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INTESTINAL PEPTIDES AS CIRCULATING HORMONES: RELEASE OF TACHYKININ-RELATED PEPTIDE FROM THE LOCUST AND COCKROACH MIDGUT

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Summary

Tachykinin-related peptides (TRPs) in the locust *Locusta migratoria* and the cockroach *Leucophaea maderae* have stimulatory effects on some muscles that are not innervated by TRP-containing neurons. Thus, these tissues may be affected by circulating TRPs. Here, we have investigated whether the midgut is the source of circulating TRPs.

TRP-immunoreactive material in the locust midgut is found only in the endocrine cells of the gut epithelium. In both species of insect, the endocrine cells contain several isoforms of TRPs, as determined by immunocytochemistry and a combination of chromatography (HPLC) and enzyme immunoassay (ELISA).

The release of TRPs was investigated by ELISA using isolated midguts of the locust and cockroach. Elevated levels of K^+ in the bathing saline induced the release of TRP from the midgut of both species. To examine the release of TRPs into the circulation *in vivo*, we measured haemolymph levels of TRPs in fed and starved locusts. The concentration of TRP-immunoreactive material in fed locusts was estimated to be 0.15 nmol l^{-1}, and this increased approximately fourfold in insects starved for 24 h. In accordance with this observation, the content of TRP-immunoreactive material in the midgut was lower in starved locusts than in fed locusts. Although part of the increased blood concentration of TRPs may be due to reduced blood volume, our data suggest that TRPs are released as hormones from the midgut of the locust and cockroach and that this release may be linked to nutritional status.

Key words: midgut, neuropeptide, tachykinin, hormone, feeding, insect, locust, *Locusta migratoria*, cockroach, *Leucophaea maderae*.

Introduction

Neuropeptides are known to act both as circulating hormones and as central neuromodulators in insects and other higher coelomates (for reviews, see Gäde et al., 1998; Strand, 1999). In fact, some neuropeptides have multiple functions both within the nervous system and at peripheral targets. Among insects, it seems to be more of an exception that neuropeptides act only as locally released neuromodulators without additional functions as circulating hormones (Nässel, 1996; Gäde et al., 1998). One family of insect neuropeptide, however, has not been detected in classical neurosecretory systems in most species studied. This family comprises the tachykinin-related peptides (TRPs), or insectatachykinins, which are found primarily in interneurons and in efferent neurons innervating different types of muscle (Nässel, 1999; Nässel et al., 1998b). The TRPs display multiple actions in the nervous system and on different kinds of muscle (for reviews, see Schoofs et al., 1997; Nässel, 1999; Vanden Broeck et al., 1999). Many of these actions seem to be based on the release of TRPs from axon terminations close to the targets or even mediated by release at distinct synapse-like junctions. The evidence for such close-range action at peripheral targets is immunocytochemical. Some TRP-responsive muscles in cockroaches are directly supplied by TRP-immunoreactive (TRP-IR) axon terminations (Muren et al., 1995; Nässel et al., 1998a), and the hormone-producing glandular cells of the locust corpora cardiaca are contacted by TRP-IR terminals forming synaptoid junctions (Nässel et al., 1995; Vullings et al., 1998).

There are, however, several TRP-responsive muscles in cockroaches and locusts that are not innervated by TRP-IR axons: the Malpighian tubules of locusts respond to locust TRPs (LomTKs) with writhing movements (Coast, 1998), muscle contractions in the oviducts of both the locust and cockroach are stimulated by TRPs (Kwok et al., 1999; Nässel, 1999) and the locust hindleg extensor tibiae muscle responds to LomTKs with an increase in twitch amplitude and in the relaxation rate of twitch tension (Evans, 1994). In other insects, the abdominal heart also responds to TRPs with an acceleration of contractions, but it lacks innervation by TRP-IR axons (Sliwowska et al., 2001; N. J V. Skaer, N. J. Tublitz, H. F. MacGraw, D. R. Nässel and S. H. P. Maddrell, in preparation). These different types of muscle are therefore likely to be activated by circulating TRPs. This assumption is intriguing because there is no evidence in locusts or cockroaches for the

presence of TRPs in typical neurosecretory cell systems in the brain or ventral nerve cord (Nässel et al., 1998b). Only in one moth species and two coleopteran insect species is there immunocytochemical evidence for TRPs in cells of the protocerebral median neurosecretory cell group with axons to the neurohaemal release sites in the storage lobes of the corpora cardiaca and around the aorta (Kim et al., 1998; Sliwowska et al., 2000).

What is the likely source of circulating TRPs in locusts and cockroaches? In the central nervous system of these insects, there is an abundance of TRP-IR interneurons (see Nässel et al., 1998b). These interneurons do not seem to have processes close enough to the circulation to be likely candidates as sources of circulating TRPs. In the cockroach *Leucophaea maderae*, there are, however, some efferent TRP-IR neurons with axons running over the surface of the hindgut and midgut, and in the American cockroach *Periplaneta americana* TRP-IR axons have been found on the foregut muscle layer (Muren et al., 1995; Nässel et al., 1998a). These axons are in contact with the open circulation and could be potential sources of hormonal TRPs. However, in locusts, where the best evidence exists for an *in vitro* action of TRPs on target muscle lacking TRP-IR innervation, such efferent axons have not been detected. When screening for further potential sources of circulating TRPs in locusts, the only tissue that captured our interest was the midgut. This portion of the intestine was found to contain numerous TRP-IR cells with morphologies suggestive of an endocrine function.

The insect midgut is known to contain a multitude of peptides, including TRPs, and these have been localized to cells of endocrine type (see Iwanaga et al., 1981; Montuenga et al., 1989; Zitnan et al., 1993, Lundquist et al., 1994; Muren et al., 1995; Veenstra et al., 1995; Yu et al., 1995; Pabla and Lange, 1999). Endogenous TRPs have been identified in the midgut of *Leucophaea maderae* and the locust *Schistocerca gregaria*; some of these are specific to the midgut and are not found in the brain (Muren and Nässel, 1996b; Muren and Nässel, 1997; Veelaert et al., 1999). The functions of the midgut peptides are not known, although a few recent publications suggest local roles associated with midgut function: FLRFamide-related peptides and TRPs regulate midgut contractions (Lange and Orchard, 1998; Pabla and Lange, 1999), and sulfakinins and myosuppressins control the release of digestive enzymes (Nachman et al., 1997). Functions related to feeding have also been suggested by the fluctuating peptide content of insect midgut endocrine cells during starvation, after feeding with different nutrients and while being parasitized by wasps (Zitnan et al., 1995; Kingan et al., 1997; Zudaire et al., 1998). Furthermore, injection of peptides of the sulfakinin family has been shown to reduce food intake in locusts (Wei et al., 2000). In insects in which neuropeptides have been found in axons forming extensive plexa on the midgut surface, for instance in the cockroaches *Leucophaea maderae* and *Diploptera punctata*, it has been suggested that these peptides may be released into the circulation (Muren et al., 1995: Yu et al., 1995; Winther et al., 1999). Release of allatostatins from the midgut of *D. punctata* has been demonstrated *in vitro* (Yu et al., 1995). There is, however, no experimental evidence for the release of peptides from midgut tissue, where peptides are found only in endocrine cells.

