J. exp. Biol. **193**,307–319 (1994)
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HAEMOLYMPH AND TISSUE TITRES OF ACHETAKININS IN THE HOUSE CRICKET *ACHETA DOMESTICUS*: EFFECT OF STARVATION AND DEHYDRATION

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Accepted 21 April 1994

Summary

Achetakinin-like immunoreactive material in tissues and haemolymph of adult male crickets was quantified by radioimmunoassay. Achetakinin-like material was found in the brain, suboesophageal ganglia and the thoracic and abdominal ganglia, but the largest amount was within the retrocerebral complex. A Ca^{2+}-dependent release of achetakinin-like immunoreactive material was demonstrated from retrocerebral complexes incubated *in vitro* in saline containing a high concentration of K^{+}. The concentration of achetakinin-like material in haemolymph from fed crickets was estimated to be 2.8 $nmol\,l^{-1}$ and increased more than 10-fold in insects starved for 48 h without access to water. The presence of achetakinin-like material in haemolymph suggests that these peptides are released *in vivo* and function as circulating neurohormones.

Introduction

Recent advances in techniques for the purification and characterization of peptides have greatly facilitated the identification of insect neuropeptides, which are frequently present in small amounts. A major restriction on the numbers of identified sequences has been the lack of suitable robust bioassays for screening fractions for biological activity. The hindgut of the cockroach *Leucophaea maderae* has proved a remarkably productive bioassay, in terms of both the absolute number of peptides isolated and the different classes of peptide that influence spontaneous contractile activity (Holman *et al.* 1990). Using this bioassay, more than 40 sequences, representing four distinct families of peptides, have been identified from insects as diverse as cockroaches and mosquitoes (Holman *et al.* 1990). There is no obvious reason why *Leucophaea* hindgut should possess such a diversity of receptor types, if indeed these peptides act at different receptors. Furthermore, extracts of whole bodies or heads have frequently been used as starting material for extraction of active materials, raising questions as to the cellular origin of identified peptides. With the availability of synthetic peptides, it has been possible to test for biological activity in other assays and to raise antibodies both for immunocytochemical studies and for the development of specific immunoassays with which to measure haemolymph and tissue titres.

Key words: achetakinins, radioimmunoassay, diuretic peptides, neurohormones, *Acheta domesticus*, house cricket.

In at least two instances, peptides identified using the cockroach hindgut assay have been shown to have other sites of action. The C-terminal pentapeptide sequence of leucopyrokinin is identical to that of the pheromone biosynthesis activating neuropeptide (PBAN) from the silkworm *Bombyx mori* and stimulates pheromone production (Kuniyoshi *et al.* 1992). Additionally, myokinins from *Leucophaea* (leucokinins), the house cricket *Acheta domesticus* (achetakinins), the locust *Locusta migratoria* (locustakinin) and the mosquito *Culex salinarius* (culekinins) have been shown to stimulate ion and fluid transport by isolated Malpighian tubules and are therefore potential regulators of diuresis (Hayes *et al.* 1989, 1994; Coast *et al.* 1990; G. M. Coast, unpublished observation). These actions can be relatively specific; for example, leucopyrokinin has no effect on fluid secretion by cricket Malpighian tubules but, unlike achetakinins, stimulates contractions of cricket oviduct (Nachman *et al.* 1992).

A feature of these myoactive peptides is that the minimal sequence required for biological activity is that of the five residues at the C terminus. In the myokinins, this ‘active core’ region is highly conserved and has the generalised sequence Phe-X^1-X^2-Trp-Gly-NH_2, where X^1 is Asn, His, Phe, Ser or Tyr, and X^2 is Pro or Ser. For the present study, antibodies were raised to the C-terminal core of achetakinin-I (AK-I), Phe-Tyr-Pro-Trp-Gly-NH_2, and used to develop a sensitive radioimmunoassay (RIA) with which to measure haemolymph and tissue titres. Achetakinin-like immunoreactivity was found to be widespread in the central nervous system, with large amounts in the retrocerebral complex, from where material could be released by a Ca^{2+}-dependent mechanism following depolarization in high concentrations of K^+. Achetakinin-like immunoreactive material was also detected in samples of haemolymph, and the titre changed in response to the feeding status of the insect, indicating that these peptides could function as circulating neurohormones.

