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SPECIES-SPECIFIC ACTION AND DISTRIBUTION OF TACHYKININ-RELATED PEPTIDES IN THE FOREGUT OF THE COCKROACHES *LEUCOPHAEA MADERAE* AND *PERIPLANETA AMERICANA*

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Summary

Nine tachykinin-related peptides (TRPs) have been isolated from the brain and intestine of the cockroach *Leucophaea maderae*. In the present investigation, two of the nine TRPs, LemTRP 1 and 5, were tested for their ability to stimulate contractions in the foregut of the cockroaches *L. maderae* and *Periplaneta americana in vitro*. The two LemTRPs and the related locust peptide locustatachykinin I (LomTK I) induced contractions in the foregut of *P. americana* in a dose-dependent manner, but had no myostimulatory action in *L. maderae*. A half-maximal response for the LemTRPs and LomTK I was obtained at 5×10^{-9} mol l^{-1}. In both species, the neuropeptide proctolin stimulated foregut contractions. Using an antiserum to LomTK I, we demonstrated that in both species there are LomTK-like-immunoreactive (LomTK-LI) cell bodies and fibers within the ganglia and nerves of the stomatogastric nervous system. However, correlated with the species-specific action of the TRPs, we found efferent LomTK-LI nerve fibers supplying muscle fibers in the foregut of *P. americana*, but not in *L. maderae*. In both cockroach species, there is a rich supply of proctolin-immunoreactive fibers to the foregut muscle. Some of the LomTK-LI fibers supplying the *P. americana* foregut muscle contain co-localized proctolin immunoreactivity. These fibers appear to be derived from a large cell body in the frontal ganglion which also displayed co-localized immunoreactivities. Since TRP-containing neurons are restricted to the nerves and ganglia of the stomatogastric nervous system both in *P. americana* and *L. maderae*, TRPs may be involved in the control of foregut movements in both species, but in *P. americana* the control may be more complex with the additional peripherally projecting LomTK-LI neurons.

Key words: tachykinin-related peptide, proctolin, *Leucophaea maderae*, *Periplaneta americana*, neuropeptide, myotropic peptide, intestine, nervous system.

Introduction

Several years ago, substance P, a member of the tachykinin neuropeptide family, was found to induce contractions in the isolated hindgut as well as in the oviduct of the cockroach *Periplaneta americana* (Penzlin *et al.* 1989). At the time, tachykinin-related peptides (TRPs) had not been identified in insects, but a number of other insect neuropeptides were known that could stimulate or inhibit visceral muscle contractions (see Cook *et al.* 1989, 1990; Holman *et al.* 1990; Osborne *et al.* 1990). Since the late 1980s, the hindgut of the cockroach *Leucophaea maderae* and the oviduct of the locust *Locusta migratoria* have been explored extensively both as muscle contraction bioassays to isolate further myotropic neuropeptides and to analyse the mechanisms of action of myotropic peptides (Holman *et al.* 1991; Schoofs *et al.* 1993; Orchard and Lange, 1995; Osborne, 1996). With the aid of the *L. maderae* hindgut contraction assay, the first insect peptides related to tachykinins were isolated from extracts of the locust brain and retrocerebral complex (Schoofs *et al.* 1990*a*,*b*). These so-called locustatachykinins (LomTK I–IV) are potent stimulators of contractions of both the cockroach hindgut and the locust oviduct.

Only recently were the endogenous TRPs of *L. maderae* characterized. Nine peptides with structural similarities to the LomTKs and with some resemblance to the vertebrate tachykinins were isolated from the brain and midgut of *L. maderae* (Muren and Nässel, 1996*b*, 1997). These peptide isoforms, designated *Leucophaea* tachykinin-related peptides 1–9 (LemTRP 1–9), consist of 9–19 amino acids and are characterized by a C-terminal hexapeptide sequence GFX_1GX_2Ramide (where X_1 is F, H, L, M or Q, and X_2 is M, T or V). The LemTRPs were purified by monitoring the chromatographic fractions both in a radioimmunoassay (RIA) employing an antiserum to LomTK I and in a bioassay recording changes in spontaneous contractile activity of the

hindgut of *L. maderae*. All nine LemTRPs increased the amplitude and frequency of spontaneous contractions of the hindgut muscle, and at higher concentrations they increased the muscle tonus (Muren and Nässel, 1996*b*, 1997; Winther *et al.* 1998).

Are the LemTRPs also active on the cockroach foregut muscle? This was proposed for LomTK I–IV in tests of the locust foregut (Schoofs *et al.* 1990*a*,*b*). The muscle of the locust foregut has been extensively used to analyse the myotropic actions of monoamines and several neuropeptides, and it is clear that foregut contractions may be regulated by several neuroactive compounds (Osborne *et al.* 1990; Osborne, 1996). The foregut of *L. maderae* has also been shown to respond to several neuropeptides (Cook *et al.* 1989, 1990, 1993; Cook and Wagner, 1990; Wagner and Cook, 1993; Duve *et al.* 1995). Immunocytochemistry and RIA indicated the presence of LemTRPs in the stomatogastric nervous system and the pharyngeal dilator muscles of *L. maderae* (Muren *et al.* 1995; Muren and Nässel, 1996*a*), but no direct innervation of foregut muscle by LomTK-like-immunoreactive (LomTK-LI) fibers could be seen in this species. However, in the present investigation, we found that in another cockroach, *Periplaneta americana*, LomTK-LI fibers are present in the muscle layer of the foregut, suggesting that TRPs may have a myotropic action in this species. In the present paper, we compare the distribution of immunoreactive TRPs and the actions of two of the LemTRPs and LomTK I on the foreguts of *L. maderae* and *P. americana*. The action of proctolin and the distribution of proctolin immunoreactivity is also compared between the two species. Our principal finding is that both the distribution and action of TRPs in the foregut of the two cockroaches are species-specific.

Materials and methods

Animals

Cockroaches of the species *Leucophaea maderae* Forskål and *Periplaneta americana* L. were kept under crowded conditions at 28±2 °C in a 12 h:12 h light:dark regime (16 h:8 h L:D in Stockholm) and at 65–70 % relative humidity. Both species were kept in culture in both Jena and in Stockholm (different strains). The animals were fed on dry dog food pellets and water *ad libitum*. Between 2 and 3 weeks after the imaginal molt, the animals were taken from the stock culture. Only male cockroaches were used for experiments. These were starved for 1 day prior to experiments. For immunocytochemistry, the tissues were collected from adult cockroaches of both sexes. Additional experiments were made on the cockroach *Nauphoeta cinerea* Olivier from a stock in Jena.

Peptides and other reagents

LemTRP 1 and 5 were synthesized by Dr Å. Engström (Department of Medical and Physiological Chemistry, Uppsala University) as described in Muren and Nässel (1996*b*). LomTK I and proctolin were purchased from Peninsula (Belmont, CA). The sequences of the peptides are shown in Table 1. Unless otherwise stated, all other reagents were from Sigma (St Louis, MO, USA).

Table 1. *Amino acid sequences of peptides used in this investigation*

LemTRP 1	APSGFLGVRamide
LemTRP 5	APAMGFQGVRamide
LomTK I	GPSGFYGVRamide
Proctolin	RYLPT

Underlined amino acids in the tachykinin-related peptides (TRPs) are in identical positions.

