4°C. The supernatant was collected and evaporated for 2 h in a rotary evaporator at room temperature. The lyophylised supernatant was then dissolved in 150 μl assay buffer (0.05 mol l^{-1} sodium acetate, pH 5.8) of the *Biotrak* enzyme immunoassay. The cAMP and cGMP concentration measurements were carried out according the protocols of the manufacturer for each assay. The acetylation procedure was performed because of its ability to detect small amounts of cyclic nucleotides. Parallel to each measurement, a standard measurement with known concentrations of cAMP or cGMP standards was carried out. Each sample and standard value derives from at least one double measurement.

Statistics

Pooled data are presented as mean ± s.e.m. Tests for statistical significance were performed using Student's *t*-test and were always compared to controls. $P<0.05$ is considered significant and N is the number of experiments. Asterisks in figures always illustrate statistical significance compared to control conditions (without peptides, inhibitors or activators).

Results

Proctolin does not increase cAMP concentration in Idotea muscle

Effects of second messenger pathway-dependent modulators may be transient and only observable during narrow time periods. Therefore, we investigated the intracellular cAMP concentrations of the fibres for different incubation times. There was an observed decline of the cAMP concentration with increasing periods of incubation in either ASW or proctolin (1 μmol l^{-1}) and application of proctolin did not significantly change the cAMP concentration compared to controls. The cAMP concentration 1 min after dissection was 13.41±3.05 (N=2, data not shown), declining to 0.26±0.03 (N=7) after 15 min in ASW (Fig. 1A). When 1 μmol l^{-1} proctolin was applied for 1 min immediately after dissection, the cAMP concentration was 13.51±4.39 (N=2, data not shown) declining to 0.27±0.02 (N=8) after 15 min in 1 μmol l^{-1} proctolin (Fig. 1A). The biogenic amine octopamine was used as a positive control for cAMP production because it stimulates adenylate cyclase coupled receptors and elevates the intracellular cAMP concentration in several arthropod muscles (Goy et al., 1984; Groome and Watson, 1989; Lange and Orchard, 1986; Mazzocco-Manneval et al., 1998; Nykamp and Lange, 2000). In *Idotea* fibres a 15 min application of octopamine (10 μmol l^{-1}) caused an almost threefold significant increase of the cAMP concentration ($P<0.01$, N=5, Fig. 1A). To exclude receptor desensitisation by 1 μmol l^{-1} proctolin in *Idotea* fibres, the intracellular cAMP concentration was measured after incubating the fibres with a lower proctolin concentration. 1 nmol l^{-1} proctolin did not cause a significant change in cAMP concentration compared to controls (0.24±0.08 pmol cAMP mg^{-1} protein, N=2; data not shown).

In order to adapt conditions in tension measurements, which were obtained after about 10 min of experimental setup and during 5 min application of high K^+, cAMP concentration was determined after equilibrating preparations for 10 min in ASW before application of drugs or ASW for controls. When

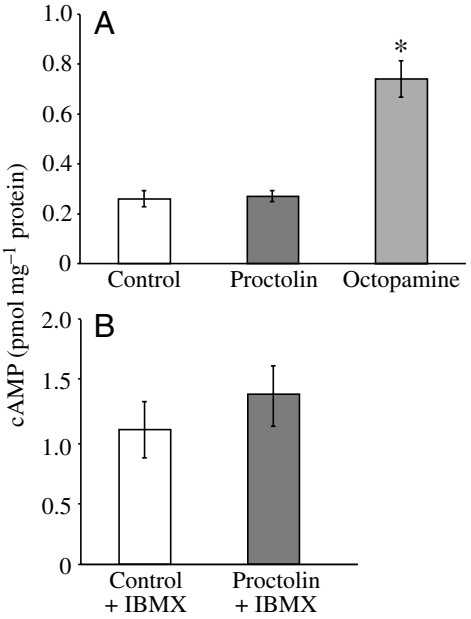

Fig. 1. Effects of proctolin and octopamine on the cAMP concentration in *Idotea* muscle fibres. (A) Without preequilibration in ASW, the cAMP concentration does not change significantly after 15 min of exposure to 1 μmol l^{-1} proctolin (*N*=8), whereas 15 min of exposure to 10 μmol l^{-1} octopamine increases the cAMP concentration significantly (*N*=5). (B) In fibres equilibrated for 10 min in ASW after dissection, 3 min of simultaneous exposure to 1 μmol l^{-1} proctolin and the phosphodiesterase inhibitor 0.5 mmol l^{-1} IBMX (*N*=8) does not change the cAMP concentration compared to controls with IBMX alone (*N*=8). **P*<0.05.

proctolin was applied either for 0.5 min or for 3 min after equilibration, the cAMP concentration was not significantly changed compared to controls (Table 1).