Materials and methods

Insects

Acheta domesticus were reared as described by Clifford *et al.* (1977). They were fed a diet of turkey starter crumbs with water provided *ad libitum*. Newly emerged adults were isolated daily and kept in cohorts of identical age and sex. Unless otherwise stated, adult male crickets of 6–12 days old were used in these studies. Haemolymph volume was estimated from the difference between the body mass of the intact insect and that after exsanguination by cutting open the abdomen and drying the internal tissues gently with a soft tissue. The specific gravity of cricket haemolymph is 1.037±0.0047 (mean ± S.E.M., N=8). This method was preferred to the use of [^{3}H]inulin, because preliminary studies in dehydrated animals showed that the volume marker did not distribute evenly throughout the haemolymph compartment.

Peptides

Achetakinins I–V and leucopyrokinin were generous gifts from Dr Ronald J. Nachman and Dr G. Mark Holman (USDA, College Station, Texas), and *Locusta* tachykinin-IV was obtained from Dr Lillian Schoofs (Leuven, Belgium). Peptide stock solutions in

methanol were routinely quantified by measuring tryptophan fluorescence (excitation wavelength 280 nm; emission wavelength 348 nm; Luminescens Spectrometer, Perkin Elmer LS-5).

Antibodies

Antibodies were raised commercially by Cambridge Research Biochemicals (Cambridge, UK) using New Zealand white rabbits. An analogue (Cys-Lys-Ala-Phe-Tyr-Pro-Trp-Gly-NH_2) was prepared which incorporated the C-terminal pentapeptide core of AK-I (Phe-Tyr-Pro-Trp-Gly-NH_2). This was conjugated *via* the free –SH group of cysteine to keyhole limpet haemocyanin activated with *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester. Antisera were divided into aliquots of 60 μl and stored at -70 °C. Dilutions of antisera in phosphate-buffered saline [50 mmol l^{-1} phosphate buffer, pH 7.4, 1 % bovine serum albumin (BSA), 0.1 % Tween 20, 1 mmol l^{-1} EDTA and 0.9 % NaCl; PBS] were prepared just before use.

Radioimmunoassay

Radioiodinated AK-I core was used as a radioligand probe in the RIA: $Na^{125}I$ (74 TBq $mmol^{-1}$) was purchased from Amersham International and ^{125}I was incorporated into the tyrosyl residue of the core peptide using Iodogen reagent (Sigma, Dorset, UK). The mono-iodinated peptide was isolated by reverse-phase HPLC using an ABI C_{18} column (4.6 mm×250 mm) (Applied Biosystems Inc., Warrington, UK).

For the RIA, samples of 40 μl (standards, tissue extracts or haemolymph) were incubated at 4 °C overnight with 50 μl of diluted antisera and 10 μl of the radioligand (approximately 15 000 cts min^{-1} $tube^{-1}$) in 1.6 ml polypropylene microcentrifuge tubes. The antibody was used at a dilution of 1:60, which bound approximately 50 % of the total radioactivity. Free and bound peptide were separated by adding 60 μl of activated charcoal (2 % charcoal, 1 % BSA, 0.5 % NaN_3, 1 % dextran in 50 mmol l^{-1} phosphate buffer, pH 7.4) followed by centrifugation at 4 °C for 10 min at 12 000 revs min^{-1}. Radioactivity in the supernatant was measured using an LKB 1275 MiniGamma counter. The RIA was standardised using AK-I core peptide in the range 0.5 fmol to 50 pmol, and the bound:free ratios were fitted to a smoothed spline using the program MultiCalc (Pharmacia).

