

THE NEUROPEPTIDE PROCTOLIN INDUCES PHOSPHORYLATION OF A 30 kDa PROTEIN ASSOCIATED WITH THE THIN FILAMENT IN CRUSTACEAN MUSCLE

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Summary

In the isopod *Idotea emarginata*, the neuropeptide proctolin is contained in a single pair of motoneurones located in pereion ganglion 4. The two neurones supply dorsal extensor muscle fibres of all segments. Proctolin ($1 \mu\text{mol l}^{-1}$) potentiates the amplitude of contractures of single extensor muscle fibres elicited by 10 mmol l^{-1} caffeine. In western blots of myofibrillar proteins isolated from single muscle fibres and treated with an anti-phosphoserine antibody, a protein with an apparent molecular mass of 30 kDa was consistently found. The

phosphorylation of this protein was significantly increased by treating the fibres with proctolin. After separation of myofibrillar filaments, a 30 kDa protein was found only in the thin filament fraction. This protein is phosphorylated and detected by an antiserum against crustacean troponin I.

Key words: *Idotea emarginata*, nervous system, motoneurone, modulation, actin, myosin, troponin I, contraction, caffeine.

Introduction

The penta-peptide proctolin (amino acid sequence RYLPT) was initially isolated from the hindgut of the cockroach *Periplaneta americana* (Brown and Starrat, 1975) and was subsequently localised in nervous systems of a number of arthropods. In insects and crustaceans, it is contained within identified neurones as a cotransmitter being released in the central nervous system, at neuromuscular synapses and from neurohaemal organs (O'Shea, 1985; Siwicki et al., 1987; Orchard et al., 1989). Proctolin causes graded contractures of some visceral and skeletal muscles in insects and crustaceans (Schwarz et al., 1980; Orchard et al., 1989; Baines and Downer, 1992). It also potentiates contractions of skeletal or visceral muscles either when produced myogenically (Evans and Myers, 1986; Lange et al., 1986), or upon activity of motoneurones or upon depolarisation by high K^+ or current (Orchard et al., 1989; Baines and Downer, 1992; Belanger and Orchard, 1993; Erxleben et al., 1995; Facciponte et al., 1996; Jorge-Rivera et al., 1998).

The mechanisms underlying the potentiations involve both pre- and postsynaptic actions. Presynaptically, proctolin can increase transmitter output at some neuromuscular endings (Belanger and Orchard, 1993; Pasztor and Golas, 1993; Rathmayer et al., 2001). Postsynaptically, proctolin closes non-voltage-dependent K^+ channels (Erxleben et al., 1995; Baines et al., 1996; Walther et al., 1998) resulting in an increase of input resistance and time constant of the muscle membrane and thus in stronger summation of excitatory junctional potentials. It also stimulates an influx of Ca^{2+} into muscle fibres (Baines and

Downer, 1992; Wilcox and Lange, 1995) by increasing the open probability of voltage-sensitive sarcolemmal Ca^{2+} channels (Bishop et al., 1991a; Erxleben and deSantis, 1998). The pre- and postsynaptic effects of proctolin add synergistically to potentiate contractions of muscle fibres (for summary of the combined effects in muscle fibres of the isopod *Idotea emarginata* and *Idotea baltica*, see Rathmayer et al., 2001).

There is some indication that the effects of proctolin on sarcolemmal ion channels may be mediated by protein phosphorylation via a cAMP signalling pathway: activators or inhibitors of this pathway mimic or inhibit the effects of proctolin, respectively (Evans, 1984; Bishop et al., 1991a; Erxleben et al., 1995; Erxleben and deSantis, 1998). In some muscles, however, proctolin increases inositol trisphosphate (InsP_3) and may activate protein kinase C because InsP_3 and phorbol esters mimic the potentiating effect of proctolin on contractions (Baines and Downer, 1992; Lange and Nykamp, 1996; Wegener and Nässel, 2000). The action of proctolin on sarcolemmal ion channels with different ion selectivity, the variety of possible signalling pathways and the finding that multiple proctolin receptor subtypes exist in insect visceral muscles (Baines et al., 1996; Mazzocco-Manneval et al., 1998) suggest that additional intracellular targets could be involved in mediating the proctolin-induced potentiation of contractions. Since it is now widely accepted that muscle contractions are modulated by reversible phosphorylation of target proteins in the sarcolemma, sarcoplasmic reticulum (SR) and myofilaments, for example, myosin light chains (Bárány

and Bárány, 1980; Horowitz et al., 1996, for review), we studied the effect of proctolin on phosphorylation of myofibrillar proteins in a crustacean.

The marine isopod *I. emarginata* has the advantage that several neuropeptides can be localised in identified motoneurons (Kreissl et al., 1999; Rathmayer et al., 2001) and the effect of modulators on isolated membrane currents under voltage-clamp conditions and the effects on contractions can be analysed in single identified muscle fibres (Erxleben et al., 1995; Kreissl et al., 1999; Rathmayer et al., 2001). In the present study we show that proctolin also potentiates muscle contractures elicited by caffeine, which is known to induce Ca^{2+} release from internal stores via activation of the ryanodine-sensitive Ca^{2+} channel of the SR of crustacean muscles (Lea, 1996; Zhang et al., 1999). By studying phosphorylation of myofibrillar proteins we show that proctolin induces phosphorylation of an intramuscular 30 kDa protein. After separation of myofilaments, a 30 kDa protein was found only in the thin filament fraction. This protein is phosphorylated and detected by an antiserum against lobster troponin I₃. We also show that proctolin immunoreactivity is associated with extensor muscle fibres of *I. emarginata* and identify two single motoneurons as the centre of origin of this peptidergic innervation.

Materials and methods

Animals and saline

All experiments were performed on adult males of *Idotea emarginata* Fabricius 1798 raised in the animal facility of the University of Konstanz.