A possible proctolin-induced increase of cAMP could be masked by phosphodiesterases. To prevent this masking, the unselective phosphodiesterase inhibitor IBMX (0.5 mmol l^{-1}) was applied during the 3 min incubation period after equilibration. IBMX by itself increased the cAMP concentration in the *Idotea* fibres by 125% from 0.49±0.05 to 1.10±0.20 pmol cAMP mg^{-1} protein (Table 1, Fig. 1B). When fibres were incubated for 3 min with proctolin (1 μmol l^{-1}) in the presence of IBMX, no significant change in cAMP concentration was detected (Fig. 1B). 3 min application of octopamine (10 μmol l^{-1}) however, tested once in the presence of IBMX, caused a 8.2-fold increase in cAMP concentration by elevating the cAMP concentration to 8.99 pmol mg^{-1} protein (data not shown).

The proctolin-induced potentiation of contracture is not inhibited by H89, a PKA inhibitor

Exposing isolated short extensor muscle fibres of *Idotea* to high K^+ (30 mmol l^{-1}) induced a contracture of the fibres resembling that described for tonic flexor muscles in *Procambarus clarkii* (Bishop et al., 1987).

Idotea muscle fibres had resting potentials ranging from –64 to –88 mV, with a calculated mean of –70.1±1.2 mV (*N*=49). In the presence of normal saline, application of proctolin (1 μmol l^{-1}) for 5 min had no effect on tension or membrane potential (data not shown). A 5 min application of high-K^+ saline elicited a graded slow depolarisation by 24.98±1.55 mV (*N*=8) in the muscle fibres (Fig. 2A). This was accompanied by graded contractures of the fibres with a maximum amplitude of 142.35±48.49 μN (*N*=8). The contractures started at –63.9±9.9 mV (*N*=42) and persisted as long as high K^+ was present. On washing with normal saline, tension returned to the starting value. Proctolin (1 μmol l^{-1}) increased the amplitude of K^+-induced contracture but did not influence K^+-induced depolarisations (Fig. 2A,B). After washing for 30 min with saline, the amplitude of subsequent K^+-induced contracture returned to the level recorded before proctolin treatment. Although the maximal amplitude of K^+ contractures varied between experiments, this potentiating effect was consistently observed. On average, the amplitudes of K^+-induced contractures were significantly increased by 47±15.98% (*N*=8) in the presence of proctolin (Fig. 2B).

H89 (20 μmol l^{-1}), an inhibitor of PKA (Geilen et al., 1992), did not prevent the potentiation of contracture by proctolin (Fig. 2C,D). The potentiation of contracture was even about threefold higher in the presence of proctolin and H89 compared to controls (252±62.87%, *N*=4). In the absence of proctolin, H89 did significantly increase the amplitudes of K^+-induced contractures to 117±9% (*N*=6).

Table 1. *Summary of cAMP concentrations with a 10 min period of equilibration in ASW after dissection and different incubation times*

Incubation time (min)	[cAMP] (pmol mg^{-1} protein)	
	Control	Proctolin
0.5	0.91±0.17 (4)	0.91±0.22 (3)
3	0.49±0.05 (4)	0.56±0.08 (3)

Values are means ± s.e.m.
Number of experiments (*N*) is shown in parentheses.

Proctolin induces a decrease in the cGMP concentration, which is mediated by PKC

To test if proctolin influences the cGMP concentration in the *Idotea* muscle fibres, we measured intracellular cGMP concentrations after proctolin incubation. Proctolin reduced the cGMP concentration in the muscle fibres by 50% compared to controls (*P*<0.01, Fig. 3A). While the cGMP concentration of controls was at 24.81±1.3 fmol cGMP mg^{-1} protein (*N*=6), 1 μmol l^{-1} proctolin induced a decrease of the cGMP concentration to 12.32±2.1 fmol cGMP mg^{-1} protein (*N*=6) after an incubation time of 15 min.