Sequences of TRPs are taken from Muren and Nässel (1996*b*) and Schoofs *et al.* (1990*a*).

Bioassay

To test for bioactivity, the isolated foreguts were ligated anteriorly at the hypopharynx as well as in the region posterior to the ingluvial ganglion; the portion posterior to the second ligature was cut off (Fig. 1). The anterior part was mounted for tests as described by Penzlin (1994). In brief, the foregut was suspended in a glass chamber (5 mm in diameter) containing 2 ml of saline consisting of 9.0 g l^{-1} NaCl, 0.2 g l^{-1} KCl, 0.2 g l^{-1} $CaCl_2$, 4.0 g l^{-1} glucose and 10 ml of 0.1 mol l^{-1} sodium phosphate buffer (pH 7.1). A water jacket allowed control of bath temperature at 28 °C. To monitor longitudinal contractions, the intestine was connected at one end by a cotton thread to the lever of an isotonic transducer (photoelectric detection of motion) with a counterbalance of approximately 70 mg. The other end was attached to a hook fixed at the bottom of the chamber. Contractions in the circular musculature were monitored by cutting a ring out of the foregut at its broadest part and suspending the ring between two hooks, one of which was attached to the lever of the transducer and the other to the bottom of the chamber.

The peptides were applied in 20 µl volumes to obtain the desired concentration in the bath. The isotonic contraction of the foregut was measured in arbitrary units (millimetres of response on the plotter paper). Each point of the dose–response curves is based on at least ten preparations. At least six replicates were carried out for the other experiments described.

Immunocytochemistry

A rabbit antiserum (code 9207-7) raised against LomTK I of *Locusta migratoria*, as described by Nässel (1993*a*), was used for immunocytochemistry. This LomTK I antiserum detects the different known insect TRPs to varying degrees (Nässel, 1993*a*; Lundquist *et al.* 1994*b*; Muren and Nässel, 1996*a*,*b*, 1997). A rabbit antiserum raised against proctolin and characterized previously (Eckert and Ude, 1983; Bartos *et al.* 1994) was also applied. Both antisera were used at a dilution of 1:1000 or 1:2000 (in 0.01 mol l^{-1} phosphate-buffered saline with 0.5 % bovine serum albumin and 0.25 % Triton X-100).

Foreguts with associated stomatogastric nervous systems were tied with a cotton thread at one end, inflated with saline

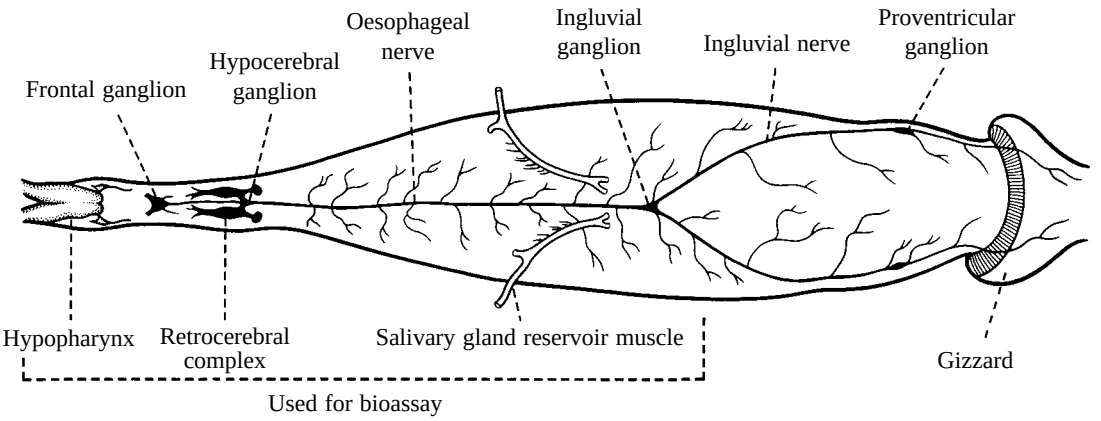

Fig. 1. Drawing of the foregut of *Periplaneta americana* in dorsal view (anterior to the left). The figure displays the innervation of the foregut by the stomatogastric nervous system. The retrocerebral complex includes the corpora cardiaca and corpora allata. Only the anterior portion, as indicated, was used for bioassay. (Magnified about 10×.)

by means of a syringe and then tied at the other end. The inflated and distended foreguts were immersed in 4% paraformaldehyde in 0.1 mol l^{-1} sodium phosphate buffer for 10 min. Thereafter, they were cut open and spread out in dishes coated with Sylgard (Dow Corning, Bruxelles, Belgium) and postfixed for a further 4 h in the same fixative. Immunocytochemistry was performed on the whole free floating foreguts as described previously (Eckert and Ude, 1983; Muren *et al.* 1995; Muren and Nässel, 1996*a*) using either detection by the peroxidase anti-peroxidase (PAP) method or by fluorescence with fluorescein isothiocyanate (FITC), tetramethyl rhodamine isothiocyanate (TRITC) or Cy3-tagged secondary immunoglobulins (reagents from DAKO, Copenhagen or Dianova, Hamburg). Further details are given in the next section. Each antiserum was applied to at least eight intestines (with associated ganglia) of each species (both PAP and fluorescence methods). Preabsorption controls were made by incubating 1 ml of the diluted primary antiserum with 20–50 nmol of the respective peptide and then applying them for immunocytochemistry to three intestines of each species (further specificity tests of the antisera have been made previously: Eckert and Ude, 1983; Lundquist *et al.* 1994*b*; Muren *et al.* 1995; Muren and Nässel, 1996*a*,*b*).

Double-labelling immunocytochemistry

For simultaneous detection of TRPs and proctolin, we performed double-labelling immunocytochemistry on wholemounts and cryostat sections. Cryostat sections (25 µm thick) were made from paraformaldehyde-fixed brains, frontal ganglia and suboesophageal ganglia of *P. americana*. In the double-labelling experiments, we utilized the rabbit antiserum to proctolin and one of two approaches for the detection of TRPs. The first was to use the rabbit anti-LomTK I (9207-7) which had been biotinylated according to Harlow and Lane (1988) and Würden and Homberg (1993). The biotinylation was kindly performed by Dr S. Würden, Regensburg, Germany. The anti-proctolin was detected with goat anti-rabbit antiserum tagged with AMCA (aminomethyl coumarin acetic acid; Dako) and the anti-LomTK with streptavidin tagged with Texas Red (Amersham) according to Nässel (1993*a*). The second approach was to use a rat monoclonal antibody to substance P (Accurate Chemicals, Westbury, NY, USA; see Cuello *et al.* 1979). This antibody has been shown to cross-react with invertebrate TRPs and to label neurons identical to those displaying LomTK-like immunoreactivity in insects and crabs (Nässel, 1993*b*; Blitz *et al.* 1995). The anti-substance-P was detected with FITC-tagged goat anti-rat immunoglobulin (Sigma) and the anti-proctolin was detected with TRITC-labelled swine anti-rabbit immunoglobulin (Dako). Each combination of antiserum and detection method was applied to at least three preparations (frontal ganglia as well as intestines with ganglia). The double-labelled specimens were analyzed and photographed with a Zeiss Axiophot microscope equipped with appropriate filters.