The present paper demonstrates for the first time the release of peptides from the locust midgut. We induced the release of locust TRPs (LomTKs) from the isolated midgut *in vitro* and also demonstrated different levels of LomTKs in haemolymph collected from starved and fed insects. Furthermore, we have demonstrated the release of TRPs from the isolated midgut of *Leucophaea maderae*.

Materials and methods

Animals

Cockroaches of the species *Leucophaea maderae*, obtained from laboratory stock, were fed dry dog chow and water *ad libitum*. African migratory locusts *Locusta migratoria* were purchased from Blades Biological (Cowden, Kent, UK) and fed fresh wheat and oat bran. Both species were kept at 26 °C on a 12 h:12 h light:dark photoperiod. In all experiments, adult animals of both sexes were used unless stated otherwise.

Immunocytochemistry

The intestines (midguts) of adult male and female locusts and cockroaches were removed, ligated at the cut ends, and inflated with 0.1 mol l^{-1} sodium phosphate buffer. These inflated guts were immersed for fixation in 4% paraformaldehyde in 0.1 mol l^{-1} sodium phosphate buffer for 30 min. The intestines were cut along their length and pinned out on a Sylgard-coated dish in the same fixative for another 2 h and then washed in phosphate buffer.

To detect TRPs in the locust intestine, we used an antiserum raised against LomTK-I (code 9207; Nässel, 1993). This antiserum has been extensively characterized (Nässel, 1993: Lundquist et al., 1994; Muren and Nässel, 1996a). We previously used antisera to *Leucophaea maderae* TRPs (LemTRP-2 and LemTRP-3) to detect specific peptide isoforms in the cockroach midgut (Winther et al., 1999). Here, we produced two more antisera to *Leucophaea maderae* TRPs. One (code M9838) was raised in guinea pig to an N-terminal hexapeptide sequence of LemTRP-1, synthesized with a C-terminal Cys residue (APSGFLC) to enable coupling of bovine serum albumin (BSA) to the SH group of the Cys (using maleimide-coupled BSA). Antigen production and immunization was performed as described previously (Winther et al., 1999). We also raised a rabbit antiserum (code K9836) to the full sequence of LemTRP-1 (APSGFLGVRamide) coupled to BSA with carbodiimide (as described previously; Nässel, 1993). Immunization was performed at Euro-Diagnostica (Malmö, Sweden) (as described by Winther et al., 1999).

The antisera were employed for immunocytochemistry at the following dilutions: anti-LomTK-I at 1:1000, rabbit anti-LemTRP-1 at 1:6000 and guinea pig anti-LemTRP-$1_{1–6}$ at 1:2000. The antisera were diluted in dilution buffer (DiB)

consisting of 0.01 mol l^{-1} phosphate-buffered saline (PBS) with 0.5 % BSA and 0.1 % Triton X-100. Tissues were incubated in antisera for 2–4 days at 4 °C, washed in DiB and incubated for 24 h in peroxidase-conjugated IgGs (secondary antiserum): swine anti-rabbit or rabbit anti-guinea pig (Dako, Copenhagen, Denmark). Detection of peroxidase was performed with 0.03 % diaminobenzidine and 0.01 % hydrogen peroxide in 0.05 mol l^{-1} Tris-HCl buffer (for a detailed protocol, see Winther et al., 1999). The specificity of these antisera was tested immunocytochemically on gut tissue by preabsorption with the respective antigen or full-length peptide (20 and 50 nmol ml^{-1} diluted antiserum).

Preparation of the midgut and release experiments

Prior to dissection, animals were cold-anaesthetized. Locust tissues were dissected in locust saline (Orchard and Lange, 1987): 150 mmol l^{-1} NaCl, 10 mmol l^{-1} KCl, 4 mmol l^{-1} $CaCl_2$, 2 mmol l^{-1} $MgCl_2$, 4 mmol l^{-1} $NaHCO_3$, 5 mmol l^{-1} Hepes, 90 mmol l^{-1} sucrose, 5 mmol l^{-1} trehalose, pH 7.2. Cockroach tissues were dissected in cockroach saline: 150 mmol l^{-1} NaCl, 12 mmol l^{-1} KCl, 10 mmol l^{-1} $CaCl_2$, 2 mmol l^{-1} $MgCl_2$, 4 mmol l^{-1} Hepes, pH 7.2 (Thompson et al., 1987). The midguts of both species were ligated with cotton threads just in front of the gastric caeca and behind the Malpighian tubules. The Malpighian tubules were carefully removed. For each release experiment, five midguts were collected and kept in saline on ice. The release experiments were, in principle, conducted according to the method of Yu et al. (Yu et al., 1995) in continuously aerated locust or cockroach saline at 4 °C and in the presence of a mixture of peptidase inhibitors (Complete Mini, EDTA-free; Boehringer, Mannheim, Germany). The tissues were transferred sequentially to normal saline (three incubations of 1 h), then to high-K^+ saline (100 mmol l^{-1} KCl with the additional KCl replacing NaCl) for 1 h and again to normal saline (two incubations of 1 h). After each incubation, the saline was collected and purified on Sep-Pak C-18 Classic cartridge (Waters; Milford, MA, USA) as described below. After release experiments, the midguts were stored at −70 °C until the total content of LomTK-immunoreactivity was determined.

Tissue extraction and purification

Extraction of peptides from the midguts was performed in ice-cold extraction solvent (according to the method of Bennett et al., 1981) consisting of 1 mol l^{-1} HCl, 5 % formic acid, 1 % NaCl, 1 % trifluoroacetic acid (TFA). The tissues were homogenized with a Teflon pestle in a glass tissue homogenizer (10 µl of solvent per milligram of tissue). The homogenate was briefly sonicated on ice and then centrifuged at 11 600 ***g*** for 15 min at 4 °C. The supernatant was removed and kept on ice. The pellet was resuspended in ice-cold extraction solvent, sonicated and centrifuged for another 15 min. The two supernatants from one gut equivalent were combined and purified on a Sep-Pak C-18 Classic cartridge. Prior to use, the cartridge was activated with 10 ml of acetonitrile (CH_3CN) and equilibrated with 20 ml of 0.1 % aqueous TFA. After loading the sample, the cartridge was washed with 5 ml of 0.1 % aqueous TFA, followed by a wash with 5 ml of 10 % CH_3CN containing 0.1 % TFA. The sample was then eluted with 2 ml of 40 % CH_3CN containing 0.1 % TFA. This fraction was freeze-dried prior to determination of the peptide content in the enzyme-linked immunosorbent assay (ELISA; see below).