When fibres were incubated for 15 min with proctolin (1 μmol l^{-1}) in the presence of the PKC inhibitor BIM-1 (10 nmol l^{-1}) or separately with BIM-1 (10 nmol l^{-1}), the intracellular cGMP concentrations of the fibres were not

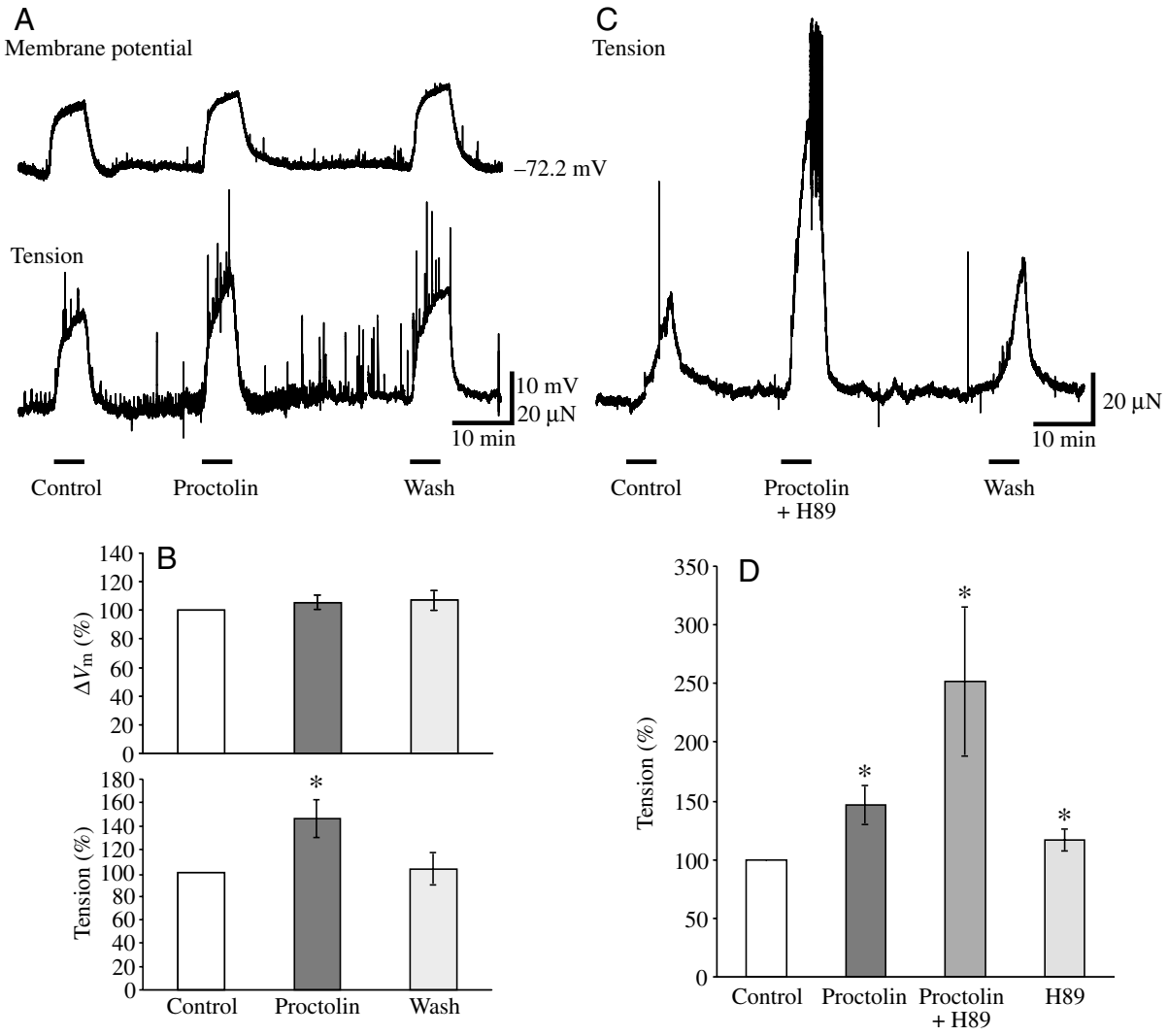

Fig. 2. Proctolin enhances the amplitude of high (30 mmol l^{-1}) K^+-induced contractures of *Idotea* muscle fibres. (A) Membrane potential (top) and tension (bottom) measured simultaneously before application of proctolin (control), in the presence of 1 µmol l^{-1} proctolin, and after washing off proctolin for 30 min. Control shows one of three K^+-contractures prior to the peptide tests. (B) Normalised membrane potentials and tensions (*N*=8) of the experiments as shown in A, indicating no significant change in membrane potential (top) and a 47% increase in the amplitudes of K^+-contractures with the peptide (bottom). (C) K^+-induced contractures measured before exposure to proctolin (control), after a 5 min application of 1 µmol l^{-1} proctolin in the presence of the PKA inhibitor H89 (1 µmol l^{-1}) for 5 min, and after washing off proctolin and H89 for 30 min. (D) Summary of independent experiments showing the maximal amplitude of contractures normalised to the controls. 1 µmol l^{-1} proctolin increases the amplitudes of contractures significantly (*N*=8). In the presence of 20 µmol l^{-1} H89, proctolin increases the amplitudes of contractures significantly compared to controls without H89 (*N*=4). H89 alone significantly increases the amplitudes of contractures (*N*=6). **P*<0.05.