For all experiments, the saline had a composition of (in mmol l^{-1}): 490 NaCl, 8 KCl, 10 CaCl_2 , 12 MgCl_2 , 10 Hepes. Proctolin (Sigma, Deisenhofen, Germany) was dissolved in distilled water at a concentration of 1 mmol l^{-1} . Samples of the stock solution were stored at -20°C and diluted in saline prior to experiments.

Preparation and protocols for immunocytochemistry

Anatomy and preparation of the dorsal extensor muscles have been described previously (Kreissl et al., 1999). Specimens were fixed in 4% paraformaldehyde, 0.5% glutaraldehyde in 100 mmol l^{-1} Tris (pH 7.4) for 3 h at room temperature (approximately 22°C) and washed in saline-substituted Tris buffer (SST: 160 mmol l^{-1} NaCl, 50 mmol l^{-1} Tris, pH 7.6) with 0.1% Triton X-100 (TRX; washing buffer) with a last wash continued overnight. The preparation was enzymatically permeabilised for 20 min with 0.5 mg collagenase, 0.5 mg hyaluronidase in $1 \text{ ml } 10 \text{ mmol l}^{-1}$ Tris (pH 7.4), and extensively rinsed in washing buffer for 6 h. Non-specific binding of the primary antiserum was blocked by preincubation in blocking solution (SST, 0.1% TRX, 0.5% BSA) for 1 h and microbial contamination of the specimens was prevented by adding 0.02% sodium azide (Fluka, Buchs, Switzerland) to incubation and washing solutions. Incubation of the specimens in the polyclonal anti-proctolin serum, diluted

1:20,000 in blocking solution, was continued for 48 h. The antiserum was a kind gift from H.-J. Agricola (University of Jena) and was previously used for crayfish (Utting et al., 2000). After washing for 2.5 h, the biotinylated anti-rabbit secondary antibody (ABC kit, Vector Laboratories, Burlingame, CA, USA) was applied at a dilution of 1:1,000 in blocking solution overnight. After three washing cycles in washing buffer without sodium azide, 2 h each, the horseradish peroxidase-containing ABC-complex (each compound diluted 1:500 in washing buffer) was allowed to bind for 12 h. Colour development was obtained with 0.001% diaminobenzidine and 0.002% ammonium nickel sulphate (Fluka, Buchs, Switzerland) in 10 mmol l^{-1} Sorensen's phosphate buffer (pH 7.6) and in the presence of 0.0001% H_2O_2 . Omitting the anti-proctolin serum and preincubating the primary serum with $10 \mu\text{mol l}^{-1}$ proctolin resulted in no staining. Backfills of motoneurons with Lucifer Yellow and immunofluorescent labelling were performed as described previously (Kreissl et al., 1999). Conventional micrographs were taken using epifluorescence with the appropriate filter combinations. To merge images of double-stained tissue, the micrographs were digitised and modified only to enhance contrast using Adobe Photoshop.

Contraction measurements

Isometric contraction measurements were performed at 18°C with the short extensor fibres 1 and 2 of pereion segment 7 as described previously (Erxleben et al., 1995; Kreissl et al., 1999). Contractions were evoked by application of 10 mmol l^{-1} caffeine (Sigma, Deisenhofen, Germany) in saline. After each caffeine contracture, the fibres were kept for 20 min in saline to allow Ca^{2+} reloading into the SR. Before each proctolin application, at least three control measurements were taken. Only preparations with an equal peak amplitude of caffeine contractures were used. When caffeine contractures in the presence of proctolin were performed, the muscles were incubated in proctolin ($1 \mu\text{mol l}^{-1}$) for 25 min before the addition of caffeine.

Preparation of dorsal extensor fibres for biochemistry

For biochemical investigations the animals were chilled and the sternites of the pereion, 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. After exposure of the extensor muscle fibres, saline was replaced for 15 min with fresh saline at 18°C , which contained either $1 \mu\text{mol l}^{-1}$ proctolin in tests or no proctolin in controls. Subsequently, the preparation was cooled on ice and the long fibres 5 (for nomenclature and anatomy, see Kreissl et al., 1999) were dissected and immediately frozen and stored in liquid nitrogen.

SDS electrophoresis

Fibres of the controls and tests were pooled in separate groups. The fibres were homogenised for 20 min in ice-cold extraction buffer (in mmol l^{-1} : 100 $\text{Na}_4\text{P}_2\text{O}_7$, 2.5 EDTA, 15

MgCl₂, 1 Pefabloc, 5 DTT; modified according to Martinez et al., 1990). Ten fibres homogenised in 140 µl extraction buffer, yielded a protein concentration of 3–4 mg ml⁻¹. After homogenisation, the homogenate was centrifuged for 4 min at 1200 g to remove insoluble material. The supernatant was analysed for protein content (Bradford, 1976). The protein concentration of different samples on each gel was kept identical in order to enable comparisons between the samples. The supernatant of the homogenate was mixed with extraction buffer to obtain the desired protein concentration. Sample buffer (60 mmol l⁻¹ Tris, 10 % glycerol, 5 % SDS, 400 mmol l⁻¹ 2-mercaptoethanesulfonic acid (MESNA), 0.04 % Bromophenol Blue; pH 6.8) was added in a ratio of 1:1. In addition, 100 mmol l⁻¹ Pefabloc and 490 mmol l⁻¹ DTT were added in a ratio of 1:100. To solubilize proteins, samples were heated to 100 °C for 10 min. Prior to electrophoresis, the samples were centrifuged for 3 min at 1200 g.

SDS-gel electrophoresis was performed using 15 % polyacrylamide gels. For each sample, about 30 µg of protein were electrophoretically separated. Electrophoresis was run in a Mini-Protean II chamber (Bio-Rad, München, Germany) at 180 V and at room temperature. Gels were silver stained (Heukeshoven and Dernick, 1988).