Preparation of haemolymph

Haemolymph samples were collected from male locusts (1–2 weeks old). We separately sampled fed locusts and locusts starved for 24 h. For each sample, haemolymph from 10 insects was collected using microcapillary tubes and pooled in tubes kept on dry ice. To every pooled sample, 500 µl of 10 % CH_3CN with 0.1 % TFA was added. The samples were centrifuged at 11 600 ***g*** for 10 min at 4 °C. The supernatants were purified on Sep-Pak C-18 Light cartridges. Cartridges were activated with 3 ml of CH_3CN equilibrated with 6 ml of 0.1 % aqueous TFA. The samples were loaded onto the cartridge, washed with 1.5 ml of 10 % CH_3CN containing 0.1 % TFA and eluted with 1 ml of 40 % CH_3CN with 0.1 % TFA. The eluate was freeze-dried before ELISA analysis. Midguts from the locusts used for haemolymph sampling were dissected, extracted and purified as described above. We also collected and pooled haemolymph from 18 starved, male locusts. This sample was treated in the same way as the other haemolymph samples, but also separated on HPLC before determining peptide content in the ELISA (see below).

Enzyme-linked immunosorbent assay (ELISA)

Synthetic LomTK-I was conjugated to bovine thyroglobulin (1:10) using 0.1 % glutaraldehyde in 0.1 mol l^{-1} phosphate-buffered saline (PBS, pH 7.4) for coupling. The reaction was performed at room temperature (21–23 °C) overnight and stopped by addition of sodium metabisulphite (final concentration 53 mmol l^{-1}). The conjugate was concentrated by dialysis against PBS at 4 °C overnight. The optimal concentration of LomTK-I conjugate (125 ng ml^{-1}) and dilution of antiserum (1:80 000) were determined using checkerboard titration. The LomTK-I conjugate was coated on microtitre plates, 100 µl per well (Maxisorp immunoplate; Nunc, Roskilde, Denmark), in coating buffer (5.5 mmol l^{-1} Na_2CO_3, 35 mmol l^{-1} $NaHCO_3$, 0.02 % NaN_3, pH 9.6) and incubated for 3 h at room temperature. Non-specific binding to the microtitre plates was blocked with blocking buffer (137 mmol l^{-1} NaCl, 20 mmol l^{-1} Tris, 0.02 % NaN_3, 1 % gelatin, pH 7.4) for 1 h at room temperature. After washing three times with 300 µl of wash buffer (WB; 137 mmol l^{-1} NaCl, 20 mmol l^{-1} Tris, 0.02 % NaN_3, 0.1 % gelatin, 0.05 % Tween 20, pH 7.4), freeze-dried samples were reconstituted in WB and added to the wells (50 µl per well). Synthetic LomTK-I was used as a standard. LomTK-I antiserum (code 9207/7; Nässel, 1993) diluted in WB was added to each well to give a final dilution of 1:80 000 (50 µl per well). The microtitre plates were incubated overnight at 4 °C and then for 1 h at room temperature. Following three washes with WB, 100 µl of goat

anti-rabbit antiserum labelled with alkaline phosphatase (Pierce, Rockford, IL, USA) was added at a final dilution of 1:4000, and the mixture was incubated for 2 h at room temperature. After washing three times with WB, we added *p*-nitrophenyl phosphate (1 mg ml^{-1}, Pierce) in substrate buffer (0.05 mmol l^{-1} $MgCl_2$, 9 % diethanolamine, 0.02 % NaN_3, pH 9.8). The colour reaction was stopped by the addition of 3 mol l^{-1} NaOH (100 µl per well). Plates were monitored at 405 nm in an ELISA plate reader (Labsystems Multiskan Plus, Helsinki, Finland).

All tissue and haemolymph samples were assayed in duplicate and at a range of dilutions. The cross-reactivity of synthetic peptides to LomTK-I antiserum was determined using ELISA. Synthetic LomTK-I and -II were obtained from Peninsula, and synthetic LomTK-III and -IV were kindly provided by Drs R. Kwok and I. Orchard (Department of Zoology, University of Toronto, Canada). Synthetic LemTRP-1, LemTRP-3, LemTRP-4 and LemTRP-5 were synthesized by Dr Å. Engström, Department of Medical Biochemistry, Uppsala University (see Muren and Nässel, 1996b).

Reverse-phase high-performance liquid chromatography (HPLC)

Freeze-dried samples of either three locust midgut equivalents or haemolymph pooled from 18 starved locusts were reconstituted in 100 µl of 10 % CH_3CN containing 0.1 % TFA and separated on a Waters HPLC system with a U6K injector, a 600E system controller and a 486 ultraviolet detector (set at 214 nm). For separation, the samples were loaded onto a Supelcosil C-8 reverse-phase column (LC-8-DB, 3 µm, 4.6 mm×150 mm; Supelco, Bellefonte, PA, USA) at room temperature. Samples were eluted with 15 % CH_3CN with 0.1 % TFA for 8 min, followed by a linear gradient of 15 % to 20 % CH_3CN with 0.1 % TFA for 13 min. Isocratic conditions (20 % CH_3CN with 0.1 % TFA) were run for 13 min and then followed by a linear gradient of 20 % to 40 % with 0.1 % TFA for 18 min. Fractions were collected at a flow rate of 1 ml min^{-1} and stored at −70 °C until further processing. Prior to assay using ELISA, the samples were freeze-dried. To establish the different retention times of the LomTKs, synthetic LomTK-I–IV (100 pmol of each) were chromatographed and detected by ultraviolet absorbance.

Statistical analyses

Student's *t*-test or analysis of variance (ANOVA) and the Bonferroni test were performed using Prism 2.0 (GraphPad, CA, USA). Differences were considered significant at $P<0.05$. Results are presented as means ± S.E.M.