significantly different from controls (Fig. 3A). These cGMP concentrations were 33.80±7.6 fmol cGMP mg^{-1} protein (*N*=6) and 27.70±3.19 fmol cGMP mg^{-1} protein (*N*=8), respectively.

To determine whether the proctolin-induced decrease of cGMP concentration is mediated by the activation of phosphodiesterases, the fibres were preincubated for 10 min with the unselective phosphodiesterase inhibitor IBMX (0.5 mmol l^{-1}) in ASW. The cGMP concentration of control fibres after applying IBMX for 3 min was 148.53±6.28 fmol cGMP mg^{-1} protein (*N*=3), indicating that IBMX inhibits cGMP degradation. The cGMP concentration of fibres stimulated with proctolin and IBMX for 3 min was 90.13±11.46 fmol cGMP mg^{-1} protein (*N*=4). The reduction by 39% in proctolin-stimulated fibres in the presence of IBMX was significant compared to control fibres with IBMX (*P*<0.05, Fig. 3B). This result shows that proctolin also induces a decrease in cGMP concentration if the hydrolysation of cGMP is inhibited by a phosphodiesterase inhibitor.

8-Bromo cGMP, a cGMP-analogue, decreases the proctolin-induced potentiation of contractures

To investigate if the proctolin-induced potentiation of contracture depends on the proctolin-induced reduction of the cGMP concentration, we applied the membrane-permeable and