Preparation of actomyosin, myosin and thin filaments

The preparation of actomyosin is a variant of the technique of Offer et al. (Offer et al., 1973). 25 identified muscle fibres (fibre 5) were transferred after dissection into 400 µl slightly modified Guba–Straub solution (in mmol l⁻¹: 300 NaCl, 100 NaH₂PO₄, 50 Na₂HPO₄, 1 MgCl₂, 10 Na₄P₂O₇, 10 EDTA, 15 NaN₃, 100 MESNA); 12 µl Pefabloc (100 mmol l⁻¹) was added and the fibres were homogenised for 15 min on ice. The extraction was continued by vigorous stirring for 90 min at 4 °C. The muscle homogenate was then centrifuged at 15 000 g for 10 min. The supernatant fluid was removed and 4 ml of 1 mmol l⁻¹ EDTA, 100 mmol l⁻¹ MESNA were added to the supernatant. The proteins were allowed to precipitate at 4 °C for 1 h. Following precipitation, the homogenate was centrifuged again for 10 min at 15 000 g, the pellet was dissolved in 500 µl 100 mmol l⁻¹ Na₄P₂O₇, 4 mmol l⁻¹ EDTA, 3 mmol l⁻¹ DTT, 12 mmol l⁻¹ ATP and gently stirred for 1 h at 4 °C. Following centrifugation for 2 h at 105 000 g, the supernatant, which contained actomyosin, was prepared for SDS gel electrophoresis as described above.

Myosin and myosin light chains were isolated according to the method of Kendrick-Jones et al., for invertebrate muscle fibres, with slight modifications (Kendrick-Jones et al., 1976). 50 fibres (fibre 5) were homogenised in 100 µl 6 mol l⁻¹ guanidine-HCl, 5 mmol l⁻¹ DTT, 2 mmol l⁻¹ EDTA, 10 mmol l⁻¹ (pH 8.0) for 10 min. The extract was then stirred overnight at 4 °C. The extract was mixed with 100 µl cold distilled water and 800 µl cold ethanol was added slowly to precipitate the myosin heavy chains. After stirring for 30 min at 4 °C, the homogenate was centrifuged for 30 min at 3200 g and 4 °C. The pellet contained the myosin (mostly myosin heavy chains and less myosin light chains) and was dissolved

in cold extraction buffer (in mmol l⁻¹: 100 Na₄P₂O₇, 2.5 EDTA, 15 MgCl₂, 1 Pefabloc, 5 DTT; modified according to the protocol of Martinez et al., 1990). To obtain the myosin light chains, the ethanol was removed from the supernatant with a rotary evaporator at room temperature, and guanidine-HCl was removed by dialysis against 25 mmol l⁻¹ Tris-HCl, pH 8.0, 0.5 mmol l⁻¹ DTT.

Proteins of the thin filament were prepared following the procedure for crayfish abdominal muscle (Watanabe et al., 1982). The fibres were homogenised in 50 mmol l⁻¹ KCl, 1 mmol l⁻¹ MgCl₂, 6.7 mmol l⁻¹ sodium phosphate buffer, 100 µmol l⁻¹ EDTA, 5 mmol l⁻¹ ATP (pH 6.0). The extract was centrifuged at 85 000 g for 20 min and the supernatant was further centrifuged at 105 000 g for 2 h. The pellet was suspended in 50 mmol l⁻¹ KCl, 1 mmol l⁻¹ MgCl₂, 100 µmol l⁻¹ CaCl₂, 500 µmol l⁻¹ DTT, 10 mmol l⁻¹ Tris (pH 6.8).

Immunoblotting

After electrophoresis, proteins were transferred to nitrocellulose membranes (NC membranes) using a Trans-Blot SD Semi-Dry Transfer Cell (1 h at room temperature, 0.8 mA cm⁻²; Bio-Rad, München, Germany). The NC membrane was equilibrated for 5 min in Tris-saline-Tween (TST: 100 mmol l⁻¹ Tris, pH 7.6, 0.9 % NaCl, 0.01 % Tween 20) and then kept in blocking buffer for 2 h.

For blots probed with the anti-phosphoserine antibody, a blocking buffer with 0.5 % bovine serum albumin (BSA), 1 % polyvinyl-pyrrolidone (PVP), 1 % polyethylene glycol (PEG 3500), 0.2 % Tween 20, 10 mmol l⁻¹ NaF in phosphate-buffered saline (PBS: 1.9 mmol l⁻¹ NaH₂PO₄, 8.1 mmol l⁻¹ Na₂HPO₄, 154 mmol l⁻¹ NaCl, pH 7.4) was used. After removal of the blocking buffer, the NC membrane was incubated overnight at room temperature in blocking buffer containing 1 µg ml⁻¹ anti-phosphoserine monoclonal antibody (clone 4A3; Biomol, Hamburg, Germany). The NC membrane was then washed for 1 h with TST and incubated for 90 min at room temperature in biotinylated anti-mouse immunoglobulin M diluted in blocking buffer (1:500). The NC membrane was washed again for 1 h with TST and treated with an avidin-biotin-horseradish peroxidase complex (ABC kit, Vector Laboratories, Burlingame, CA, USA) diluted in TST (1:500). Subsequently, the NC membrane was washed with Tris-saline for 1 h, incubated with a developing solution (ECL, Pierce, Rockford, IL, USA) for 5 min and placed against autoradiographic film for exposure. The densitometric evaluation of the phosphorylated bands was carried out according to the BIOMETRA evaluation-technique allowing the determination of the extinction of a fixed area. This area was constant for all the evaluated proteins. Deviations were calculated as standard deviations (S.D.). Statistical evaluation was carried out using a Student's *t*-test, assuming statistical significance when *P* < 0.05.