Results

Actions of TRPs and proctolin on the foregut of P. americana

The anterior portion of the foregut used in all experiments is indicated in Fig. 1. The foregut of *P. americana* responded to LemTRPs 1 and 5 and to LomTK I with an increase in amplitude of spontaneous contractions at lower concentrations (10^{-10} mol l^{-1}) and by an increased tonus at higher concentrations (10^{-7} mol l^{-1}; Fig. 2). The response is transient and the tonic component lasts only 2–3 min. The action of the peptides on the foregut was tested both with the ingluvial ganglia and oesophageal and gastric nerves attached and after careful removal of these structures; the response to peptide application was the same in both cases. The dose–response characteristics of LomTK I and LemTRPs 1 and 5 on foregut contractions were determined for concentrations ranging between 10^{-6} and 10^{-10} mol l^{-1} (Fig. 3). All TRPs tested gave a small response at a concentration of 10^{-10} mol l^{-1}. The concentration producing a half-maximal response (EC_{50}) was found to be approximately 5×10^{-9} mol l^{-1} for all three peptides. For comparison, proctolin was applied at a concentration of

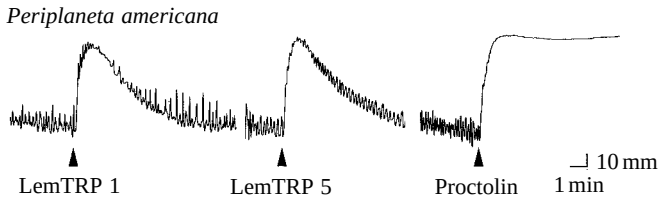

Fig. 2. Myograms showing the action of LemTRPs and proctolin on contractions of the anterior portion of the foregut (stomodeum) of *Periplaneta americana*. The peptides were added at a concentration of 10^{-7} mol l^{-1} (peptide application at arrowhead). The LemTRPs induce phasic responses of the foregut muscle, whereas the response to proctolin is tonic. No wash was made during the course of the responses shown here. Thus, the phasic reponses seen for LemTRPs may be due to desensitization. The contractions are measured in arbitrary length units.

10^{-7} mol l^{-1}. This dose induced a strong tonic contraction of the foregut which lasted at least 10 min (Fig. 2). All experiments were performed on at least 10 intestines.

Contractions of circular muscle fibers were monitored using rings cut out of the foregut and suspended by two hooks, one of which was attached to the lever of the transducer. Responses to applied LemTRPs could be registered (Fig. 4) but, owing to the fragility of the preparations, it was not possible to obtain responses reproducible enough to construct dose–response curves. Proctolin again induced a long-lasting tonic contraction.

To test whether TRPs potentiate the response to proctolin, LomTK I was applied to the foregut at low concentrations (10^{-9} mol l^{-1} or 5×10^{-9} mol l^{-1}) and proctolin (10^{-8} mol l^{-1}) was added after 5 min (without an intervening wash), as shown in Fig. 5A. The response to LomTK I combined with proctolin was not significantly different from that to proctolin alone (Fig. 5A,B), indicating that there is no potentiation (N=10–15).

Action of peptides on the foregut of L. maderae

In contrast to *P. americana*, no consistent responses were obtained when applying LomTK I (Fig. 6), LemTRP 1 and LemTRP 5 (not shown) to the foregut of *L. maderae* at concentrations of 10^{-9} to 10^{-6} mol l^{-1}. At least ten intestines were used for the different concentrations of the three peptides. Proctolin at 10^{-8} and 10^{-7} mol l^{-1}, however, did elicit contractions in the foregut similar to those in *P. americana* (Fig. 7). The foregut of the cockroach *Nauphoeta cinerea* showed no response to either LomTK or LemTRPs (Fig. 6), but responded to proctolin.

The organization of the stomatogastric nervous system in cockroaches

The stomatogastric nervous systems of the two cockroach species are in general terms organized in the same manner (Willey, 1961; Gundel and Penzlin, 1978; Penzlin, 1985). The foregut and stomatogastric nervous system of *P. americana* are shown in Fig. 1. An unpaired frontal ganglion is connected to the tritocerebrum of the brain by two frontal connectives and to the remaining stomatogastric ganglion chain by an unpaired

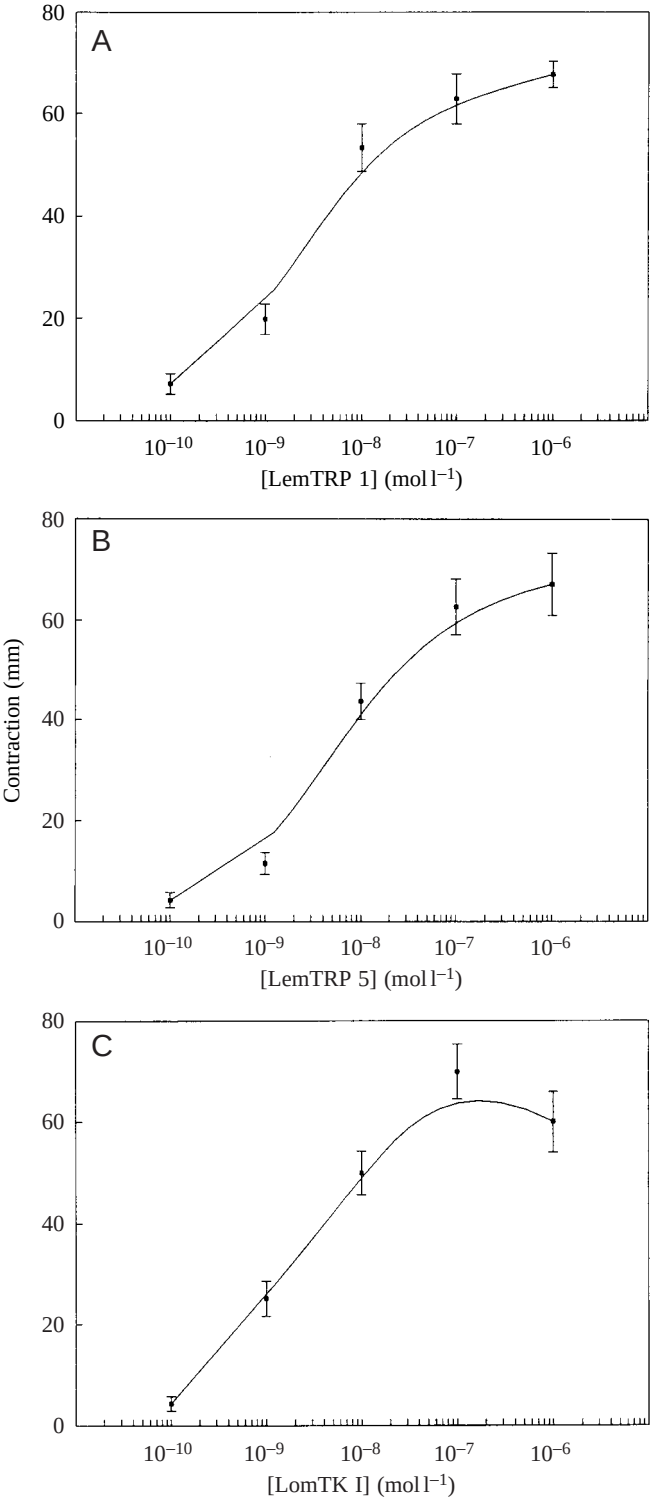

Fig. 3. Dose–response curves for tachykinin-related peptides (TRPs) in assay of contractions of the anterior part of the foregut (stomodeum) of *Periplaneta americana*. The peptides LemTRP 1 (A), LemTRP 5 (B) and LomTK I (C) were tested at concentrations ranging from 10^{-10} to 10^{-6} mol l^{-1}. The amplitudes of the peak contractions are plotted in arbitrary units (given in mm on plotter paper). The curves were fitted using Harvard Graphics 2.3. Each point represents measurements of at least 10 preparations ± S.E.M.