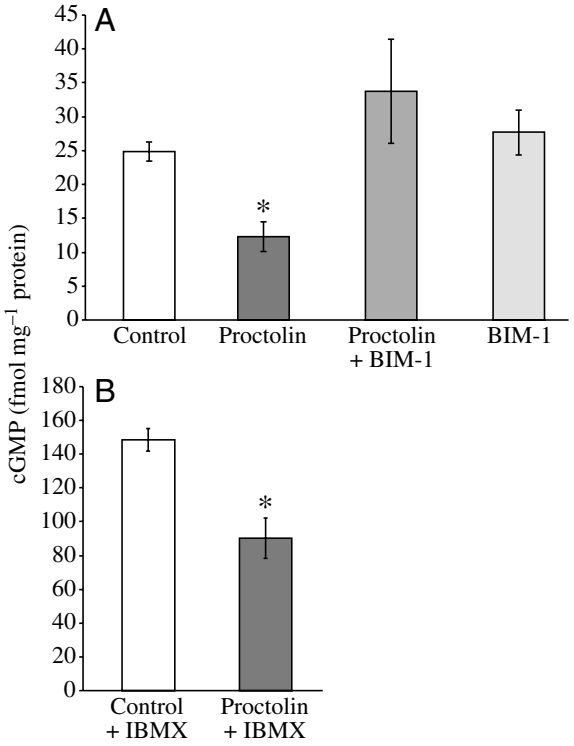

Fig. 3. Effect of proctolin on the cGMP concentration of *Idotea* muscle fibres. (A) The cGMP concentration is not reduced significantly after 15 min exposure to 1 μmol l^{-1} proctolin (N=6). In the presence of the PKC inhibitor BIM-1 (10 nmol l^{-1}), 15 min application of proctolin has no effect on the cGMP concentration (N=6). The cGMP concentration is not reduced in the presence of BIM-1 (N=8). (B) After a preincubation for 10 min with the unselective phosphodiesterase inhibitor IBMX (0.5 nmol l^{-1}), application of proctolin in the presence of IBMX for 3 min reduces the cGMP concentration in the fibres significantly compared to the cGMP concentration of control with IBMX (N=4, N=3, respectively). *P<0.05.

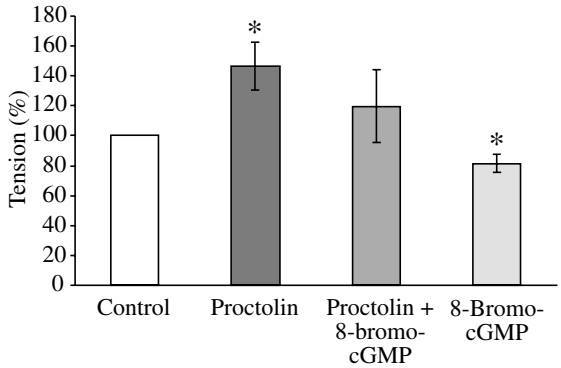

Fig. 4. The cGMP-analogue 8-bromo-cGMP reduces K^+-induced contractures and the proctolin-induced increase. Summary of independent experiments showing the maximum amplitude of contractures normalised to the controls: in the presence of 1 μmol l^{-1} proctolin (N=8), in the presence of proctolin and 20 nmol l^{-1} 8-bromo-cGMP (N=8) and in the presence of the cGMP analogue (N=8) during high K^+-saline application for 5 min. *Significantly different from controls, P<0.05.

phosphodiesterase-resistant cGMP analogue 8-bromo-cGMP in combination with proctolin under high-K^+-induced contracture conditions. In the presence of proctolin (1 μmol l^{-1}) and 8-bromo-cGMP (20 nmol l^{-1}) the amplitudes of K^+-induced contractures (119.61±24.32%; N=8) were not significantly different from controls (Fig. 4). 8-Bromo-cGMP (20 nmol l^{-1}) reduced the contracture amplitudes significantly to 81.46±6.09% (N=8; P<0.05).

Proctolin potentiates K^+-induced contractures via a PKC-dependent pathway

Applying the PKC inhibitor BIM-1 to the muscle fibres prevented the contracture-increasing effect of proctolin (Fig. 5). The amplitudes of the K^+-induced contractures in the presence of proctolin (1 μmol l^{-1}) and BIM-1 (10 nmol l^{-1}) did not significantly differ from controls (105±13.14%, N=5). The inhibitor BIM-1 had no significant effect on the amplitudes of the K^+-induced contractures (102±12.9%, N=4). Addition of 1 μmol l^{-1} PMA, a PKC activator, to the muscle fibres significantly potentiated the K^+-induced contractures by 51±13.6% (N=7; Fig. 5). This effect of PMA was not reversible within 60 min. The potentiation of contracture by PMA is similar to the potentiation induced by proctolin.

Discussion

Cellular mechanisms in postsynaptic modulation of contractions by proctolin

In this study, the role of the cyclic nucleotides cAMP and cGMP as mediators of the potentiating effect of proctolin on muscle contractures was investigated. By possibly binding to a G-protein-coupled receptor (Egerod et al., 2003; Johnson et al., 2003), proctolin might exert its actions through subsequent