For blots probed with the anti-troponin I₃ antiserum (Sohn et al., 2000) a blocking buffer with 5 % nonfat milk powder in TST was used. After blocking, blots were incubated for 1 h with the anti-troponin I₃ antibody, diluted 1:5000 in TST. The

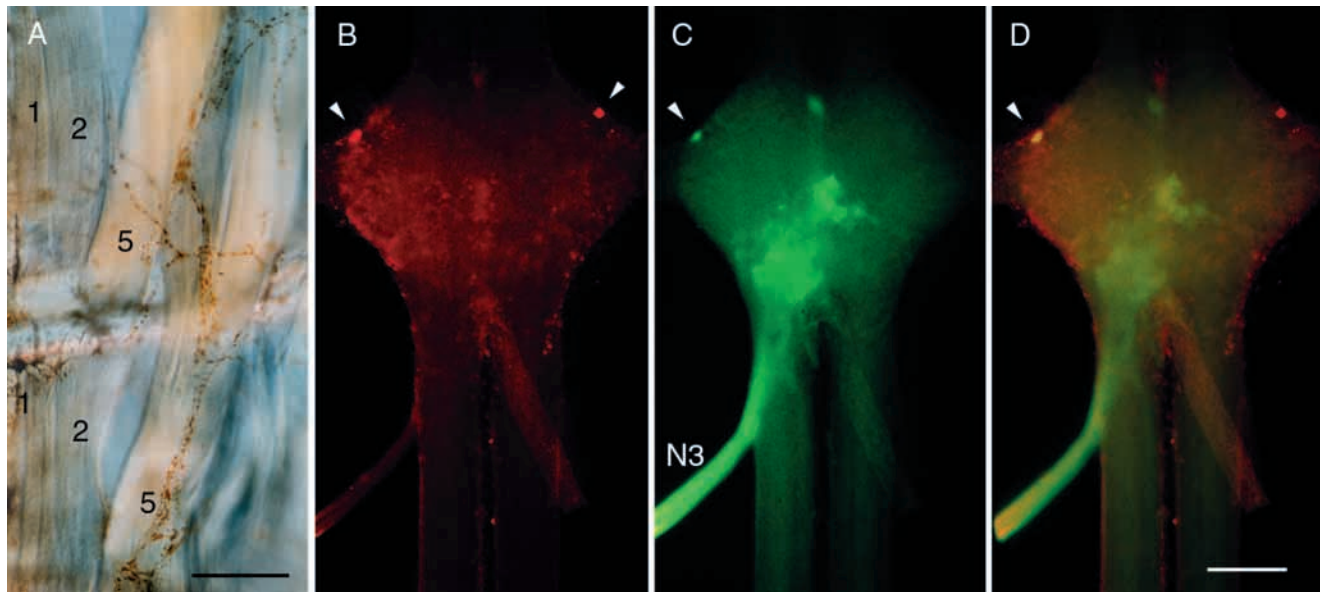


Fig. 1. Proctolin immunoreactivity on extensor muscles and in pereion ganglion 4 of *I. emarginata*. (A) Immunostained varicose axons extend along the inner layer of long fibres in pereion segment 7 and pleon segment 1. Numbers indicate identified muscle fibres. Anterior is up in all parts of the figure. Scale bar, 200 μm . (B) Proctolin immunostaining reveals a symmetric pair of neurons (arrowheads) in the anterior part of the ganglion in pereion segment 4. (C) Somata of efferent neurons projecting through N3 in the same ganglion, stained by retrograde labelling with Lucifer Yellow (arrowhead indicates the anterior motoneurone). (D) Double exposure of the specimen reveals the double labelled proctolin-ir motoneurone (arrowhead). Scale bar, 100 μm .

binding of the troponin I₃ antibody was detected as above, using a biotinylated anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA) and ECL.

Results

Proctolin-immunoreactive innervation of dorsal extensor muscles

The bilaterally symmetric dorsal extensor muscles in each segment of *I. emarginata* consist typically of a superficial dorsal layer of three to four short fibres spanning one segment, and an internal layer of two to four fibres (depending on the segment) spanning two segments (for details, see Kreissl et al., 1999). A single proctolin-immunoreactive (proctolin-ir) axon accompanies the internal layer of long muscle fibres of all pereional segments. This axon runs within the dorsal longitudinal nerve which contains the axons of other segmental motoneurons and of intersegmental modulatory neurons. The proctolin-ir axon forms numerous ovoid varicosities but only very few arborisations with bouton-like endings directly on the muscle fibres. In the pereion segment 7 and in the pleon segments 1 and 2, a plexus of varicose immunoreactive axons is present on all extensor muscle fibres (Fig. 1A). This meshwork is not in direct contact with the muscle fibres and may be a neurohaemal release site for proctolin.

The extensor muscle fibres are innervated by motoneurons located in the segmental ganglia. Their axons project through the posterior segmental nerves 3 (N3). In all seven specimens examined, a symmetric anterior lateral pair of proctolin-ir somata was detected only in pereion segment 4 (Fig. 1B).

Backfilling extensor motoneurons in pereion segment 4 with Lucifer Yellow through N3 visualised several groups of motoneurons and a single ipsilateral neurone in the anterior lateral part of the ganglion (Fig. 1C). Double exposure of ganglia containing backfilled somata, which were subsequently stained using indirect rhodamine immunofluorescence, revealed that this anterior lateral motoneurone contains proctolin (Fig. 1D). Thus the proctolin-ir innervation of the extensor muscles in all segments is provided by a single pair of central neurons in pereion ganglion 4.