Results

Immunocytochemistry

The distribution of LomTK-immunoreactive (LomTK-IR) material in cells of the intestine of *Locusta migratoria* was determined using an antiserum to LomTK-I known to cross-react to varying degrees with all insect TRPs (Nässel, 1993; Lundquist et al., 1994; Muren et al., 1995; see also below). Numerous LomTK-IR cells were found in the midgut (Fig. 1A–C). The morphology of these cells suggests that they are endocrine in nature, with a broader region close to the outer surface of the intestine and a thinner portion extending towards the gut lumen (Fig. 1D,E). As reported previously (Pabla and Lange, 1999), there are LomTK-IR cells both in the major portion of the midgut (Fig. 1A) and in the gastric caeca (not shown). Furthermore, there are slightly larger LomTK-IR cells in the six ampullae of the Malpighian tubules (Fig. 1B,C). The larger cells were not described by Pabla and Lange (Pabla and Lange, 1999), but similar endocrine cells in the locust ampullae have been identified in a study using antisera to locust diuretic hormone and the mammalian tachykinin substance P (Montuenga et al., 1996).

Endocrine cells reacting with antisera to LomTK and other TRPs have been described in *Leucophaea maderae* (Muren et al., 1995; Winther et al., 1999). In addition, it was found that the midgut surface is supplied by a plexus of TRP-containing axons. Winther et al. (Winther et al., 1999) demonstrated, using HPLC combined with radioimmunoassay, that the axons contain LemTRP-1, -3, -4 and -5, but not LemTRP-2, whereas the endocrine cells of the midgut proper contain LemTRP-2 and -3 (it was only possible to assay these using immunocytochemistry). Now, with new specific antisera, we can show that LemTRP-1 is also present in the endocrine cells of the midgut proper (Fig. 1F). This was revealed by the application of two different antisera to LemTRP-1; one raised against the N terminus and the other against the C terminus of LemTRP-1. Also, the axons on the surface of the midgut muscle layer reacted with the LemTRP-1 antisera (Fig. 1G), confirming the earlier biochemical data.

Cross-reactivity of LomTK-I antiserum in ELISA

We tested the cross-reactivity of the LomTK-I antiserum with the different LomTK isoforms (LomTK-I, -II, -III and -IV) using ELISA (Fig. 2; sequences displayed in Table 1). LomTKs were tested in serial dilutions at concentrations ranging between 10^{-13} and 10^{-7} mol l^{-1}. The antiserum recognized LomTK-IV to a lesser degree than the other LomTKs (1 % of the LomTK-I binding to the antiserum calculated from the EC_{50} values).

The cross-reactivity of the LomTK-I antiserum was also tested with the different TRP isoforms of the *Leucophaea maderae* gut, LemTRP-1, -3, -4 and -5 (see Table 2). LemTRP-

Table 1. *Amino acid sequence of LomTKs*

Peptide	Sequence
LomTK-I[1]	GPSGFYGVRa
LomTK-II[1]	APLSGFYGVRa
LomTK-III[2]	APQAGFYGVRa
LomTK-IV[2]	APSLGFHGVRa

Sequences are from [1]Schoofs et al., 1990b and [2]Schoofs et al., 1990a.

