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QUANTITATIVE DETERMINATION OF MYOTROPIC NEUROPEPTIDE IN THE NERVOUS SYSTEM OF THE COCKROACH *LEUCOPHAEA MADERAE*: DISTRIBUTION AND RELEASE OF LEUCOKININS

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Summary

Being interested in the distribution and function of insect myotropic peptides, we developed a sensitive radioimmunoassay (RIA) using an antiserum directed against the cockroach neuropeptide leucokinin I. The levels of leucokinin immunoreactivity were measured in extracts of different portions of the nervous system, in the retrocerebral complex and in the hemolymph of the cockroach *Leucophaea maderae*. The brain contains about 1.9pmol of immunoreactive material while the ganglia of the ventral nerve cord each contain less than 10% of this amount. Large amounts of leucokinin immunoreactivity (6.6pmol per tissue) were found in the corpora cardiaca–corpora allata (CC–CA) complex and in the hemolymph the titer was in the nanomolar range. When levels of leucokinins were compared in male and female cockroaches, no differences could be detected in any of the sample tissues. A calcium-dependent release of leucokinin-immunoreactive material could be induced from isolated CC–CA complexes by high-potassium depolarization. The amount of released immunoreactivity, as measured in the bathing saline by RIA, was about 2% of the extractable leucokinin immunoreactivity in the CC–CA complex. In *Leucophaea maderae*, the leucokinins apparently act both as neuroactive substances in the central nervous system and as neurohormones released into the circulation.

Introduction

Insect neuropeptides often exist in multiple forms within a given species, for example the FMRFamide-related peptides in *Drosophila melanogaster* (Nambu *et al.* 1988; Schneider and Taghert, 1988), and these different forms are likely to have diverse functions as neurotransmitters, neuromodulators and neurohormones (see Walker, 1992). We are interested in the function of another diverse group of insect neuropeptides, the leucokinins.

The leucokinins I–VIII are myotropic neuropeptides isolated from head extracts of the cockroach *Leucophaea maderae* (Holman *et al.* 1986*a*,*b*, 1987*a*,*b*, 1990). Leucokinin I

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has the amino acid sequence Asp-Pro-Ala-Phe-Asn-Ser-Trp-Gly-NH_2 and the other leucokinins all share the C-terminal pentapeptide -Phe-X-Ser-Trp-Gly-NH_2, where X denotes His, Asn, Ser or Tyr. Peptides of the leucokinin family have also been isolated from brain and corpora cardiaca of a cricket (Holman *et al.* 1990) and a locust species (Schoofs *et al.* 1992) using the same cockroach hindgut contraction assay to monitor fractions during purification (Holman *et al.* 1990, 1991). In addition to their myotropic action, some peptides of this family have also been shown to have a diuretic effect on the excretory Malpighian tubules of crickets and mosquitos (Hayes *et al.* 1989; Coast *et al.* 1990).

An antiserum raised against leucokinin I conjugated to bovine serum albumin showed that leucokinin immunoreactivity is distributed in neurons and neurosecretory cells throughout the cockroach nervous system (Nässel and Lundquist, 1991; Nässel *et al.* 1992). The antiserum we used most extensively (code 9027) appears to recognize the preserved C terminus of the leucokinins and thus seems to label not only leucokinin I, but also other members of this peptide family (Nässel *et al.* 1992). Consequently, we have employed this leucokinin antiserum to detect leucokinin-like peptides in neurons of several other insect species including dipteran flies, crickets, locusts and moths (Nässel and Lundquist, 1991; Cantera and Nässel, 1992; Cantera *et al.* 1992; Nässel, 1993).

The widespread distribution of leucokinin immunoreactivity in the cockroach nervous system suggests multiple functions for the leucokinin peptides, both in the central nervous system and at innervated peripheral targets such as the heart and spiracles. It has also been shown that the neurohemal areas of the corpora cardiaca and the abdominal transverse nerves contain leucokinin-immunoreactive varicose fibers (Nässel *et al.* 1992), suggesting a homonal release of peptide (Raabe, 1989). As proposed earlier (Cook *et al.* 1990), the leucokinin action on targets such as Malpighian tubules and visceral muscles may be hormonal, since these tissues are not directly innervated by leucokinin-immunoreactive fibers (Nässel *et al*. 1992). It would thus be of great interest to demonstrate release of leucokinins from neurohemal structures as a link in establishing their hormonal role(s).