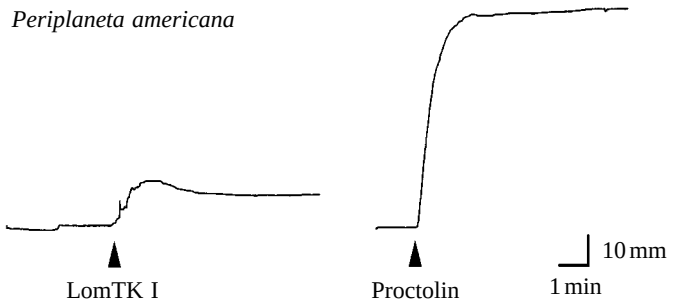

Fig. 4. Typical responses of circular muscle of anterior foregut to LomTK I (10^{-6} mol l^{-1}) and proctolin (10^{-7} mol l^{-1}). To obtain these recordings, a broad ring of foregut was cut out and suspended in the transducer as described in Materials and methods. Similar experiments were performed approximately six times for each peptide. The contractions are measured in arbitrary length units.

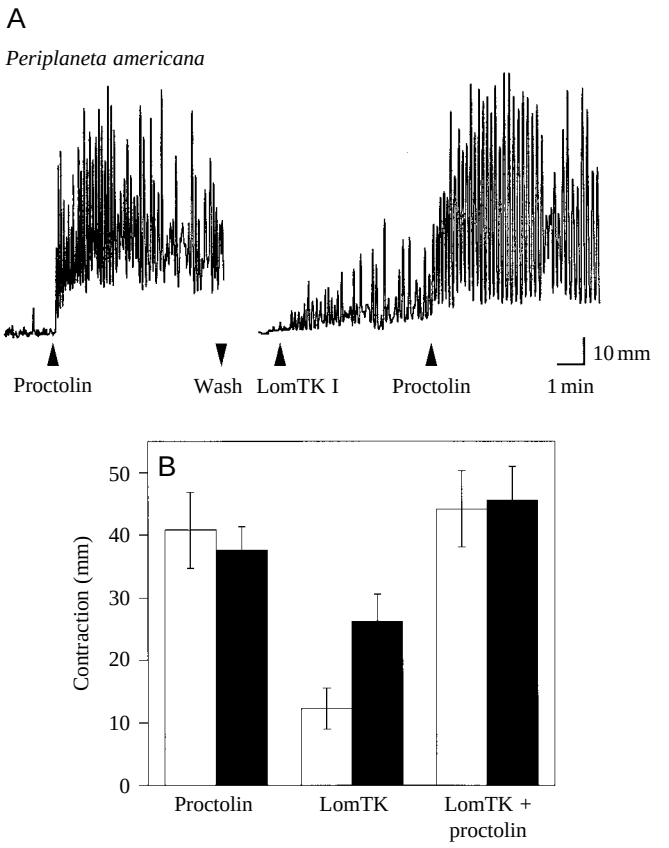

Fig. 5. Experiment to test whether LomTK I potentiates the response to proctolin. (A) Myograms showing typical responses to combined application of LomTK I and proctolin. In this recording, proctolin (10^{-8} mol l^{-1}) was first applied alone, followed by thorough washing. LomTK I (5×10^{-9} mol l^{-1}) was then applied, followed by proctolin (10^{-8} mol l^{-1}) after 5 min. The response to the second application of proctolin was similar to the first. (B) Graphical representation of responses to proctolin alone, to LomTK I alone and to proctolin added to the foregut in the presence of LomTK I (LomTK+proctolin). The experimental regime was as in A and was performed at two concentrations of LomTK I: 10^{-9} mol l^{-1} (open columns) and 5×10^{-9} mol l^{-1} (filled columns). The responses were measured as the mean amplitude of the peak response (compared with the spontaneous contractions) as described by Cook and Wagner (1990). Values are means ± S.E.M., N=10 for the open columns, N=15 for the filled columns. There was no significant difference between the application of proctolin alone and of proctolin plus LomTK I at any concentration (Student's paired t-test; P>0.05). The contractions are measured in arbitrary length units.

recurrent nerve that runs along the dorsal portion of the foregut. The recurrent nerve connects to the hypocerebral ganglion, which in turn is connected to the ingluvial ganglion *via* the oesophageal nerve. From the ingluvial ganglion, two nerves emerge posteriorly, the ingluvial nerves (also termed gastric nerves), which run on the dorso-lateral surface of the foregut. At this point, there is a difference between the two species. In the posterior portion of the each of the ingluvial nerves of *P. americana* there is a ganglion, termed the proventricular ganglion, situated close to the gizzard (Fig. 1), whereas in *L. maderae* a row of swellings is seen in each of the ingluvial nerves instead of distinct ganglia. Each of these swellings contains small clusters of neuronal cell bodies (see below). The foregut muscle fibers are innervated from neurons with cell bodies at different locations: in the frontal ganglion and other ganglia of the stomatogastric nervous system and, possibly, in the tritocerebrum of the brain.

Distribution of LomTK-like immunoreactivity

Immunocytochemistry revealed some major differences between the two cockroach species in the distribution of LomTK-like-immunoreactive (LomTK-LI) material in structures associated with the foregut. The distribution of proctolin-like immunoreactivity, in contrast, was similar (except posteriorly in the ingluvial nerves as described below). The observations are based on at least ten intestines for each species (using both PAP and fluorescence methods).

In the stomatogastric nervous system of *P. americana*, LomTK-LI cell bodies were only found in the frontal ganglion (3–4 cell bodies). Immunoreactive fibers were seen in the frontal connectives, the recurrent nerve, the hypocerebral ganglion, the oesophageal nerve, the ingluvial ganglion (Fig. 8A), the ingluvial nerves (Fig. 8B) and the proventricular ganglia. The LomTK-LI fibers arborize in these ganglia, especially in regions where neuronal cell bodies are located. In many places, the LomTK-LI fibers form varicosities close to the cell bodies of other unidentified neurons in the ingluvial nerves (Fig. 8B). Immunoreactive axons emerge from branches of the nerves and supply the circular muscle fibers of the foregut (Fig. 8C, see also Fig. 10D). The density of the innervation of this muscle layer by LomTK-LI fibers is higher in the anterior than in the posterior of the foregut. The only longitudinally oriented muscle fibers that are directly innervated by LomTK-LI fibers are seen in a separate muscle closely attached to the foregut. This is the so-called salivary gland reservoir muscle (Fig. 1) attaching the reservoir to an apodeme at the pronotum and to the dorsal wall of the foregut. The muscle consists of a few groups of longitudinal muscle