Proctolin potentiates caffeine-induced contractures

When isolated short extensor muscle fibres of *I. emarginata* were exposed to 10 mmol l⁻¹ caffeine, a contracture of the fibres resembling that described for other crustacean muscles was observed (Huddart, 1969; Chiarandini et al., 1970). The contracture persisted as long as caffeine was present (50 s). During a washing period of 20 min, tension returned to the starting values, before further caffeine exposure. Proctolin (1 $\mu\text{mol l}^{-1}$) increased the rate of development and the amplitude of the caffeine-induced contracture (Fig. 2A). After washing for 35 min with saline, the amplitude of subsequent caffeine-induced contractures returned to the level recorded before proctolin treatment (Fig. 2A,B). Although the maximal amplitude of caffeine contractures varied between experiments, this potentiating effect was consistently observed. On average, the amplitudes of caffeine contractures were significantly increased by $27 \pm 7.6\%$ (mean \pm s.d., $N=4$, $P < 0.05$ Student's *t*-test) in the presence of proctolin. Application of proctolin (1 $\mu\text{mol l}^{-1}$) alone had no effect on resting tension.

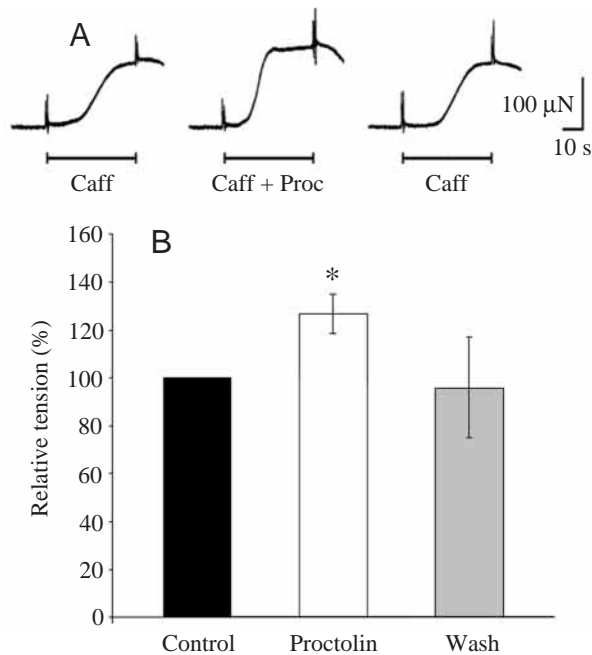


Fig. 2. Effect of proctolin on caffeine-induced contractures. (A) Potentiation of caffeine (10 mmol l^{-1}) contracture by $1\text{ }\mu\text{mol l}^{-1}$ proctolin. Two controls are shown, before and after the exposure to proctolin. (Caff, caffeine; Proc, proctolin). (B) Averages of normalised tensions obtained from 4 experiments. Control represents the average of three caffeine contractures per experiment prior to the proctolin tests. Values are means \pm S.D., * $P < 0.05$, Student's t -test.

Proctolin induces phosphorylation of a 30 kDa protein

The electrophoretic separation of proteins of identified long extensor muscle fibres on a 15% SDS gel yielded a number of protein bands with high resolution in the 15–100 kDa range (Fig. 3A). To detect a change in phosphorylation of muscle proteins due to exposure to proctolin, phosphorylated proteins were identified in western blots using an anti-phosphoserine antibody. Immunoblots with a positive control (provided by Biomol, Hamburg, Germany) showed the expected labelling. In western blots of *I. emarginata* muscle proteins treated with the anti-phosphoserine antibody, several bands were labelled indicating that these proteins are phosphorylated at a serine residue. However, not all proteins detected by silver staining were labelled in the immunoblot (Fig. 3B). Densitometry of the immunoblot of control samples (Fig. 3C) shows different intensities of antibody staining on a number of protein bands. In western blots of proteins isolated from fibres incubated with proctolin ($1\text{ }\mu\text{mol l}^{-1}$), a considerable increase in phosphorylation was evident in a protein band with the apparent molecular mass of 30 kDa ($N=11$). This is evident by comparing the phosphorylation of the 30 kDa protein in the western blots (Fig. 3B, arrow) and the two densitometric traces (Fig. 3C, arrow). The differences in phosphorylation of the 31, 35 and 36 kDa proteins seen in Fig. 3B occurred only in this experiment and are thus not a consistent effect of proctolin.

For a quantitative analysis of the proctolin effect, the relative change of extinction was determined by comparing the extinctions of protein bands in the controls and in the proctolin-treated sample of each experiment. To prove that the different intensity of staining was not due to differences in protein concentrations of the samples, the phosphorylated 70 kDa protein was analysed as a control. The relative change of