A radioimmunoassay (RIA) was developed to study the turnover and release of leucokinins in the cockroach *Leucophaea maderae*. With this RIA we determined the amounts of leucokinins in different dissected portions of the central nervous system (CNS) and corpora cardiaca–corpora allata complexes. Calcium-dependent release of leucokinin-immunoreactive material was evoked from isolated corpora cardiaca–corpora allata complexes by high-potassium depolarization *in vitro* and quantified by RIA of the bathing saline.

Materials and methods

Animals

Adult male and female cockroaches of the species *Leucophaea maderae* Fabricius were used in all experiments. The insects were kept at room temperature, 22˚C (16h:8 h L:D), and fed dry dog food and water *ad libitum*. All experimental animals were killed in the morning after 2h of light.

Tissue extraction and preparation

Extraction of whole heads was, with some modifications, carried out according to the method described by Brodin and co-workers (1983, 1986). Cockroaches were frozen at −80°C, their heads were cut off and batches of 20 heads (approximately 800mg) were ground into powder in liquid nitrogen using a ceramic mortar. The frozen powder was transferred to borosilicate glass tubes, suspended in 8ml of boiling distilled water, and the homogenate was kept boiling for 20min. The suspension was agitated every 3min using a vortex mixer and a steel rod in the glass tube. After boiling, the homogenate was cooled on ice for 30min, centrifuged (2900 ***g*** at 4°C for 15min) and the supernatant removed and saved on ice. The pellet was resuspended in 8ml acetic acid (1.0mol l^{-1}), boiled for 20min, cooled on ice, stirred, centrifuged and the supernatant withdrawn, as above. The two supernatants were pooled and further clarified by centrifugation (12000 ***g*** at 40°C for 15min) and the resulting supernatant was lyophilized. For further purification, the lyophilized extract of 20-head-equivalents was dissolved in 5ml of 0.1% trifluoroacetic acid (TFA; Sigma, St Louis, MO) and applied to an activated and equilibrated Sep-Pak C18 cartridge column (Waters/Millipore). Following a 20ml rinse with 0.1% TFA, the material was eluted from the Sep-Pak column with 4ml 70% acetonitrile (CH_3CN; Merck) containing 0.1% TFA. The eluted extract was divided into 1ml samples, containing 5 head-equivalents each, and lyophilized.

For extraction of dissected tissue, the following protocol was used. Cockroaches (5 in each batch) were cooled on ice and their heads were cut off. The brain, corpora cardiaca–corpora allata (CC–CA) complex and suboesophageal ganglion (SOG) were quickly dissected from each head in cold phosphate buffer (0.1mol l^{-1}, pH7.4) and frozen on dry ice. For dissection of the body ganglia, the thorax and abdomen were pinned down to a Sylgard (Dow Corning)-covered Petri dish. The cockroaches were opened dorsally and the ganglionic chain was taken out with the three thoracic ganglia (T1–T3) separated, the five unfused abdominal ganglia (A2–A6) connected, and the terminal abdominal ganglion (TG) separated. All tissues were stored in polypropylene tubes on dry ice. The dissected tissues were extracted in 2ml of boiling water and acetic acid (1.0mol l^{-1}) using the method described above. The RIA determinations of leucokinin immunoreactivity were made separately for tissue from males and females. Since no significant differences in amounts were detected, the values from the two sexes were pooled.

For comparison, extraction with methanol:acetic acid:water (90:9:1) was also carried out on dissected tissues, according to the method described originally for the isolation of the leucokinins by Holman and coworkers (Holman *et al.* 1986*a*,*b*, 1987*a*,*b*).

A total of 300 μl of hemolymph was collected from six cockroaches using a microcapillary tube; this was added to 400 μl of 0.9% NaCl containing 0.5% BSA, and lyophilized. Two such samples were collected for independent assays.

Release experiments

The composition of the experimental salines is given in Table 1. These salines are slightly modified from the ones used by Carroll *et al.* (1986).