Sample preparation

Tissue and haemolymph samples were collected into 200 μl of acidified methanol (methanol:water:acetic acid, 90:9:1), sonicated on ice for 30 s, and centrifuged for 10 min at 12 000 revs min^{-1} and 4 °C. Supernatants were taken to dryness in a vacuum centrifuge (Hetovac) and stored at -20 °C prior to the determination of peptide content in the RIA.

Reverse-phase high performance liquid chromatography

Fifty retrocerebral complexes (corpora cardiaca/corpora allata/hypocerebral ganglion) were extracted in acidified methanol and fractionated on a C_{18} column with a gradient of 0 % to 100 % solvent B over 60 min (solvent A: 0.1 % trifluoroacetic acid, 5 % acetonitrile, 95 % water; solvent B: 0.1 % trifluoroacetic acid, 60 % acetonitrile, 40 %

water). Absorbance was monitored at 215 nm. The flow rate was 1 ml min^{-1}, and fractions were collected automatically at 1 min intervals. For the RIA, samples (100 μl) of the fractions were taken to dryness in a vacuum centrifuge and dissolved in 40 μl of incubation buffer.

Statistics

Results are presented as the mean ±1 S.E.M. Statistical analyses (analysis of variance, ANOVA) were performed using StatView (BrainPower). Differences were considered significant for values of $P<0.05$.

Results

Assessment of antisera

Antisera recognising AK-I core peptide were obtained from four rabbits (R2–R5). The binding curves for all four antisera were similar in shape, and the dilution of antisera that bound 50 % of the ligand (15 000 cts min^{-1} tube^{-1}) was 30-fold for R2 and R3 and 60-fold for R4 and R5. Antiserum R5 was selected for the development of an RIA. Displacement of the radioligand by unlabelled core peptide (0.05 fmol 100 μl^{-1} to 250 pmol 100 μl^{-1}) is shown in Fig. 1. The linear part of the curve encompasses the range 0.5 fmol 100 μl^{-1} to 5 pmol 100 μl^{-1} (5 pmol l^{-1} to 50 nmol l^{-1}). The minimum detectable level of AK-I core peptide, defined by the lowest concentration for which the ratio bound:free cts min^{-1} differed by more than 2 standard deviations from the controls (no added peptide), was 5 fmol 100 μl^{-1}. Thus, although the antibodies in R5 antiserum

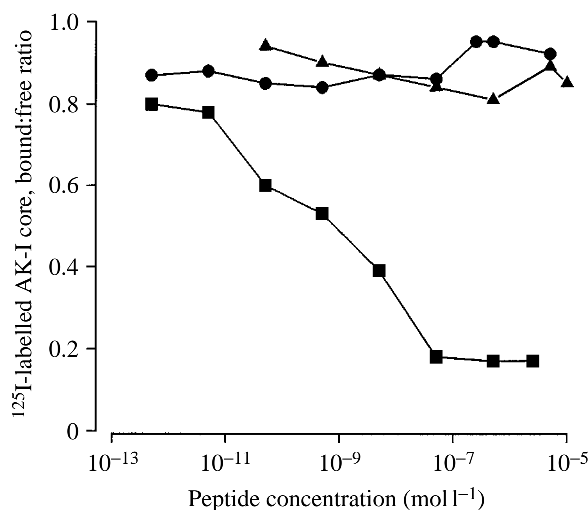

Fig. 1. Competitive inhibition of the binding of ^{125}I-labelled ligand to antibodies in antiserum R5 by unlabelled AK-I core peptide (squares) and by two unrelated myoactive peptides, leucopyrokinin (circles) and *Locusta* tachykinin-IV (triangles). Points are the mean of four determinations. Concentrations of AK-I core peptide greater than 0.5 pmol l^{-1} displace the radioligand bound by R5 antiserum, but leucopyrokinin and *Locusta* tachykinin-IV are without effect even at 10 μmol l^{-1}.

are of low titre (the working titre is a 60-fold dilution of the antiserum), they have a high affinity for the antigen.