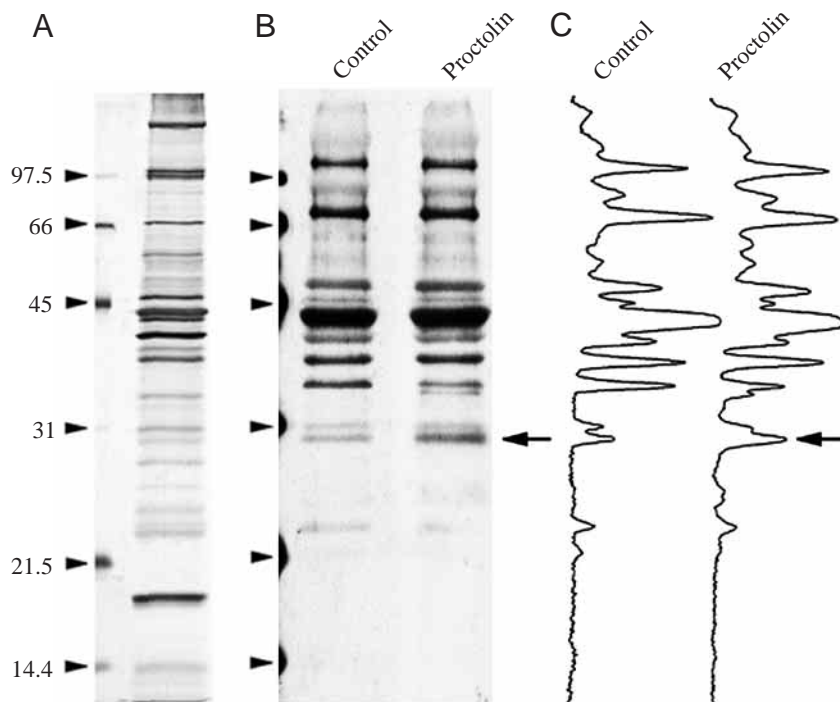


Fig. 3. (A) Electrophoretic separation of *I. emarginata* extensor muscle fibres on a 15% SDS gel. The positions of molecular mass standards (kDa) are indicated. (B) Effect of proctolin ($1\text{ }\mu\text{mol l}^{-1}$) on serine phosphorylation of *I. emarginata* extensor muscle proteins. The 30 kDa protein band of the proctolin-treated sample (arrow) shows an increased phosphorylation signal compared to the 30 kDa protein band of the untreated sample. Molecular mass standards are indicated by arrowheads. Although differences in phosphorylation of a 31 kDa, 35 kDa and a 36 kDa protein are seen in this experiment, these effects do not occur consistently as an effect of proctolin incubation. (C) Densitometric analysis of B. The 30 kDa protein band is marked (arrow).

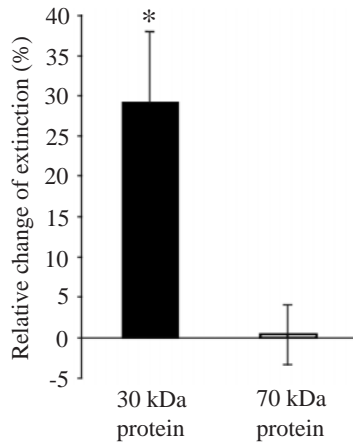


Fig. 4. Changes in relative extinction of the 30 kDa protein and a 70 kDa protein (for comparison) upon treatment with $1 \mu\text{mol l}^{-1}$ proctolin. Summary of 11 experiments. The relative changes in the extinctions of the 30 kDa and the 70 kDa band of proctolin-treated samples and untreated samples were compared in each experiment. The extinctions result from immunoreactivity of the anti-phosphoserine antibody. Values are means \pm s.d., * $P < 0.05$, Student's t -test.

extinction of the 70 kDa protein bands in proctolin-treated samples and in untreated samples was only small and in both directions. On average, the difference was only $0.4 \pm 3.7\%$ (Fig. 4) and statistically not significant ($N=11$, $P > 0.05$ Student's t -test). The proctolin effect on the phosphorylation of the 30 kDa protein was quantitatively analysed in the same 11 experiments. In seven of these experiments, the extinction in the proctolin-treated samples was increased by 26% to 89% over the untreated samples. In two experiments, no obvious differences in the extinction of the 30 kDa protein band was detected, and in two other experiments the extinction of the proctolin-treated samples was slightly smaller (6%, 9%) than that of the untreated samples. On average, the extinction of the 30 kDa protein band of the proctolin-treated samples was significantly increased by $29 \pm 8.9\%$ ($N=11$, $P < 0.05$, Student's t -test). These results show that proctolin induces phosphorylation of a 30 kDa protein in the extensor muscle fibres of *I. emarginata* (Fig. 4).

The phosphorylated 30 kDa protein is associated with the thin filament and troponin I immunoreactive