Table 1. *Composition of experimental salines used in release experiments*

	[Salt] (mmol l^{-1})		
Experimental saline*	NaCl	KCl	$CaCl_2$
Preincubation buffer I	140	–	5
Preincubation buffer II	54	93	–
Incubation buffer	47	93	5
Rinse buffer	147	–	–

*All salines contained 5mmol l^{-1} Hepes, 7mmol l^{-1} KOH, 1mmol l^{-1} $MgCl_2$, 5mmol l^{-1} trehalose, 5 mmol l^{-1} glucose, 10mmol l^{-1} sucrose, and 0.5% BSA, pH6.7.
Preincubation buffer I contained 7mmol l^{-1} K^+, 5mmol l^{-1} Ca^{2+}.
Preincubation buffer II contained 100mmol l^{-1} K^+, no Ca^{2+}.
Incubation buffer contained 100mmol l^{-1} K^+, 5mmol l^{-1} Ca^{2+}.

Four separate release experiments were performed, and for each experiment isolated CC–CA complexes from eight animals were used. The CC–CA complexes were rapidly dissected in cold calcium-free saline (rinse buffer), washed three times in rinse buffer and then transferred to the wells of a tissue culture plate and preincubated for 1h at 20°C in 100 μl of either low-potassium, calcium-containing saline (preincubation buffer I; four complexes per experiment) or high-potassium, calcium-free saline (preincubation buffer II; four complexes per experiment). After this preincubation, the tissues were transferred to two new culture wells and incubated in high-potassium, calcium-containing saline (incubation buffer) for 1h at 20°C. Following incubation, the CC–CA complexes were discarded and the different incubation buffers were transferred to microcentrifuge tubes and lyophilized for RIA analysis.

Radioimmunoassay

To determine the amount of leucokinin immunoreactivity in extracts and in salines from release experiments, an RIA was developed that could measure concentrations in the picomolar (fmol ml^{-1}) range. The RIA was, with some modifications, performed according to the protocol described by Brodin *et al.* (1983).

The leucokinin antiserum was raised in a rabbit immunized with synthetic leucokinin I (Peninsula Laboratories, Belmont, CA) conjugated to bovine serum albumin (BSA) using 1,5-difluoro-2,4-dinitrobenzene (Nässel and Lundquist, 1991). The antiserum used in all experiments was from blood taken 8 weeks after a booster injection (code 9027/8). Further details on the antiserum production and characterization are given by Nässel and Lundquist (1991) and Nässel *et al.* (1992). As a radioligand we used synthetic leucokinin I labeled with ^{125}I-Bolton–Hunter reagent (Amersham, UK) (Bolton and Hunter, 1973).

The total incubation volume in each assay tube was 1100 μl, containing 100 μl of either sample (i.e. extracts and incubation buffers) or standard (synthetic peptide) dissolved in 0.9% NaCl containing 0.5% BSA (sample buffer) and 500 μl each of antiserum and radioligand (adjusted to 1000ctsmin $^{-1}$ tube $^{-1}$). Both antiserum and radioligand were dissolved in barbital buffer (0.02mol l^{-1}, pH8.6) containing 0.8% BSA. The antiserum was diluted 1:10^6, giving an initial specific binding of approximately 40% of the total radioactivity. Non-specific binding in the absence of antiserum was calculated for each

measurement. Standard measurements of synthetic leucokinin I were made at ten different concentrations, ranging between 0.3 and 300fmol ml^{-1} (ED_{80}=10fmol ml^{-1}, ED_{20}=210fmol ml^{-1}). Samples were incubated for 72h at 4°C and the separation of free radioligand from antibody-bound radioligand was made by the addition of 500 μl of Sepharose-coupled, sheep anti-rabbit antibodies (Pharmacia decanting suspension 3; Kabi/Pharmacia) to each assay tube followed by a 30min incubation at 4°C. The assay tubes were centrifuged (1000 ***g***, 4°C), the supernatants discarded and the radioactivity in the bound fraction determined in a LKB Wallac 1272 Clinigamma counter (Kabi/Pharmacia).