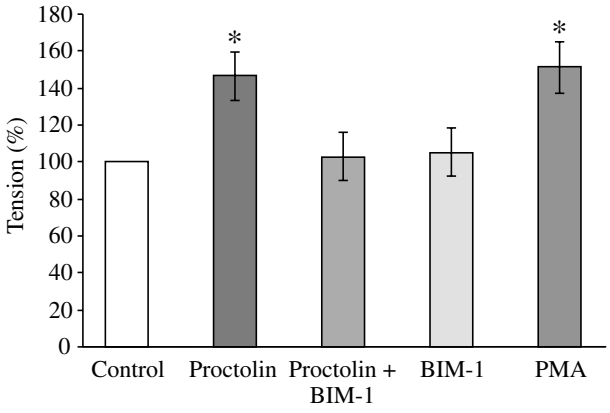

Fig. 5. The proctolin-induced potentiation of K^+-induced contracture is prevented by the PKC inhibitor BIM-1 and mimicked by the PKC activator PMA. Summary of independent experiments showing the maximum amplitude of contractures normalised to controls: in the presence of 1 μmol l^{-1} proctolin (N=8), in the presence of proctolin and 10 nmol l^{-1} BIM-1 (N=5), in the presence of BIM-1 (N=4) and in the presence of 1 μmol l^{-1} of the PKC activator PMA (N=7) during high K^+-saline application for 5 min. *Significantly different from controls, P<0.05.

induction of a signalling cascade modulating a number of different cellular targets. The peptide exerts its postsynaptic action in arthropod muscles through several cellular mechanisms, which determine the amplitude of contractions. It is well established that contractions of crustacean muscles are dependent on influx of extracellular Ca^{2+} (Gainer, 1968; Ashley and Ridgway, 1970; Atwater et al., 1974) and subsequent Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum (Goblet and Mounier, 1986; Györke and Palade, 1992).

In *Idotea,* proctolin affects sarcolemmal processes as well as intracellular processes, which are independent of depolarization of the membrane. Contractures that are induced by Ca^{2+} release from intracellular stores due to caffeine application are increased by 27% in the presence of proctolin (Brüstle et al., 2001). The intracellular mechanisms might include an augmented Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum (SR), as in the cockroach hyperneural muscle (Wegener and Nässel, 2000) and the modulation of Ca^{2+} sensitivity of sarcoplasmic proteins (Brüstle et al., 2001).

However, it is unlikely that activation of the proctolin receptor only exerts its effect on tension downstream of depolarization-dependent mechanisms, because in the present study proctolin increases high K^+-induced contractures by 47%. Presumably, the modulation of intracellular mechanisms is accomplished by mechanisms that are activated by depolarization of the sarcolemma.

Contractions that are evoked by depolarization-induced activation of sarcolemmal ion channels are strongly potentiated in the presence of proctolin (Bishop et al., 1987; Bishop et al., 1991; Erxleben et al., 1995). In voltage clamp studies, short depolarizing steps elicited contraction of *Idotea* muscle fibres only at membrane potentials above –40 mV, corresponding to the activation threshold of L-type Ca^{2+} channels (Erxleben et al., 1995; Weiss et al., 2001). Currents through these channels are enhanced by proctolin (Rathmayer et al., 2002b). In our experiments, contractures had already been obtained with long-lasting high K^+-induced depolarisations of membranes to potentials well below –40 mV. Sporadic openings of single L-type Ca^{2+} channels already occur about 25 mV above membrane resting potential (Erxleben and Rathmayer, 1997). We assume that within long-lasting K^+-induced depolarisations these openings allow for Ca^{2+} influx contributing to enhancement of contractures by proctolin. Another possible explanation is the presence of other Ca^{2+} currents, which were inaccessible in preceding studies. A Ca^{2+} current activated by multiple peptides and well below the activation threshold of L-type currents was previously found in neurons in the stomatogastric ganglion of a crab (Swensen and Marder, 2000).

In addition to the enhancement of sarcolemmal Ca^{2+} channels, proctolin closes non-voltage-dependent K^+ channels in *Idotea* and in locusts (Erxleben et al., 1995; Walther et al., 1998). Consequently, proctolin should depolarise the membrane potential of muscle fibres. In our study, proctolin neither depolarised the resting membrane potential nor increased the magnitude of the depolarisation induced by 30 mmol l^{-1} KCl. We assume that proctolin activates additional outward currents, counteracting the depolarising effect of the Ca^{2+}-influx and of the closure of non-voltage-dependent K^+-channels. However, Ca^{2+}-dependent depolarisation of the sarcolemmal membrane was observed after application of crustacean FMRF-related peptide DF_2 to *Idotea* fibres (Weiss et al., 2003). The differential modulation of ion channels in identical cells may indicate that the two peptides confer their action through different signalling pathways.