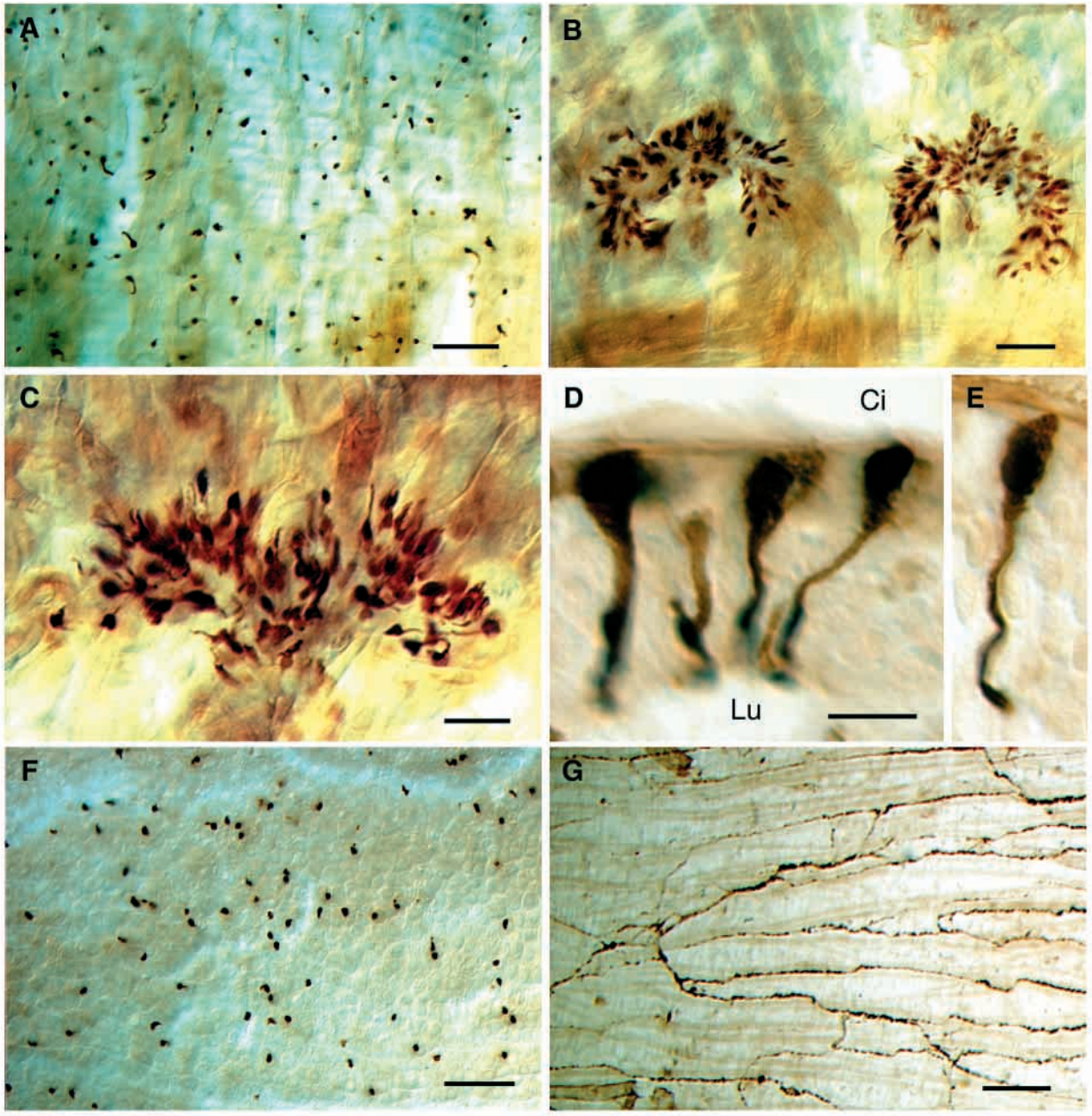

Fig. 1. Immunocytochemical localization of tachykinin-related peptides (TRPs) in the midguts of the locust *Locusta migratoria* and the cockroach *Leucophaea maderae*. All panels, except D and E, are spread wholemount preparations (peroxidase-based detection). (A) LomTK-immunoreactive (LTK-IR) endocrine cells of the midgut of *Locusta migratoria* seen from the outer surface of the intestine. (B) Two ampullae of Malpighian tubules (*Locusta migratoria*) with numerous LTK-IR endocrine cells. (C) Higher magnification of another ampulla (*Locusta migratoria*) with immunoreactive endocrine cells. (D,E) Transverse cryostat sections of locust midgut with LTK-IR endocrine cells. Note the enlarged portion towards the outer surface of the gut and the circulation (Ci) and the slender extension towards the gut lumen (Lu). (F) Endocrine cells of the cockroach midgut reacting with an antiserum to the N-terminal portion of LemTRP-1. (G) The same LemTRP-1 antiserum also labels a plexus of varicose neuronal processes on the surface of the muscle layer in the cockroach midgut. Scale bars: A,B,F,G, 100 μm; C, 50 μm; D,E, 10 μm.

2, which is also present in the gut, was not tested since it is an N-terminally extended form of LemTRP-1 with an identical C-terminal nonapeptide. The LemTRPs were tested at concentrations ranging between 10^{-11} and 3×10^{-6} mol l^{-1}. The LemTRPs were recognized to lesser extent than the LomTKs, with cross-reactivity ranging from 14.4 to 0.0015 % of the binding to LomTK-I (Table 2).

In both species of insect, it is thus likely that the LomTK antiserum recognizes all the isoforms, but to differing degrees. The ELISA measurements of released material are therefore an approximate estimate of a cocktail of TRP isoforms. Values given for the amount of TRP-IR material have not been corrected for cross-reactivity, so the actual amounts of peptide released are probably higher than those given.

Table 2. *EC_{50} values and cross-reactivity with LomTK-I antiserum of the different synthetic LemTRPs measured using ELISA*

Peptide	Sequence	EC_{50} value (mol l^{-1})[1]	Cross-reactivity (%)[2]
LomTK-I	GPSGFYGVRa[3]	5.6×10^{-10}	100
LemTRP-1	APSGFLGVRa[4]	3.9×10^{-9}	14.4
LemTRP-3	NGERAPGSKKAPSGFLGTRa[4]	3.7×10^{-5}	1.5×10^{-3}
LemTRP-4	APSGFMGMRa[4]	6.6×10^{-6}	8.5×10^{-3}
LemTRP-5	APAMGFQGVRa[4]	5.7×10^{-7}	0.1

[1]Samples were measured in duplicate. Curves were fitted to a four-parameter logistic equation from which the EC_{50} values were calculated (GraphPad Prism 2.0).

[2]The cross-reactivity is calculated from the EC_{50} values, with the EC_{50} value of LomTK-I as 100 %.

Sequences are from [3]Schoofs et al., 1990a; [4]Muren and Nässel, 1996b.

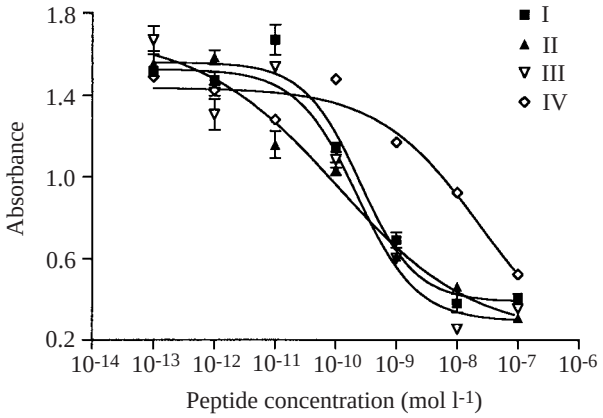

Fig. 2. ELISA was performed to determine the cross-reactivity of LomTK-I antiserum with synthetic LomTK-I–IV. Serial dilutions of LomTK-I (I), LomTK-II (II), LomTK-III (III) and LomTK–IV (IV) were added at concentrations ranging from 10^{-7} to 10^{-13} mol l^{-1}. The EC_{50} values are 2.5×10^{-10} mol l^{-1} for LomTK-I, 1.1×10^{-10} mol l^{-1} for LomTK-II, 2.3×10^{-10} mol l^{-1} for LomTK-III and 2.4×10^{-8} mol l^{-1} for LomTK-IV. Samples were assayed in triplicate, and each point represents the mean ± S.E.M. Curves were fitted to a four-parameter logistic equation from which the EC_{50} values were calculated (GraphPad Prism 2.0).

HPLC separation followed by ELISA identifies several LomTK isoforms in the locust midgut

We wanted to determine how many isoforms of LomTKs are likely to be present in the locust midgut (and possibly released from it). HPLC separation of midgut extracts combined with ELISA revealed the presence of at least four LomTK isoforms. The immunoreactive fractions of separated midgut extract eluted with retention times corresponding to synthetic LomTK-I–IV (Fig. 3). The earliest peak (fraction 4, labelled A in Fig. 3) does not correspond to any of the synthetic peptides and is likely to be the incompletely characterized LomTK-V (Schoofs et al., 1993; Kwok et al., 1999). A possible sixth isoform is also present in the gut, eluting between LomTK-IV and LomTK-II (Fig. 3, labelled B). This isoform may correspond to a new N-terminally extended TRP similar to that identified in the midgut of *Schistocerca gregaria* (Veelaert et al., 1999). Interestingly, only five LomTKs have been identified in the locust brain. Thus, it is possible that there is an extended midgut-specific TRP, similar to findings in the cockroach *Leucophaea maderae* (Muren and Nässel, 1996b).