Tissues from four to six animals were pooled in each assay experiment. All measurements were in triplicate and the experiments for quantification of the different tissues were run at least four different times (*N*=4).

The cross-reactivity of the leucokinin I antiserum was determined with the following peptides in concentrations ranging between 10^{-5} and 10^{-15} mol l^{-1}: substance P (Sigma), leucokinins I, II and VI, locustatachykinin I, kassinin and FMRFamide (all Peninsula). The peptides were all initially dissolved in 0.01mol l^{-1} acetic acid and then further diluted in sample buffer.

Results

Cross reactivity of the leucokinin antiserum in radioimmunoassay

Using the RIA, we first tested the cross reactivity of the leucokinin antiserum with different concentrations of leucokinins I, II and VI and locustatachykinin I (Fig. 1). We found that the antiserum binds equally well to the three different leucokinins, but no binding to locustatachykinin I was seen. In another RIA experiment, we showed that the antiserum does not cross-react with synthetic substance P, kassinin or FMRFamide (Fig. 2).

Radioimmunoassay of tissue extracts

The binding of radioligand to the leucokinin antiserum was assayed in competition with serially diluted synthetic leucokinin I, as compared to competition with serially diluted *Leucophaea maderae* brain and head extracts. As seen in Fig. 3, the resulting binding curves were found to be parallel, indicating that the RIA measures leucokinins in the tissue extracts.

The amount of leucokinin immunoreactivity in different dissected parts of the nervous system was calculated from RIA measurements of extracts. The tissue extracts were serially diluted (three dilutions, each in triplicate) and each ganglion or tissue part was assayed at least four times independently.

The amounts of leucokinin immunoreactivity extracted from the whole heads, the different ganglia and the corpora cardiaca–corpora allata (CC–CA) complexes are given in Table 2. In all experiments we assayed tissue from male and female cockroaches separately, but found no significant difference in leucokinin immunoreactivity between sexes. Hence, we pooled the values. It is clear that the brain contains about 10 times more immunoreactivity than any of the ventral cord ganglia. The CC–CA complexes were

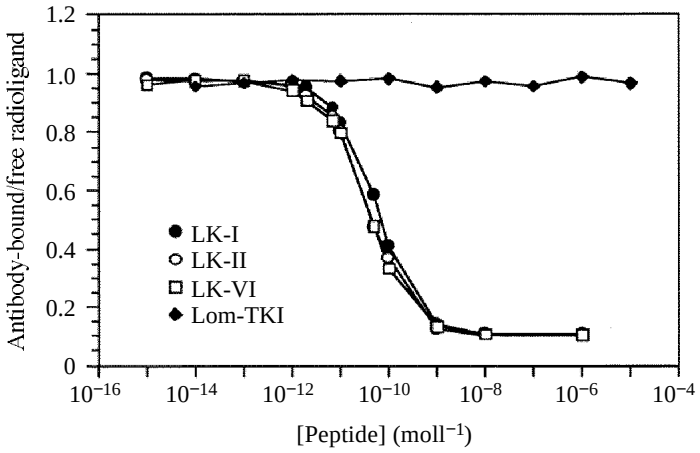

Fig. 1. Cross-reactivity of leucokinin I antiserum and the synthetic peptides leucokinins I, II and VI (LK-I, II and VI) and locustatachykinin I (Lom-TKI) added in concentrations ranging between 10^{-5} and 10^{-15} mol l^{-1}. The binding curves show that the three different leucokinins bind equally well to the antiserum whereas locustatachykinin I does not bind at all.