Postsynaptic mechanisms of proctolin are independent of PKA activation by cAMP

Intracellular signalling pathways involved in the proctolin-induced increase of contractions in the extensor muscles of *Idotea emarginata* by modulation of several cellular targets (Brüstle et al., 2001; Erxleben et al., 1995; Erxleben and Rathmayer, 1997; Rathmayer et al., 2002a,b) are not well understood so far. Several lines of evidence from studies with different arthropod muscles led to the suggestion that an increase of the second messenger cAMP and possibly subsequent activation of PKA might mediate the postsynaptic effects of proctolin on contraction amplitude (Bishop et al., 1991; Evans, 1984; Swales and Evans, 1988). In *Idotea,* agonists of the cAMP signalling pathway mimicked and antagonists of the cAMP/PKA signalling pathway counteracted the effects of proctolin on sarcolemmal Ca^{2+} and K^+ currents, respectively. It was suggested that proctolin increases contractions in *Idotea* by PKA-dependent phosphorylation of postsynaptic cellular targets (Erxleben et al., 1995; Rathmayer et al., 2002b). To test this assumption, we investigated the role of PKA in the regulation of depolarization-evoked contractures and measured intracellular concentration of the cyclic nucleotide cAMP. Evidence that proctolin does not activate PKA in *Idotea* muscle comes from experiments where we studied the proctolin-induced potentiation of muscle tension in the presence of the PKA inhibitor H89. We show that the inhibition of PKA increases rather than decreases the amplitude of evoked contractures and that the potentiation of high K^+-induced contractures by proctolin was strikingly increased rather than abolished by H89. An increase in contraction amplitude upon inhibition of PKA was previously observed in an insect visceral muscle (Wegener and Nässel, 2000). The inhibitory effect of the unspecific protein kinase blocker H7 on proctolin-increased Ca^{2+} currents (Rathmayer et al., 2002b) could either be due to effects of protein kinases other than PKA or to converging action of different protein kinases on the same current. The increase in contraction force in the presence of PKA inhibitors could be due to a cross-talk regulation between different signalling cascades. In fact, our experiments demonstrate that the cellular mechanisms underlying the effects of proctolin on muscle contraction as well as on protein phosphorylation (Brüstle et al., 2001) are not dependent on activation of PKA.

We subsequently asked the question, whether the proctolin-induced signalling cascade involves an elevation of cAMP

without activation of PKA. We show that proctolin does not increase the cAMP concentration in single muscle fibres of *Idotea*. Our results imply that proctolin neither stimulates adenylate cyclase nor inhibits phosphodiesterase activity. It should be noticed that the phosphodiesterase inhibitor IBMX increases the cAMP concentration of the muscle fibre in *Idotea*, suggesting that cAMP-degrading phosphodiesterases are active at a basic level, as observed in other crustacean muscles (Goy et al., 1984). We conclude that the potentiating effect of proctolin does not rely on elevation of the cAMP concentration in the *Idotea* extensor muscle, which is consistent with findings in many other arthropod muscles. Proctolin failed to increase intracellular cAMP levels in muscles of insects and crustaceans (Baines and Downer, 1992; Evans and Myers, 1986; Goy et al., 1984; Groome and Watson, 1989; Mazzocco-Manneval et al., 1998).

However, it is evident that experimentally increased cAMP and proctolin have parallel effects on non voltage-dependent K^+ channels and on L-type Ca^{2+} channels (Erxleben et al., 1995; Rathmayer et al., 2002b). This might be explained by convergent action of different signalling cascades on the same target.

Proctolin-induced reduction of cGMP mediates potentiation of muscle contractures and is dependent on activation of PKC

It is well known from vertebrate smooth muscle that an increase in intracellular cGMP concentration leads to muscle relaxation (Lucas et al., 2000). Therefore, we considered the possibility that a decrease in cGMP concentration might accordingly cause the potentiation of muscle tension in crustacean muscle fibres. Only a few studies exist in which the intracellular cGMP concentration of arthropod muscles was measured after proctolin stimulation. We show that proctolin induces a significant decrease of intracellular cGMP concentration in muscle fibres of the isopod crustacean *Idotea emarginata*, which is consistent with previous reports that proctolin significantly reduced the cGMP concentration in *Limulus polyphemus* muscles in a dose-dependent manner (Groome and Watson III, 1989) and caused a slight decrease in guanylate cyclase activity in the brain of *Locusta migratoria* (Hiripi et al., 1979). However, proctolin failed to induce a significant decrease of the cGMP concentration in the opener muscle of the lobster walking leg (Goy et al., 1984).