While this study was close to completion, a report appeared (Pabla and Lange, 1999) on the presence of LomTK-IR endocrine cells in the midgut and in the presence of the different LomTK isoforms (I–IV) and a possible fifth isoform (possibly LomTK-V) in the midgut of *Locusta migratoria.* Our study confirms these findings and adds the immunocytochemical findings of the large LomTK-IR cells in the ampullae of the Malpighian tubules and of a possible sixth isoform present in midgut extracts of *Locusta migratoria.*

Release of LomTK-IR material from locust and cockroach midgut in vitro

For each experiment, five ligated locust or cockroach midguts were processed together in locust or cockroach saline, respectively. The saline solutions were supplemented with a mixture of protease inhibitors, and the incubation was carried out at 4 °C. The experiment was performed five times for the locust midgut and three times for the cockroach midgut. To monitor the release of LomTK-IR material, the midguts were incubated in saline for 1 h and then transferred to fresh saline. The midguts were incubated in normal saline three times for 1 h and then treated with high-K^+ saline (100 mmol l^{-1} KCl) for 1 h. Finally, the midguts were again incubated in normal saline twice for 1 h. The incubation medium was collected at the end of each 1 h incubation and assayed for LomTK-IR material using ELISA. During the first hour, there was a high spontaneous release of LomTKs (184.8±34.0 fmol per midgut) from the locust midguts (Fig. 4A). The spontaneous release then decreased to approximately half of that level during the following 2 h (i.e. 76.8±24.8 fmol per midgut). Treatment with high-K^+ saline during the fourth hour resulted in a significant elevation in the release of LomTKs to 3.5 times the spontaneous release measured in the previous hour (i.e. 249.8±51.1 fmol per midgut; $P<0.01$; Fig. 4A). The spontaneous release of tachykinins decreased during the subsequent incubations in normal saline (Fig. 4A).

In the experiments on cockroach midguts, there was a

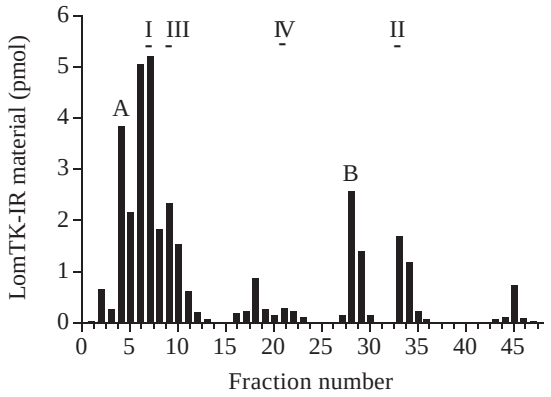

Fig. 3. An extract of three *Locusta migratoria* midgut equivalents was separated by HPLC on a C8 column. LomTK-immunoreactive (LomTK-IR) material was detected by ELISA. Fractions were collected every 30 s, except fractions 1–3 and 44–48, which were collected at 3 min intervals. Bars indicate fractions in which the synthetic peptides were detected. Note that the EC_{50} for LomTK-IV in the ELISA is only 1.0 % of the EC_{50} value for LomTK-I. The peak labelled A is likely to be the incompletely characterized LomTK-V (Schoofs et al., 1993; Kwok et al., 1999). The peak labelled B could be a possible new isoform (see Veelaert et al., 1999). However, this is just an assumption, and there are also small amounts LomTK-immunoreactive material in fraction 18 that could be a possible new midgut isoform.

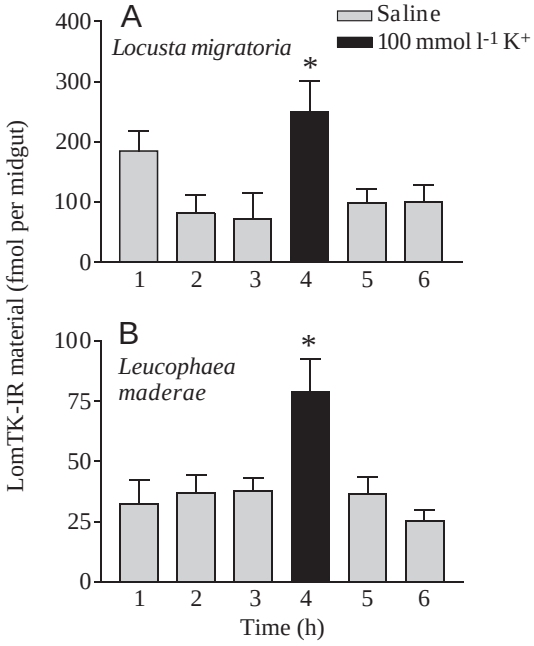

Fig. 4. K^+-induced release of LomTK-immunoreactive (LomTK-IR) material from *Locusta migratoria* and *Leucophaea maderae* midguts *in vitro*. On transfer from normal saline to high-K^+ saline (100 mmol l^{-1}), there is a 3.5-fold increase in *Locusta migratoria* (A) and a 2.1-fold increase in *Leucophaea maderae* (B) in the amount of LomTK-IR material released compared with the spontaneous release during the previous hour. The large amount of LomTK-IR material released during the first hour in A was considered to be leakage resulting from traumatic conditions and is not included in the statistical analysis. Each column represents the mean + S.E.M. of five (A) or three (B) different experiments. An asterisk indicates a significant difference (P<0.01; ANOVA and Bonferroni test) from all other values.

constant level of spontaneous release (35.9±4.0 fmol per midgut) over the first 3 h. Treatment with high-K^+ saline induced an elevated release of LomTK–IR material that was 2.1 times higher than that in the previous hour (78.9±13.6 fmol per midgut; P<0.01) (Fig. 4B). Levels of release then decreased when the midguts where transferred to normal saline (Fig. 4B).

The amount of immunoreactive tachykinins released from midguts treated with high-K^+ saline, calculated as a percentage of the total midgut content of LomTK-IR material, was 1.0±0.44 % in locust and 4.5±0.73 % in cockroach. The total content of LomTK-IR material in the gut was calculated as the sum of the LomTK-IR material released into the saline over the complete experiment and the LomTK-IR material extracted from the midguts after the incubations. Note that the LemTRPs cross-react to a much lower degree with the LomTK antiserum (Table 2). Thus, the total amount of TRPs released and remaining in the cockroach gut is probably much higher than that estimated above.