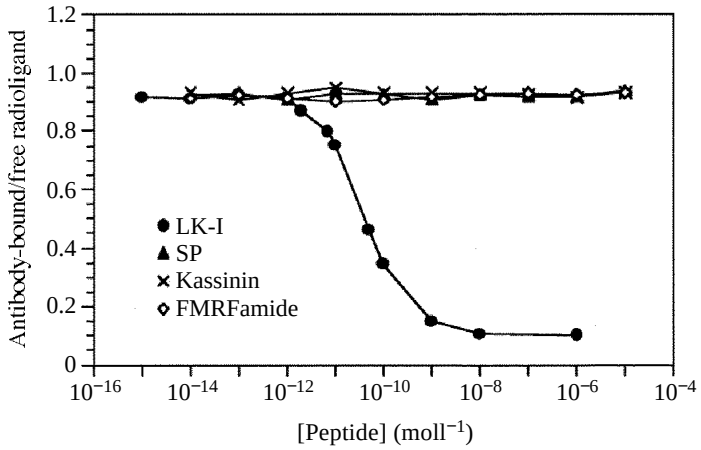

Fig. 2. Cross-reactivity of leucokinin I antiserum and the synthetic peptides leucokinin I (LK-I), substance P (SP), kassinin and FMRFamide added in concentrations ranging between 10^{-5} and 10^{-15} mol l^{-1}. Except for leucokinin I, none of these peptides showed any cross-reactivity with the antiserum.

extracted and measured as a unit and we have no values for the individual components. Each complex contains very large amounts of leucokinin immunoreactivity. This amount corresponds to 76% of the total amount of immunoreactivity extracted from whole *Leucophaea maderae* heads. The brain and suboesophageal ganglion would thus contribute only 24% of the total amount in the head.

The amount of leucokinin immunoreactivity in hemolymph samples of *Leucophaea maderae* was also measured by RIA (only two assays). These measurements gave values of 0.5 and 1.5fmol μl^{-1} of hemolymph (i.e. in the nanomolar range).

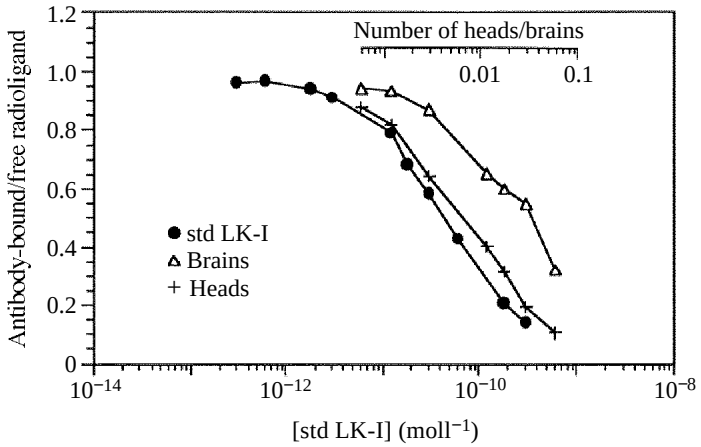

Fig. 3. Radioimmunoassay of a dilution series of *Leucophaea maderae* head/brain extracts as compared to synthetic leucokinin I (std LK-I). Inserted abscissa represents the number of head/brain equivalents.

Table 2. *Leucokinin immunoreactivity in* Leucophaea maderae *CNS and CC–CA complex*

Tissue (*N*)	Leucokinin immunoreactivity* (fmol per tissue)
Heads (4)	8650±460
CC–CA (6)	6600±360
Brain (7)	1940±160
SOG (6)	115±10
T1 (6)	153±13
T2 (6)	180±18
T3 (6)	220±19
A2–A6 (5)†	361±41
TG (5)	148±21

*Values are presented as mean ± S.E.M. of 4–7 separate experiments (*N*=4–7), each performed on tissues from 5 animals. Assays were always run on male and female tissues separately; but as no significant difference between them was detected, values were pooled.

SOG, suboesophageal ganglion; T1–T3, thoracic ganglia 1–3 (T3 contains one fused abdominal ganglion); A2–A6, unfused abdominal ganglia 2–6; TG, terminal abdominal ganglion.

†The transverse nerves and perisympathetic organs were removed before the assays. The amount per unfused ganglion can be calculated as approx. 72fmol.

We compared our extraction technique with that used originally by Holman *et al.* (1986*a*) in their isolation of leucokinins. There are no differences in the amounts of immunoreactivity extractable by the two methods (data not shown).