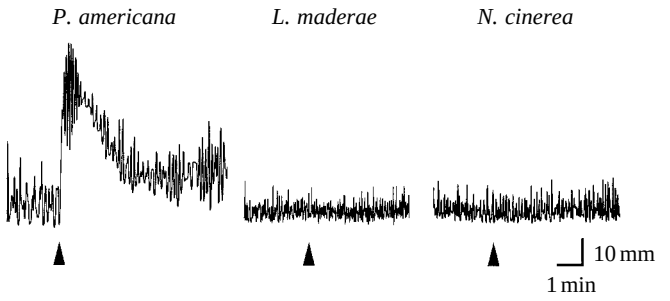

Fig. 6. Myograms showing the effect of LomTK I (10^{-7} mol l^{-1}) (applied at the arrowheads) on contractions of the anterior part of the foregut of three cockroach species, *Periplaneta americana*, *Nauphoeta cinerea* and *Leucophaea maderae*. Only in *P. americana* was there a stimulatory action of the peptide; in the other species, there was no response to any concentration tested. The contractions are measured in arbitrary length units.

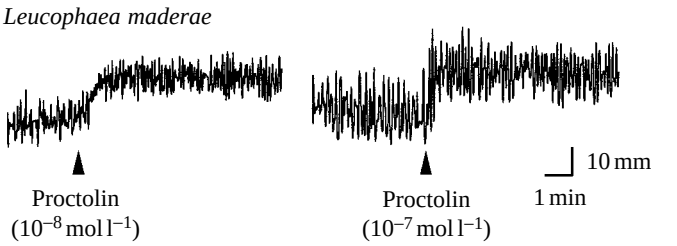

Fig. 7. The response of the foregut of *Leucophaea maderae* to proctolin at two concentrations is an increase in tonus. The contractions are measured in arbitrary length units.

fibers all of which are densely supplied by LomTK-LI fibers (see Fig. 10H).

In *L. maderae*, we found no innervation of foregut muscle fibers by axons of LomTK-LI neurons. All immunoreactivity is confined to axons within the ganglia (Fig. 8D) and the recurrent, oesophageal and ingluvial nerves (Fig. 8E). These axons form arborizations in each of the ganglia and in the regularly distributed swellings of the ingluvial nerves (Fig. 8E). The immunoreactive fibers are derived from three LomTK-LI cell bodies localized in the frontal ganglion (see Fig. 10B for *P. americana*), but probably also from cell bodies in the brain since many LomTK-LI axons can be seen in the frontal connectives. Additionally, many of the pharyngeal

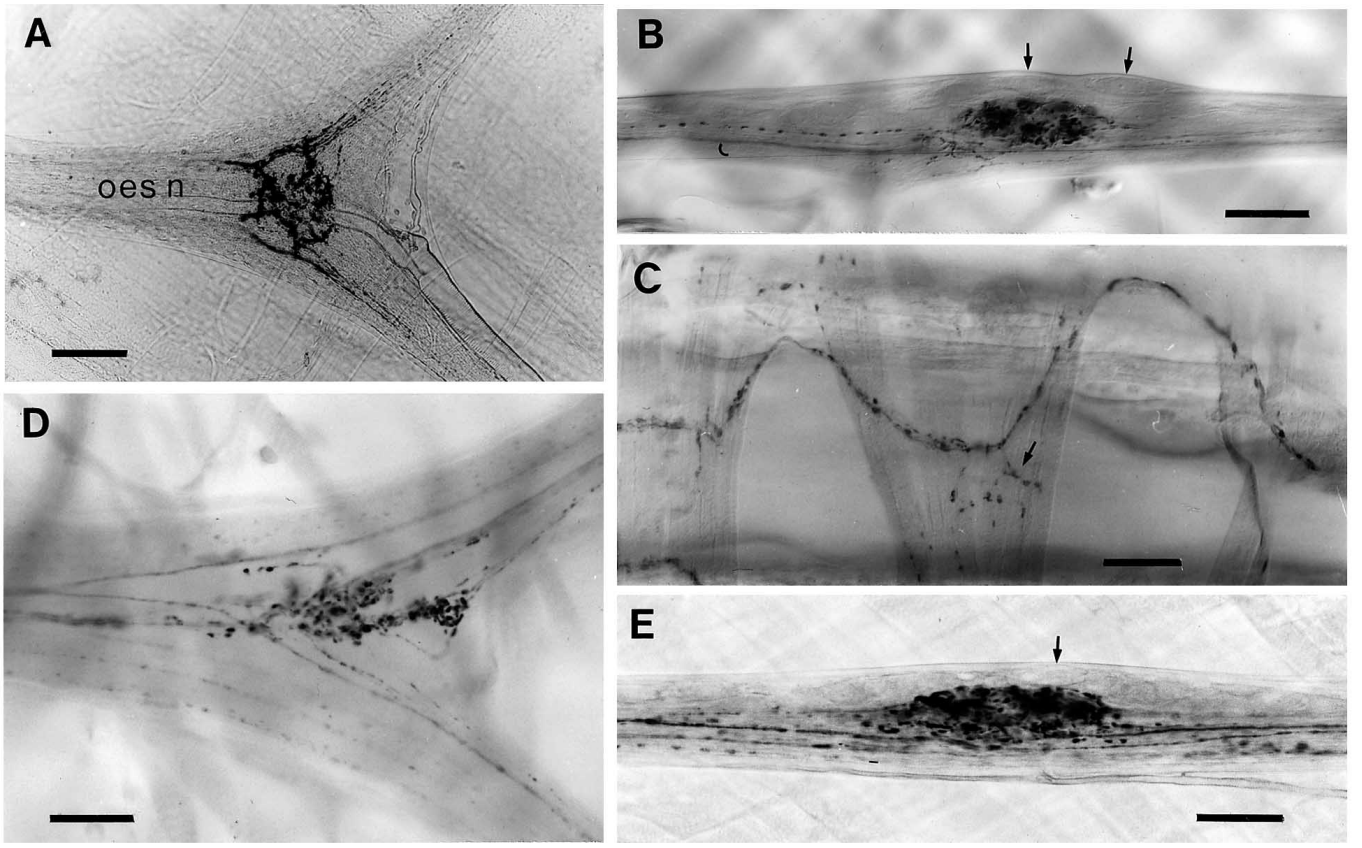

Fig. 8. Immunolabelling of foregut structures with antiserum to LomTK I. (A) Ingluvial ganglion of *Periplaneta americana* showing LomTK-immunoreactive (LomTK-LI) fibers in the oesophageal nerve (oes n), arborizing fibers in the ganglionic neuropil and fibers in the two ingluvial nerves. (B) In the ingluvial nerves of *P. americana*, the LomTK-LI fibers form varicose arborizations in neuropil-like regions of the nerve adjacent to unlabelled cell bodies (arrows). (C) Varicose LomTK-LI fibers on circular muscle of the anterior foregut (arrow) in *P. americana.* (D) In the ingluvial ganglion of *Leucophaea maderae*, the distribution of LomTK-LI fibers is similar to that in *P. americana*. (E) Varicose fibers in the ingluvial nerve of *L. maderae* arranged in a similar manner to those in *P. americana*. Scale bars, 50 μm.