Since the phosphorylated 30 kDa protein band is present in

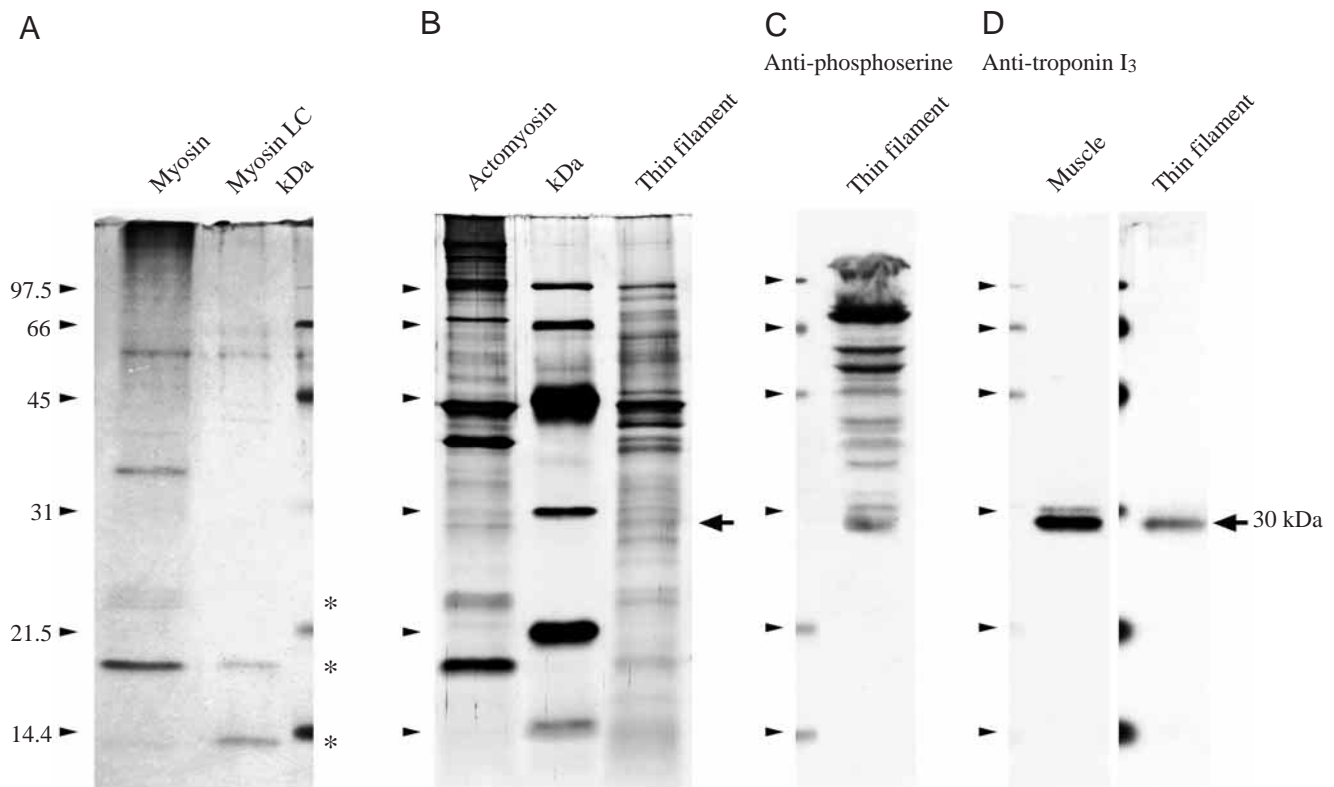


Fig. 5. Electrophoretic separation and western blot analysis of *I. emarginata* myosin, actomyosin and thin filament on 15% SDS gels. The positions of molecular mass standards (kDa) are indicated. (A) In the myosin extract three myosin light chains (myosin LC) were identified (asterisks). In myosin LC extracts obtained by guanidine-HCl precipitation two of these light chains (14 kDa and 18.5 kDa) are present. (B) The actomyosin and thin filament extracts contain a protein with the apparent molecular mass of 30 kDa (arrow), whereas no such protein is present in the myosin extract. (C) Western blot analysis with a phosphoserine antibody reveals a number of high molecular mass proteins and two proteins at 30 kDa and 31 kDa in thin filament preparations. (D) In *I. emarginata* muscle homogenates and thin filament preparations, a major band at 30 kDa (arrow) and a minor band at 31 kDa are stained in western blot analysis with antiserum against lobster troponin I₃.

preparations of sarcoplasmic proteins (Fig. 3), we assumed that it is associated with either the thin or the thick filament. To determine whether the potentiation of caffeine-induced muscle contraction is due to myosin light chain phosphorylation we identified myosin light chains of *I. emarginata*. Electrophoretic separation of the myosin preparation revealed three protein bands at 14, 18.5 and 24 kDa and a prominent band at the apparent molecular mass of 189 kDa (Fig. 5A). The molecular masses of these proteins correspond to those reported for myosin light and heavy chains of other crustacean muscles (Mykles, 1985a; Mykles, 1985b; Mykles, 1988; Li and Mykles, 1990). The protein bands at 36 kDa and 56 kDa do not correspond to any known crustacean myosin light chains and are therefore supposed to be contaminants. By subsequent treatment with guanidine-HCl and precipitation of heavy chains with ethanol, the two myosin light chains with molecular masses of 14 kDa and 18.5 kDa were further separated (Fig. 5A). A 30 kDa protein that was present in the actomyosin preparation (Fig. 5B) was not present in the myosin preparation. We therefore concluded that proctolin does not induce myosin light chain phosphorylation.

The preparation and electrophoretic separation of the thin filament proteins showed a number of different bands with apparent molecular masses between 14 and 100 kDa (Fig. 5B). Among these, two protein bands at 30 kDa and 31 kDa are visible (Fig. 5B, arrow at 30 kDa). Thus, these proteins belong to the thin filament complex, and are most probably thin-filament-associated regulatory proteins. Both are phosphorylated at a serine residue (Fig. 5C). Since in other crustacean muscles thin filament proteins with the molecular mass of approximately 30 kDa inhibit actomyosin-ATPase activity and have therefore been classified as troponin I (Regenstein and Szent-Györgyi, 1975; Mykles, 1985a; Nishita and Ojima, 1990; Miegel et al., 1992; Neil et al., 1993), we applied an antiserum, generated against lobster troponin I₃ (Sohn et al., 2000), onto *I. emarginata* muscle and thin filament preparations (Fig. 5D). The immunoblot revealed only two bands: one major band of 30 kDa and another minor band of 31 kDa. There was no signal with the anti-troponin I₃ antiserum on *I. emarginata* myosin preparations. From these results we suggest that proctolin induces phosphorylation of the 30 kDa troponin I isoform in *I. emarginata* muscle.

Discussion

Immunocytochemistry

The potentiating effects of proctolin on *Idotea* extensor muscle fibres (Erxleben et al., 1995) raised the question of whether the peptide is present in *I. emarginata* and how it reaches the fibres *in vivo*. We identified a single pair of proctolin-ir motoneurons in pereion ganglion 4, which appears to be the only central source for directly supplying proctolin to the extensor muscles in all segments. The axons of these motoneurons accompany the internal layer of long extensor muscle fibres across several segments and form a neurohaemal meshwork of varicosities in the posterior body

segments, allowing modulation of local and more distant targets. This mode of release has also been found in other arthropods (Anwyl and Finlayson, 1973; Bräunig, 1997). We have recently localised allatostatin immunoreactivity in pairs of motoneurons with similar location and morphology in all pereion ganglia except ganglion 4 of *I. emarginata* (Kreissl et al., 1999). Thus, the modulatory action of these homonomous pairs of neurons present in all pereion ganglia is exerted by different peptides which are segment-specifically expressed but applied to extensor muscle fibres in all segments.