Release of leucokinin reactivity

Since the corpora cardiaca contains large amounts of leucokinin-immunoreactive

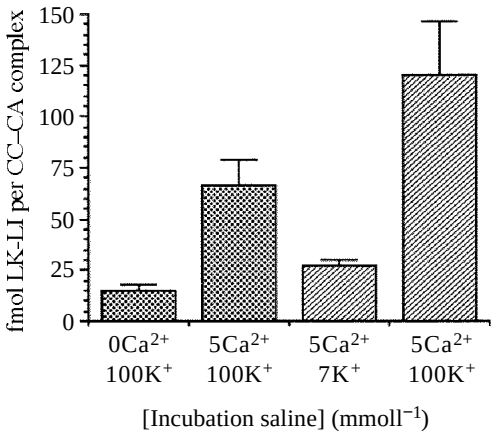

Fig. 4. Spontaneous and potassium-induced release of leucokinin-like immunoreactivity (LK-LI) from corpora cardiaca–corpora allata (CC–CA) complexes *in vitro* as determined by radioimmunoassay. Each bar represents the mean of four different experiments (N=4) ± S.E.M. Stippled bars: left bar shows the amount of LK-LI released during 1h in 100 μl of preincubation buffer II (100mmol l^{-1} K^+, no Ca^{2+}); right bar shows the amount of LK-LI released from the same tissues after a subsequent incubation for 1h in 100 μl of the incubation buffer (100mmol l^{-1} K^+, 5mmol $l^{-1}$$Ca^{2+}$). Hatched bars: left bar shows the amounts of LK-LI released during 1h in 100 μl of preincubation buffer I (7mmol l^{-1} K^+, 5mmol l^{-1} Ca^{2+}); right bar shows the amount released during 1h from the same organs transferred to 100 μ1 of the incubation buffer (l00mmol l^{-1} K^+, 5 mmol l^{-1} Ca^{2+}). There is a significant difference (P<0.05) in the amount of immunoreactivity released in the two different preincubation buffers. There is, however, no significant difference (P>0.1) in amounts of immunoreactivity in the stippled and hatched bars representing incubation in high-potassium, calcium-containing saline.

terminals (Nässel *et al.* 1992), we selected this tissue for analysis of release *in vitro.* By depolarizing with high potassium, we could induce release of leucokinin-immunoreactive material into the bathing saline as measured by RIA. As seen in Fig. 4, there was little release from CC–CA complexes after 1h in preincubation buffer II (high-potassium, calcium-free). When these tissues were transferred to the incubation buffer (high-potassium, calcium-containing), the amount of reactivity released in 1h was more than four times higher (P<0.05; paired t-test).

The spontaneous basal release from CC–CA complexes preincubated in preincubation buffer I (low-potassium, with calcium) was significantly higher (P<0.05) than the release seen in preincubation buffer II (high-potassium, calcium-free). When these complexes were transferred from preincubation buffer I to the incubation buffer, there was an increased release (by more than 4 times) of immunoreactive material (P<0.05; paired t-test). Regardless of the preincubation treatment, the complexes released similar amounts of immunoreactivity (no significant difference; P>0.1) when transferred to incubation buffer (high-potassium, with calcium). The amount of leucokinin immunoreactivity released in a calcium-dependent manner during a 1h incubation in high potassium was about 2% of the total extractable amount present in the CC–CA complex.

Discussion

We have shown here that the leucokinin antiserum cross-reacts in RIA with leucokinins I, II and VI, but not with the locust myotropic peptide locustatachykinin I (Schoofs *et al.* 1990) or with the vertebrate tachykinins tested. Similar results have been obtained in immuno-dot blots and preabsorption experiments for immunohistochemistry (Nässel and Lundquist, 1991; Nässel *et al.* 1992). It is, therefore, likely that the antiserum recognizes the preserved C terminus of the leucokinins, which is unique to this family of peptides (Holman *et al.* 1990, 1991; Schoofs *et al.* 1992). From this it follows that our leucokinin RIA probably measures the amount of several, if not all, of the leucokinins I–VIII in *Leucophaea maderae*. The amount of leucokinin-like material found in the RIA is about 8.6pmol per head, as compared with 1.76pmol per head calculated from values given for leucokinins I–VIII by Holman *et al.* (1986*a*,*b*, 1987*a*,*b*). Possibly this difference can be explained by loss of part of the peptide content during the purification steps in the leucokinin isolation.