We provide two lines of evidence suggesting that the reduction of cGMP in *Idotea* is a key event in the response of muscle fibres to proctolin. First, the membrane-permeable and phosphodiesterase-resistant cGMP-analogue 8-bromo-cGMP diminishes the amplitude of evoked contractures of the extensor muscle fibres. Second, by counteracting the proctolin-induced reduction of the intracellular cGMP concentration, 8-bromo-cGMP reduces the proctolin-induced potentiation of muscle contraction. Although our data suggest that the proctolin-induced reduction of cGMP concentration mediates at least some of the postsynaptic mechanisms leading to the proctolin-induced increase in contractures, as yet we have no evidence to determine which of the mechanisms are affected.

From vertebrate smooth muscle cells, it is known that PKC reduces the activity of the guanylate cyclase, which is responsible for the build-up of cGMP levels (Jaiswal, 1992). A role of PKC in proctolin-induced signalling cascades has already been proposed for the oviduct and the skeletal muscle of *Locusta migratoria* (Baines and Downer, 1992; Lange and Nykamp, 1996; Walther et al., 1998), the foregut of *Schistocerca gregaria* (Hinton et al., 1998) and the heart muscle of *Limulus polyphemus* (Groome and Watson, 1989). Activation of PKC also induces extracellular Ca^{2+}-dependent contraction of cockroach hyperneural muscle, resembling that evoked by proctolin (Wegener and Nässel, 2000). As known from vertebrate heart muscle cells, PKC-dependent phosphorylation increases the probability of open L-type Ca^{2+} channels (Keef et al., 2001), similar to the action of proctolin in *Idotea* muscles. The potentiation of contracture amplitude in *Idotea* by proctolin was mimicked by a PMA-induced activation of PKC and abolished by the PKC inhibitor BIM-1. It should be noted that basic levels of PKC activity do not contribute to the magnitude of K^+-induced contractures because the PKC inhibitor only reduced the contracture after applying proctolin. Activation of PKC implicates the involvement of a phospholipase C-linked receptor and therefore, an increase of intracellular $InsP_3$ concentration. The total abolishment of the proctolin-induced enhancement of contracture amplitude by the PKC inhibitor suggests that the proctolin-induced production of $InsP_3$ might only have an effect on the time course of K^+-induced contractions in skeletal muscle fibers of *Idotea*. The PKC inhibitor BIM-1 also abolished the proctolin-induced reduction of cGMP concentration, whereas the phosphodiesterase inhibitor IBMX did not prevent the reduction of intracellular cGMP levels. Thus, proctolin reduces the activity of guanylate cyclase *via* activation of PKC and does not interfere with cGMP-reducing phosphodiesterases. Activation of PKC is necessary for all cellular mechanisms contributing to the potentiation of contraction amplitude, including a reduction of cGMP concentration.

Therefore, phosphorylation of a 30 kD protein, augmentation of Ca^{2+}-release from the SR, closing of non-voltage sensitive K^+ channels and the increase in probability of open Ca^{2+} channels underlying the proctolin-induced enhancement of contracture amplitude are dependent on activation of PKC and might be independent of $Ins\mathit{P}_3$ production in skeletal muscle fibres of *Idotea*.

In conclusion, our results confirm that in a crustacean skeletal muscle, proctolin does not influence PKA-activity. Rather it induces an activation of PKC, leading to a reduction in cGMP and a potentiation of muscle contraction.

List of abbreviations

ASW	artificial seawater
BIM-1	bisindolylmaleimide-1
cAMP	3′,5′-cyclic adenosine monophosphate
cGMP	3′,5′-cyclic guanosine monophosphate
DMSO	dimethyl sulfoxide
H89	H89-dihydrochloride
IBMX	3-isobutyl-1-methylxanthin

$InsP_3$	inositol 1,4,5-trisphosphate
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol-12-myristate-13-acetate
RYLPT	myotropic pentapeptide proctolin
SR	sarcoplasmic reticulum

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