Haemolymph levels of LomTK-IR material in locusts during feeding and starvation

Adult male locusts, 1–2 weeks old, were allocated to two groups. The first group, consisting of 10 insects, had access to food, while the other group of 10 insects was deprived of food for 24 h. Neither group had access to water. Haemolymph samples were pooled for each group. Following sampling of haemolymph, the midguts were removed from the insects and peptides were extracted for identification. Haemolymph samples and midgut extracts were then assayed for LomTK content using ELISA. These experiments were performed five times (each time with 10 starved and 10 fed insects). The haemolymph level was 4.4 times higher (0.66±0.19 nmol l^{-1}) in starved insects than in fed ones (0.15±0.03 nmol l^{-1}) (Fig. 5A). The difference was statistically significant (P<0.05). The volume of haemolymph that it was possible to collect with microcapillary tubes from starved locusts decreased by 65.8 % compared with the volume from fed ones (49.7±3.9 µl per insect from fed and 17.0±1.5 µl per insect from starved). The LomTK content of the midgut was 1.6 times higher in fed insects than in starved ones (85.7±8.1 pmol per midgut in fed insects and 54.0±10.8 pmol per midgut in fed insects; P<0.05) (Fig. 5B). It appears that part of the observed increase in haemolymph concentration could be due to a reduction in haemolymph volume in starved locusts rather than to actual release of TRPs. However, the significant reduction in TRP content in the midgut tissue of starved insects appears to indicate a release of TRPs during starvation. It is also important to note that neuropeptides released into the haemolymph are subject to rapid proteolytic degradation (Li et al., 1997;

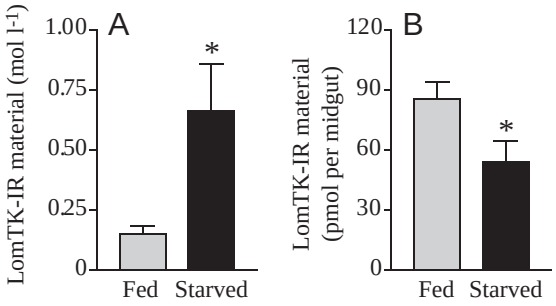

Fig. 5. LomTK-immunoreactive (LomTK-IR) material in haemolymph and midguts of locusts during feeding and starvation. Each column represents the mean + S.E.M. of five different determinations (each with 10 fed or starved insects). (A) Starvation promotes an increase in concentration of LomTK-IR material in the haemolymph. (B) The content of LomTK-IR material in midgut extract decreases during starvation. An asterisk indicates a significant difference (P<0.05, Student's t-test) between the fed and starved locusts.

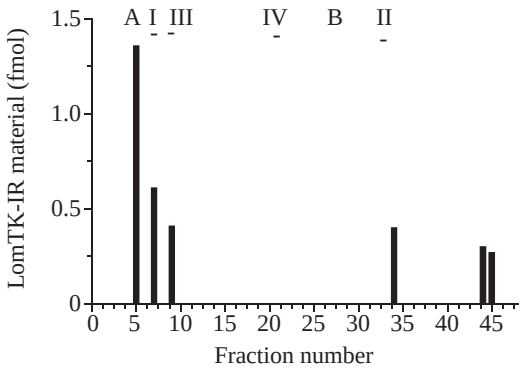

Fig. 6. Locust haemolymph samples were separated by HPLC on a C8 column, and LomTK-immunoreactive (LomTK-IR) material was detected by ELISA. Fractions were collected every 30 s, except fractions 1–3 and 44–48, which were collected at 3 min intervals. Bars indicate fractions in which the synthetic peptides were detected. Note that the EC_{50} value for LomTK-IV in the ELISA is only 1.0 % of the EC_{50} value for LomTK-I. The peak labelled A is likely to be the incompletely characterized LomTK-V (Schoofs et al., 1993). B indicates were the possible new isoform would elute (see Veelaert et al., 1999).

Oudejans et al., 1996). Thus, haemolymph levels of TRPs measured after extended periods of release are likely to be drastically underestimated.

LomTK isoforms in the haemolymph of locusts

We pooled the haemolymph samples from 18 adult male locusts that had been starved for 24 h. The pooled haemolymph sample was separated by HPLC, and the fractions were freeze-dried and assayed using ELISA to identify the different LomTK isoforms. We chose to collect haemolymph from starved locusts since the haemolymph from starved locusts has a higher concentration of LomTKs (Fig. 5). We could identify LomTK-IR material in fractions that coincided with the retention times of LomTK-I, -II and -III (Fig. 6). We could not detect any LomTK-IR material with the same retention time as synthetic LomTK-IV. Since LomTK-IV cross-reacts to a very low degree with the LomTK-I antiserum (the EC_{50} for LomTK-IV is only 1.0 % of that for LomTK-I; see Fig. 2), it is possible that LomTK-IV is present in the haemolymph but below the detection limit. LomTK-IR material also eluted in fraction 5, which probably corresponds to the partially characterized LomTK-V (Schoofs et al., 1993; Kwok et al., 1999). A possible novel sixth isoform, found in the midgut extract (see Fig. 3), could not be identified in the haemolymph. This may indicate that this isoform is released to a lower degree than the other isoforms or is not released at all.

Discussion

Our findings strongly suggest that the locust midgut is an endocrine organ releasing TRPs into the circulation: treatment with high-K^+ saline induced release of TRPs from the locust midgut *in vitro*, and we could detect TRPs in the locust haemolymph *in vivo*. This is especially intriguing since no other neurosecretory systems containing TRPs have been detected in locusts, and the only sources of TRPs in the locust midgut are the endocrine cells of the intestinal epithelium. Further support for the idea that TRPs identified in the haemolymph are released from the endocrine cells of the midgut is the finding that, while the TRP concentration in the haemolymph increased in starved locusts, the TRP content of the midgut tissue decreased. The only other demonstrations in insects of peptide release from the intestine into the circulation are cases in which the peptide is mainly, or exclusively, present in neuronal processes derived from cell bodies outside the intestine (Truman and Copenhaver, 1989; Yu et al., 1995).

We also demonstrated K^+-induced release of TRPs from the midgut of the cockroach *Leucophaea maderae in vitro*. Because of difficulties in sampling enough haemolymph from this species, we have no data on release *in vivo*. Both endocrine cells and neural processes are present in the midgut of *Leucophaea maderae*, and our experiments cannot distinguish whether one or both of these cell types are the source of release. This is similar to the situation in the cockroach *Diploptera punctata*, in which *in vitro* release of allatostatins was induced from the midgut, which contains these peptides in both axons and endocrine cells (Yu et al., 1995). It is possible that other types of peptide identified in the endocrine cells of the insect midgut (see Zitnan et al., 1993; Veenstra et al., 1995) are also released into the circulation, and that this part of the intestine is an important neurohaemal release site. Recently, it has been shown that the foregut and hindgut of the crab *Carcinus maenas* are supplied with large numbers of endocrine cells containing the peptide hyperglycaemic hormone and that this peptide is released in massive amounts during ecdysis (Chung et al., 1999; Webster et al., 2000). Thus, it may be that intestinal endocrine cells in arthropods are generally employed for release of peptides into the circulation, in addition to the better-described cerebral and thoracico-abdominal

neurosecretory cell systems and neurohaemal organs (see Raabe, 1989; Orchard, 1983; Nässel et al., 1994).