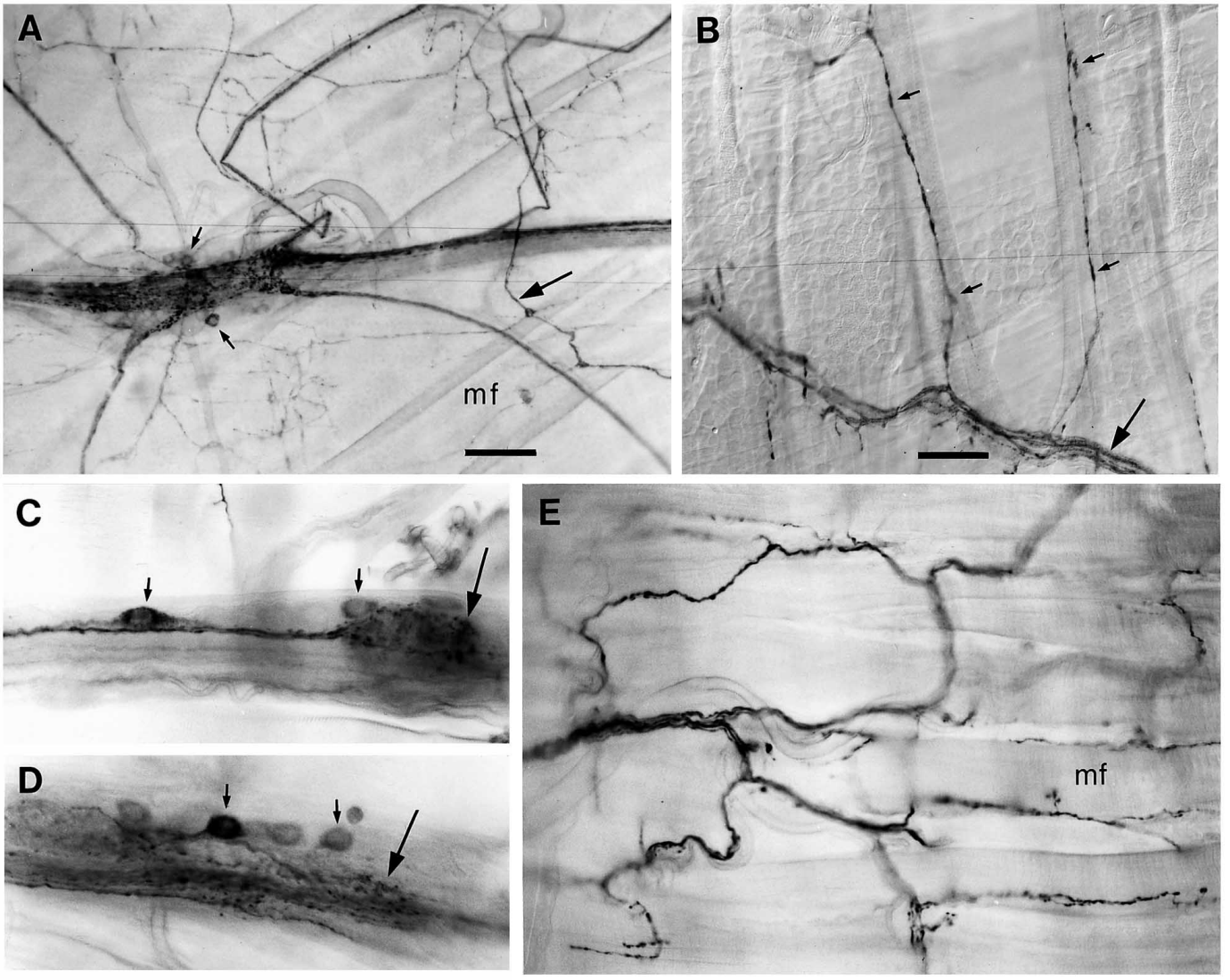

Fig. 9. Proctolin immunoreactivity in foregut-related structures of *Periplaneta americana* and *Leucophaea maderae*. (A) Proventricular ganglion of *P. americana*. In this ganglion, there are proctolin-immunoreactive cell bodies (small arrows) and arborizing fibers. Note that numerous immunoreactive nerve fibers emerge from the ganglion and innervate muscle fibers (mf), e.g. at the large arrow. (B) Proctolin-immunoreactive fibers associated with circular muscle fibers of the foregut of *P. americana*. Note that occasionally several immunoreactive fibers fasciculate in bundles (arrows). (C,D) In *L. maderae*, there are no proventricular ganglia; instead, proctolin-immunoreactive cell bodies are located in groups in the posterior ingluvial nerves. Immunoreactive cell bodies are indicated by small arrows and arborizations of fibers by larger arrows. (E) Proctolin-immunoreactive fibers associated with circular muscle fibers (mf) in *L. maderae*. In this species, there is also a rich innervation of the foregut muscle by proctolin-immunoreactive fibers. Scale bars, 100 μm (A); 50 μm (B–E).

dilator muscles are supplied by LomTK-LI fibers, as described by Muren *et al.* (1995).

Distribution of proctolin immunoreactivity

The proctolin antiserum was applied to at least ten intestines from each species. In *P. americana*, cell bodies of proctolin-like-immunoreactive (PLI) neurons are distributed at several locations in the stomatogastric nervous system. Up to ten PLI cell bodies are located in the frontal ganglion (some seen in Fig. 10B). PLI cell bodies were also seen in the hypocerebral ganglion, the ingluvial ganglion and in each of the proventricular ganglia (Fig. 9A). The PLI cell bodies are mostly located peripherally in these ganglia. In addition, there are PLI cell bodies scattered along the length of the oesophageal and ingluvial nerves, and there may be further PLI cell bodies in the brain or suboesophageal ganglion contributing fibers to the stomatogastric nervous system. Axons of the PLI neurons run along the entire stomatogastric nervous system, and arborizations are seen in the different ganglia (e.g. Fig. 9A). PLI axons supply the circular muscle fibers of the foregut (Fig. 9B) *via* the different ganglia and side branches of the nerves of the stomatogastric nervous system. This innervation of the foregut is especially dense in the anterior region (pharynx; in front of ingluvial ganglion) but is