The distribution of leucokinin immunoreactivity in the central nervous system of *Leucophaea maderae* as measured by RIA corresponds well with the immunohistochemical localization of immunoreactive material described by Nässel *et al.* (1992). Hence, the largest amount of immunoreactivity was seen in the brain, including the optic lobes (about 1900fmol per brain). Each of the thoracic ganglia contains about 10 times less immunoreactivity than the brain, while the suboesophageal and abdominal ganglia contain somewhat smaller amounts still. The terminal abdominal ganglion, which is a fusion of at least three ganglia, contained twice as much as an unfused abdominal ganglion. The value for the individual unfused ganglia (about 72fmol per ganglion) was obtained by dividing the value of the measured abdominal ganglion chains by 5. This calculation seems justified since immunocytochemistry has indicated that each of the unfused ganglia contain identical sets of leucokinin-immunoreactive neurons and processes (Nässel *et al.* 1992). We detected no difference in leucokinin levels between male and female cockroaches, in contrast to findings for Met-enkephalin-immunoreactive material in the locust nervous system (Davenport and Evans, 1986).

The brain contains a large number of leucokinin-immunoreactive neurons, many of which are neurosecretory cells with axons to the corpora cardiaca (Nässel *et al.* 1992). The terminals of these neurosecretory cells within the storage lobes of the corpora cardiaca appear to contain very large amounts of leucokinin immunoreactivity (more than 3 times the amount in the brain). Release of leucokinin-immunoreactive material from the CC–CA complex was induced with depolarization by high potassium in a calcium-dependent fashion, similar to the release of FMRFamide from the corpora cardiaca of *Manduca sexta* demonstrated by Carroll *et al.* (1986). During a 1h incubation in a high-potassium saline, about 2% of the total extractable content of immunoreactive material was released from the *Leucophaea maderae* CC–CA complex. The absolute amount of released peptide is still large (120fmol per complex) since the total amount of leucokinin immunoreactivity in the CC–CA complex is very high. In the moth *Manduca sexta*, about 16% of the FMRFamide immunoreactivity was released during a 2h incubation, but the amount of extractable immunoreactivity in the corpora cardiaca is much lower in this case

(Carroll *et al.* 1986). Another example is calcium-dependent release of prothoracicotropic hormone from potassium-depolarized *Manduca sexta* corpora cardiaca, which constituted 35% of the extractable bioactivity (Carrow *et al.* 1981).

The released leucokinins probably act as neurohormones in *Leucophaea maderae*. We could measure leucokinin immunoreactivity in the hemolymph of the cockroach in the nanomolar range, indicating release *in vivo* and suggesting a hormonal role. We need to study this *in vivo* release in more detail under different physiological conditions, for example during different states of hydration (Maddrell and Gee, 1974; Proux and Rougun-Rapuzzi, 1980; Piquot and Proux, 1987). There are no published reports on the *in vivo* hormonal action(s) of leucokinins in *Leucophaea maderae*. Some indications of a hormonal role are derived from studies of cricket and mosquito Malpighian tubules where a diuretic effect of leucokinins/achetakinins has been shown (Hayes *et al.* 1989; Coast *et al.* 1990). In addition, the myotropic action of leucokinins on muscle of hindgut, foregut and oviduct (Cook *et al.* 1989, 1990) may be hormonally mediated, since no direct innervation by leucokinin-immunoreactive fibers has been demonstrated (Nässel *et al.* 1992).

Immunocytochemistry indicated that there may be additional sites of leucokinin release (Nässel *et al.* 1992): varicose leucokinin-immunoreactive terminals were seen in the abdominal transverse nerves, in the lateral cardiac nerves and on spiracle muscles, and superficially along the posterior abdominal nerves supplying these structures. We have not yet tested whether immunoreactive material is released from these structures.

In conclusion, our results indicate that leucokinins are abundant both in neuronal systems and in sets of neurosecretory cells. Release of leucokinins from the corpora cardiaca was demonstrated. The neurohemal region of the corpora cardiaca has been previously shown to contain large amounts of leucokinin immunoreactivity, which is stored in terminals of neurosecretory cells that have their perikarya in the median and lateral neurosecretory cell groups of the brain (Nässel *et al.* 1992).

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