In the cockroach, the amount of peptide released compared with the total content of peptide in the gut was greater than in the locust. This implies that the TRP-immunoreactive neuronal processes that form a superficial plexus on the outer muscle layer of the cockroach midgut constitute an additional source of hormonal TRPs. Some other insects have been shown to have both TRP-immunoreactive endocrine cells in the intestine and cerebral neurosecretory cells with terminations in the corpora cardiaca (Kim et al., 1998; Sliwowska et al., 2000), suggesting multiple neurohormonal release sites for TRPs.

Although we could show significant K^+-induced release of TRPs *in vitro* and the probable release of TRPs into the haemolymph of locusts *in vivo*, it was not possible to correlate the amount released in these different experiments. In the *in vitro* experiments, TRP release was monitored in the presence of peptidase inhibitors over a relatively brief period. The haemolymph samples were collected after the locusts had been kept for 24 h under different conditions. It is very likely that the TRPs in the haemolymph are degraded by various released or membrane-bound peptidases, as for other circulating insect peptides (see Oudejans et al., 1996; Li et al., 1997). Three types of insect peptidase have been shown to degrade TRPs (Lamango et al., 1997; Nässel et al., 2000; R. E. Isaac and D. R. Nässel, unpublished observations) and may well also be acting on circulating peptides. As a result, it is possible that the TRP levels measured in the locust haemolymph provide a rather poor estimate of the actual amount released. Another caveat in our haemolymph peptide determinations is that, after starvation, the amount of haemolymph appears to decrease by at least 50 % (as determined by the maximum amount of haemolymph that it is possible to sample from each locust). Thus, most of the increase in TRP concentration (a 4.4-fold increase) may be an effect of decreased haemolymph volume. A similar loss of haemolymph volume was reported after exposure of crickets to desiccation (Chung et al., 1994), but in that study the amount of a diuretic hormone released was still significant after the volume loss had been subtracted. At present, we can therefore suggest that TRPs are present in the haemolymph both in fed and starved locusts, but are unable to state unequivocally that there is a significant increase in TRP release in response to starvation. We can, however, clearly demonstrate a significant decrease in the amount of TRPs in the midgut after 24 h of starvation, which is suggestive of TRP release.

What are the physiological functions of the TRPs released into the circulation from the midgut and other sites? As shown in the present study, the release of TRPs could be related to feeding status since there is a decrease in TRP levels in the midgut and there may be a higher concentration in the haemolymph of starved locusts compared with fed ones. The TRPs released from midgut endocrine cells could also have a paracrine function, acting directly on the midgut. LomTK-I–IV have been shown to stimulate contractions of the circular muscles of the locust midgut (Pabla and Lange, 1999). These findings indicate a possible role in food transport and digestion. It has been suggested that the endocrine cells of the ampullae of the Malpighian tubules of the locust might be involved in the local control of diuresis (Montuenga et al., 1989). Montuenga et al. (Montuenga et al., 1996) suggested that diuretic hormone and tachykinins released from the ampullar cells could increase the spontaneous muscle contractions of the Malpighian tubules and, thereby, increase the haemolymph flow around the gut and the Malpighian tubules. An action of LomTKs on writhing movements of Malpighian tubules was demonstrated later (Coast, 1998). Thus, the release of TRPs from the endocrine cells of the ampullae might, at least indirectly, be involved in diuresis.

It is tempting to suggest a broader functional role for the TRPs released into the circulation. They may act more generally to increase the flow of haemolymph in the body cavity by activating various visceral muscles. Different TRPs have been shown *in vitro* to induce contractions in the muscle of the oviduct, hindgut, midgut, foregut, heart and Malpighian tubules (Schoofs et al., 1990a; Schoofs et al., 1990b; Winther et al., 1998; Coast, 1998; Nässel et al., 1998a; Kwok et al., 1999; Pabla and Lange, 1999; Sliwowska et al., 2000). It has been suggested that the oviduct muscle and the abdominal aorta (heart), together with the Malpighian tubules and possibly the intestine, act in concert to increase the circulation of haemolymph, thereby enhancing the transport of neuropeptides and other hormones in the open circulation system (Coast, 1998; Kwok et al., 1999, Sliwowska et al., 2000). Furthermore, increased haemolymph flow would also increase the accessibility of the nutrients and oxygen needed in the tissues to support a higher metabolic rate.

It is clear that the midgut endocrine cells of both *Locusta migratoria* and *Leucophaea maderae* contain several isoforms of TRPs, at least four in the locust and at least three in the cockroach (this investigation; Pabla and Lange, 1999; Winther et al., 1999). Two more LomTK isoforms may be present in the locust midgut, as seen in liquid chromatography experiments (this investigation; Pabla and Lange, 1999) and as indicated in a study on another locust, *Schistocerca gregaria* (Veelaert et al., 1999). Thus, TRP release from the midgut probably involves several peptide isoforms. We have been able to identify circulating LomTK-I–III and possibly LomTK-V in locust haemolymph. We were unable to detect LomTK-IV in the haemolymph, but this could be because LomTK-IV is poorly recognized in the ELISA. The amounts of circulating LomTK-I–III are approximately the same, but we found higher levels of the presumed LomTK-V in the haemolymph. This could indicate that LomTK-V is released in larger amounts or that it is less susceptible to enzymatic degradation. Similar release of multiple peptide isoforms is likely to occur from ‘traditional’ neurosecretory release sites such as the corpora cardiaca and abdominal perisympathetic organs (see Winther et al., 1996; Predel et al., 1999; Predel et al., 2000). The functional relevance of the release of cocktails of peptide isoforms is not clear. In fact, it may be that most of the isoforms in such a cocktail are functionally redundant, as has been

shown at some target muscles for FMRFamides and myomodulins (Brezina et al., 1995; Hewes et al., 1998). It is known that the different isoforms of LomTKs and LemTRPs have very similar bioactivities on the muscles tested (Winther et al., 1998; Kwok et al., 1999; Pabla and Lange, 1999), suggesting that for TRPs as myostimulators all isoforms but one are physiologically redundant.

It is not obvious what physiological roles the hormonally released TRPs have in insects, except that they seem to act as myostimulators and that they may have some role in feeding behaviour. With the recent identification of a *Drosophila melanogaster* gene encoding a precursor with multiple TRPs expressed in the nervous system and in endocrine cells in the midgut (Siviter et al., 2000) and with two cloned *Drosophila melanogaster* receptors of TRPs available (Li et al., 1991; Monnier et al., 1992), it will be possible to investigate the physiological role of the TRP signalling system in insects using molecular techniques.

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