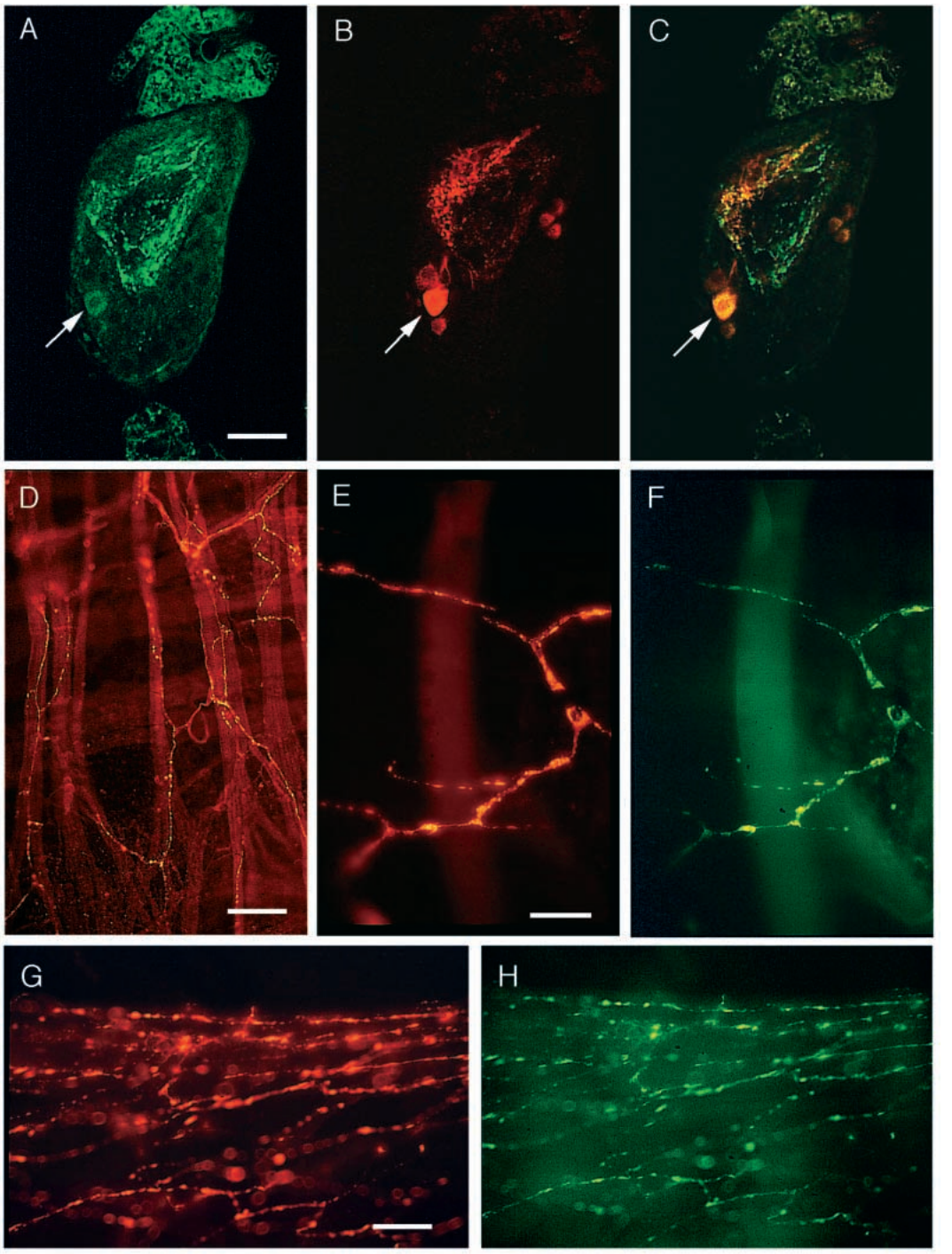

Fig. 10. Double-labelling immunocytochemistry of the same section or wholemount. We used a rat monoclonal anti-substance-P to demonstrate tachykinin-related peptide (TRP), visualized using FITC-tagged secondary antibody (green). Proctolin (rabbit antiserum) was detected using a TRITC-conjugated secondary antiserum (red). (A–C) Co-localization of TRP and proctolin immunoreactivity in a neuron of the frontal ganglion of *Periplaneta americana* as seen in a cryostat section. (A) Substance P immunoreactivity in fibers within the neuropil and in a cell body (arrow). (B) Proctolin immunoreactivity in the same section. The large cell body (arrow) and a few smaller ones contain proctolin immunoreactivity. (C) Double exposure of the same section. The yellow structures (e.g. the cell body at the arrow) contain co-localized substance P and proctolin immunoreactivities: the large cell body (arrow) and some fibers. Some fibers contain only proctolin or only substance P immunoreactivity. (D) Innervation of circular muscle fibers by varicose LomTK-like-immunoreactive processes in the foregut of *P. americana*. (E–F) Varicose nerve fibers in the foregut muscle layer which contain both proctolin immunoreactivity (red) and substance-P-like (green) immunoreactivity. (G,H) Nerve fibers in the salivary gland reservoir muscle also contain co-localized proctolin (G) and substance P (H) immunolabelling. Scales bars: A–C, 50 μm; D, 100 μm; E–H, 25 μm.

also prominent in the oesophagus and crop. The longitudinal muscle fibers do not seem to be directly supplied by PLI fibers. It was, however, possible to detect a dense PLI innervation of the longitudinally oriented muscle fibers of the salivary gland reservoir muscle (see Fig. 10G).

In *L. maderae*, the distribution of PLI fibers in the stomatogastric nervous system was very similar to that seen in *P. americana*. A major difference was that, without proventricular ganglia, most of the posterior PLI cell bodies were scattered in the ingluvial nerves (Fig. 9C,D). This was similar to the distribution of callatostatin-immunoreactive cell bodies detected in the same species (Duve *et al.* 1995). *L. maderae* also showed a dense distribution of PLI fibers on the circular muscle fibers of the foregut (Fig. 9E).

Co-localization of proctolin and TRPs in P. americana

Since the patterns of immunolabelling with antisera to proctolin and LomTK seemed to be very similar in some regions of the foregut muscle, we decided to examine whether these immunoreactivities were co-localized. The antisera to proctolin and LomTK were both produced in rabbit, so double labelling required slight modifications to the immunocytochemical protocol. We first employed double labelling with biotinylated antiserum to LomTK, detected with streptavidin/Texas Red, and the proctolin antiserum binding was visualized with AMCA-labelled secondary antiserum. The proctolin and LomTK immunoreactivities were co-localized in fibers innervating the anterior portion of the foregut: circular muscle and the salivary gland reservoir muscle (not shown). Since this approach produced relatively weak immunolabelling in the muscle layers, we used a monoclonal antibody to substance P, which is known to cross-react with invertebrate TRPs (Nässel, 1993*b*; Blitz *et al.* 1995), in combination with the rabbit anti-proctolin antibody. Exactly the same pattern of co-localized immunoreactivities was seen. As shown in Fig. 10E–H, TRP and proctolin immunoreactivities are co-localized in nerve fibers supplying circular muscle fibers and the salivary gland reservoir muscle. We screened the ganglia for cell bodies giving rise to the fibers with co-localized immunoreactivities. Only in the frontal ganglion could we find a neuronal cell body with co-localized proctolin and substance P immunoreactivity (Fig. 10A–C). This cell body gives rise to arborizations within the neuropil of the frontal ganglion and apparently to the fibers supplying the anterior foregut muscle. Other cell bodies and fibers were found that contained either proctolin or substance P immunoreactivity, but not both. The above observations are based on at least three replicates of each antiserum combination.

Discussion

This study of the foregut of two cockroach species has indicated a species-specific action of tachykinin-related peptides (TRPs) on muscle fibers of *P. americana* but not of *L. maderae*. We have also provided additional evidence that insect TRPs are involved in the control of different types of visceral muscle. Previous work has suggested a role for TRPs as myotropic peptides acting on the locust oviduct (Schoofs *et al.* 1993) and the hindgut of the cockroach *L. maderae* (Muren and Nässel, 1996*b*, 1997; Winther *et al.* 1998).