Mechanisms of proctolin-induced potentiation of contraction

Proctolin enhances contraction by synergistic pre- and postsynaptic effects. In *I. emarginata* the postsynaptic effects induced by concentrations of proctolin in the range of 2 nmol l⁻¹ to 1 μmol l⁻¹ include an increase in the input resistance of the sarcolemma by closure of non-voltage-dependent K⁺ channels and an increase of inward current through L-type Ca²⁺ channels (Erxleben et al., 1995; Rathmayer et al., 2001). The involvement of intracellular signalling pathways (Lange and Nykamp, 1996, for review; Rathmayer et al., 2001) suggests that, in addition to the effects on sarcolemmal ion channels, proctolin may also modulate Ca²⁺ release from the SR and/or Ca²⁺ sensitivity of sarcoplasmic proteins. In this study we show that proctolin, when applied at the concentration that was most effective in previous studies on this preparation (1 μmol l⁻¹), enhances caffeine contractures of *I. emarginata* extensor muscle fibres and induces the phosphorylation of a 30 kDa protein. The proctolin-induced activation of Ca²⁺ channels does not contribute to the proctolin-induced enhancement of caffeine contractures. Caffeine contractures, which develop without membrane depolarisations, are not accompanied by Ca²⁺ currents through voltage-dependent Ca²⁺ channels (Huddart, 1969; Chiarandini et al., 1970). Caffeine contractures are thought to be due to its effect on the ryanodine receptor engaged in Ca²⁺ release from the SR. Since in locust oviduct and foregut muscles proctolin causes an increase in InsP₃, and injection of InsP₃ and phorbol esters mimicked the effect of proctolin (Baines and Downer, 1992; Lange and Nykamp, 1996; Hinton et al., 1998), it was suggested by these authors that the potentiation of contractions by proctolin is mediated by activation of InsP₃-induced Ca²⁺ release from the SR. At present we have not investigated a possible potentiating effect of proctolin on caffeine contractures caused by a change in Ca²⁺ release.

However, proctolin may have yet another mechanism of action that is downstream of the Ca²⁺ release from the SR, by changing the Ca²⁺ sensitivity of regulatory proteins through phosphorylation.

Proctolin induces phosphorylation of an intramuscular protein

As the present study demonstrates, proctolin induces serine phosphorylation of a muscle protein with the apparent molecular mass of 30 kDa. No other protein shows a consistent

change in its degree of serine phosphorylation on exposure to proctolin. However, considerable variability in the proctolin-induced phosphorylation can be detected, which may reflect different endogenous proctolin levels. Erxleben et al. (Erxleben et al., 1995) reported a considerable variability among preparations of *I. baltica* for the proctolin-induced enhancement of contraction. Sometimes proctolin even failed to exert any effect on membrane properties or contraction amplitudes. The effects of proctolin was also shown to vary seasonally in crayfish (Bishop et al., 1991b).

To study phosphorylation of muscle proteins, we chose an extraction method that would isolate sarcoplasmic proteins in the range 15–200 kDa. Therefore, membrane-bound proteins such as ion channels were not present in the homogenates. In myofibrillar proteins, phosphorylation of myosin regulatory light chains, which is common in vertebrate muscle and regulates force generation (Sweeney et al., 1993; Horowitz et al., 1996, for review), has been detected in several insects (Winkelman and Bullard, 1980; Takano-Ohmuro et al., 1986). Lange and Nykamp (Lange and Nykamp, 1996) reported that a 20 kDa light chain of a locust visceral muscle is phosphorylated after proctolin exposure (10 nmol l^{-1}). In *Drosophila* flight muscle phosphorylation of a light chain enhanced both calcium-activated and magnesium-activated ATPase activities (Takahashi et al., 1990). We show that the 30 kDa protein of *I. emarginata* muscles, the phosphorylation of which is modulated by proctolin, is not a myosin light chain because the protein is not present in the myosin preparations (Fig. 5A). In the opener muscle of lobster walking legs, the biogenic amines serotonin and octopamine, but not proctolin, were found to induce phosphorylation of a 29 kDa protein (Goy et al., 1984). Although the molecular mass of this protein is similar to that of the *I. emarginata* protein, the lobster protein is soluble and not associated with myofibrils and therefore not likely to correspond to the *I. emarginata* 30 kDa protein.

In crustacean muscles, thin filament regulatory proteins in the 30 kDa range have been classified as troponin I because they inhibit actomyosin-ATPase activity (Regenstein and Szent-Györgyi, 1975; Nishita and Ojima, 1990). Five different troponin I isoforms, with molecular masses between 27 and 30 kDa, were found in lobster fast and slow muscles (Mykles, 1985a; Mykles, 1985b; Miegel et al., 1992; Neil et al., 1993). By applying an anti-troponin I₃ antiserum that was made against lobster troponin I₃ but which reacts with other troponin I isoforms, we provide evidence that *I. emarginata* muscle contains at least two troponin I isoforms. Both are phosphorylated but proctolin increases the phosphorylation of only the 30 kDa protein.

A functional role of troponin I phosphorylation is well known from studies of vertebrate heart muscle (for review see Perry, 1999). In parallel with the β -adrenergic-positive inotropic effect, the phosphorylation of troponin I leads to an reduction of the Ca^{2+} sensitivity of troponin C (Reiffert et al., 1996; Jaquet et al., 1998). It is not known if phosphorylation of troponin I in crustacean muscle takes place *in vivo*. If the analogy with vertebrate heart muscle is valid this effect of

proctolin in *I. emarginata* muscle fibres would counteract the other effects of this peptide in this preparation.

However, possible serine phosphorylation sites of troponin I from the crayfish *Astacus leptodactylus* are not thought to contribute to a control mechanism equivalent to the negative feedback mechanism in vertebrate heart muscle (Kobayashi et al., 1989). In addition, recent studies in an insect muscle showed that phosphorylation of a thin filament-associated 30 kDa protein actually increased Ca^{2+} sensitivity of the actomyosin ATPase (Weitkamp et al., 1998). The identity of this 30 kDa protein is not known but the authors assume it to be a troponin I on the basis of its molecular mass.

If the phosphorylation of the *I. emarginata* 30 kDa thin-filament-associated protein also increases Ca^{2+} sensitivity of the ATPase, this would contribute to the proctolin-induced potentiation of contractures and would be synergistic to all other effects of proctolin in this preparation.

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