It is not clear to what degree insect TRPs and mammalian tachykinins are ancestrally related, but it has been well established that tachykinins are potent spasmogens when applied to mammalian visceral muscle (Otsuka and Yoshioka, 1993; Shuttleworth and Keef, 1995). More specifically, peptides of this family are excitatory transmitters in the circular and longitudinal muscle fibers of the mammalian gastrointestinal tract (Bartho and Holzer, 1985; Maggi *et al.* 1993; Otsuka and Yoshioka, 1993; Shuttleworth and Keef, 1995). Three types of G-protein-coupled tachykinin receptor (NK 1–3) with different affinities for the five known mammalian tachykinins (Maggi, 1994; Maggi *et al.* 1994; Gerard *et al.* 1993) have been localized to the rat intestine (Grady *et al.* 1996). Two receptors, designated NKD and DTKR, which are structurally related to the mammalian tachykinin receptors, have been cloned from *Drosophila melanogaster* (Li *et al.* 1991; Monnier *et al.* 1992), but their endogenous ligands are not known. When expressed heterologously in cells, the *Drosophila* NKD receptor could be activated by the locust peptide LomTK II, and this activation could be antagonized by the substance P antagonist Spantide I (Monnier *et al.* 1992). Similarly, the contractile response of the *L. maderae* hindgut to LemTRP 1 was blocked by Spantide I (Winther *et al.* 1998). This antagonist also blocked responses to LomTK I applied to locust dorsal unpaired median neurons (Lundquist and Nässel, 1997) and to *Cancer borealis* TRP 1a (CabTRP 1a) applied to neurons of the crab stomatogastric nervous system (Christie *et al.* 1997). In all these cases, Spantide I did not affect responses to proctolin. It is noteworthy that substance P (albeit at slightly higher concentrations) has a myostimulatory action when applied to the hindgut of *P. americana* (Penzlin *et al.* 1989) and *L. maderae* (D. R. Nässel, in preparation). In *P. americana*, this action could be blocked by another peptidergic substance P antagonist (Penzlin *et al.* 1989). Taken together, these findings suggest that the invertebrate TRPs act on receptors that are similar to the vertebrate tachykinin receptors.

We found a correlation between the presence of innervation of the foregut muscle by LomTK-LI fibers and responsiveness to LomTK 1 and LemTRPs 1 and 5 in *P. americana*. In *L. maderae*, the foregut did not respond to TRPs and no LomTK-LI innervation of foregut muscle was seen. LomTK-LI fibers were, however, detected inside the ganglia and nerves of the stomatogastric nervous system in both species, suggesting that there is also some involvement of TRPs in the regulation of foregut activity in *L. maderae*. The foregut muscle of both cockroach species responded to the pentapeptide proctolin, which correlated with a distinct innervation of muscle fibers by proctolin-like immunoreactive axons. A species-specific action has also been demonstrated for the cockroach neuropeptide corazonin (Predel *et al.* 1994). In eight different cockroach species tested, corazonin stimulated the contraction

of the hyperneural muscle, but only in *P. americana* did this peptide induce contractions in heart muscle and a variety of other visceral muscles.

The native TRPs of *P. americana* have not yet been isolated. It is clear from previous studies that the LomTK antiserum cross-reacts to different extents with all nine known LemTRPs of *L. maderae* and all four LomTKs of *L. migratoria* (Muren and Nässel, 1996*a*,*b*, 1997; Passier, 1996). Thus, we can assume that the immunolabelling in the fibers in the foregut of *P. americana* represents the distribution of native TRPs. The activity of the non-native peptides LomTK I and LemTRPs on the foregut of *P. americana* is not surprising since all the insect and crustacean TRPs isolated so far stimulate contractions of the hindgut of *L. maderae* (Schoofs *et al.* 1990*a*,*b*; Lundquist *et al.* 1994*a*; Muren and Nässel, 1996*b*, 1997; Christie *et al.* 1997). Apparently the active core, which appears to reside in the C-terminal heptapeptide, is largely preserved between different TRP isoforms and different species forms. As mentioned above, it has even been shown that substance P (at a concentration of approximately 10^{-7} mol l^{-1}) stimulated contractions in the *P. americana* hindgut (Penzlin *et al.* 1989). Proctolin has been established as an important myostimulatory peptide both in the cockroach hindgut and in the locust foregut and oviduct (reviewed by Bishop *et al.* 1990; Osborne *et al.* 1990; Osborne, 1996; Orchard and Lange, 1995). As shown here, the muscle fibers of the foregut of *P. americana* and *L. maderae* are supplied by richly arborizing proctolin-immunoreactive nerve fibers derived from the stomatogastric nervous system, and in both species proctolin induces contractions. We found no evidence for any synergism (or other interaction) between proctolin and TRPs in inducing contractions of the *P. americana* foregut. Thus, there is at present no simple functional explanation for the co-localization of the two peptides in nerve fibers derived from the frontal ganglion and with terminations on muscle fibers in the anterior foregut and the salivary reservoir muscle. Further experiments are needed to address this interesting observation.

Co-localization of proctolin and TRP immunoreactivities has also been detected in the crab stomatogastric nervous system (Christie, 1995; Christie *et al.* 1993). The stomatogastric ganglion, with its rhythmically active neural network, controls the rhythmic movements of the foregut (Marder *et al.* 1994). A large number of neuropeptides, including TRPs and proctolin, are known to modulate the rhythmic networks of this ganglion (Nusbaum and Marder, 1989; Marder *et al.* 1994; Blitz *et al.* 1995; Christie *et al.* 1997). Thus, proctolin and TRPs are present in ganglia controlling foregut movements both in crabs and cockroaches and are likely to be important modulators of both rhythmic neural networks and muscle activity in these animals.

It is apparent that the foregut of cockroaches is the target of a number of neuropeptides such proctolin, allatostatins, leucokinins, leucopyrokinins, leucomyosuppressins (extended FLRFamides) and LemTRPs in addition to glutamate, serotonin and possibly other transmitters (Bishop *et al.* 1990; Cook and Wagner, 1990; Cook *et al.* 1989, 1993; Wagner and Cook, 1993; Schoofs *et al.* 1993; Duve *et al.* 1995). Of the compounds listed above, only allatostatins, proctolin, LemTRPs and leucokinins have been mapped to nerve fibers in the cockroach foregut (Duve *et al.* 1995; Bishop *et al.* 1990; Nässel *et al.* 1992; this investigation). More data on the actions of the different peptides and other transmitters are required to understand their physiological roles in the control of the foregut. In addition, the possible interactions between the different neuroactive compounds on the foregut muscle fibers and in the frontal ganglion need to be explored.

The spasmogenic action of the LemTRPs on the *P. americana* foregut is an addition to the list of actions of insect TRPs, which include stimulation of hindgut and oviduct contractions in *L. maderae*, inducement of the release of adipokinetic hormone from locust corpora cardiaca, myostimulatory action on the locust oviduct and depolarization of (and triggering of action potentials in) locust thoracic dorsal unpaired median neurons (Schoofs *et al.* 1993; Nässel *et al.* 1995; Lundquist and Nässel, 1997; Winther *et al.* 1998, D. R. Nässel, in preparation). Immunocytochemical localization of TRPs in *L. maderae* and other insects has demonstrated a widespread distribution, suggesting further roles in different circuits of the central nervous system and in the function of the midgut (Muren *et al.* 1995; Nässel *et al.* 1998) and, consequently, this family of insect peptides appears to be truly multifunctional.

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