Characterization of the antiserum

The R5 antiserum was tested in the RIA against all five naturally occurring achetakinins, together with the pentapeptide core of AK-I and two unrelated myoactive peptides, leucopyrokinin (LPK) and *Locusta* tachykinin-IV. The sequences of these peptides are given in Table 1. The antiserum was specific for peptides of the myokinin family and did not recognise leucopyrokinin or *Locusta* tachykinin-IV (Fig. 1). However, even amongst the naturally occurring achetakinins, the antiserum showed selectivity: it did not recognize AK-III, and only recognized AK-V at very high concentrations (Fig. 2). The highest affinities were for AK-I and its core peptide, which were recognized equally well. AK-II and AK-IV were also recognized, but the concentration of the peptide required for 50 % displacement of the probe was about an order of magnitude less than for AK-I or AK-I core peptide (Table 1).

Achetakinin content of tissue extracts

Tissues were removed from 8- to 10-day-old virgin male adult crickets. The RIA was used to measure achetakinin immunoreactivity in extracts of brain, suboesophageal ganglia, retrocerebral complex and the ganglia of the ventral nerve cord. In addition, achetakinins were measured in extracts of head capsules from which the brain, suboesophageal ganglia and retrocerebral complex had been removed, in flight muscles and in Malpighian tubules (tissue mass approximately equal to that of five brains). No immunoreactive material was detected in head capsules, flight muscle or Malpighian tubules. However, significant quantities were found in all extracts of nervous tissue, although there was relatively little achetakinin-like material in the abdominal nerve chain. Extracts of the retrocerebral complex contained quantities of immunoreactive material 50–100 times higher than in any other tissue (Table 2).

The retrocerebral complex of *Acheta* comprises the corpora cardiaca, corpora allata and hypocerebral ganglion. The corpora allata can be separated readily from the fused

Table 1. *Sequences of achetakinins I–V (AK I–V), leucopyrokinin (LPK) and* Locusta *tachykinin-IV (*Lom *TK-IV), together with the amount of peptide needed to displace 50 % of the counts bound by the antiserum (IC_{50})*

Peptide	Sequence	IC_{50} (mol l^{-1})
AK-I	SGADFYPWG-NH_2	2.5×10^{-13}
AK-I core	FYPWG-NH_2	3.9×10^{-13}
AK-II	AYFSPWG-NH_2	1.4×10^{-12}
AK-III	ALPFSSWG-NH_2	–
AK-IV	NFKFNPWG-NH_2	3.4×10^{-12}
AK-V	AFHSWG-NH_2	1.1×10^{-11}
LPK	*p*QTSFTPRL-NH_2	–
Lom TK-IV	APSLGFHGVR-NH_2	–

The antiserum did not recognize AK-III, LPK or *Lom* TK-IV.

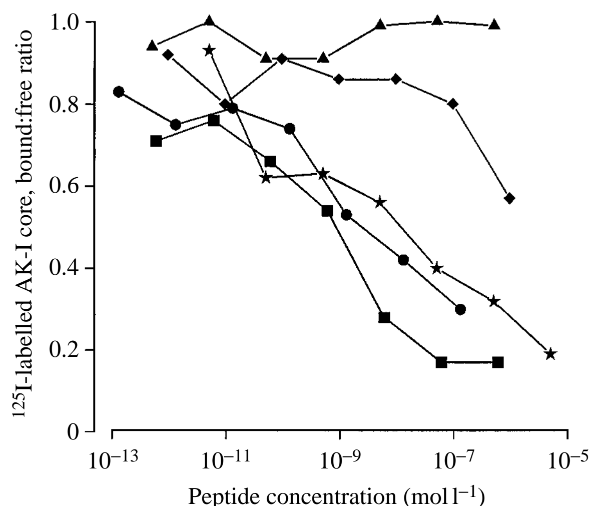

Fig. 2. Cross-reactivity of R5 antiserum with achetakinins I–V (AK-I, squares; AK-II, circles; AK-III, triangles; AK-IV, stars; AK-V, diamonds). Points are the means of four determinations. The binding curves show that the antiserum recognizes AK-I>AK-II=AK-IV ≫ AK-V, whereas AK-III is not recognized.

corpora cardiaca/hypocerebral ganglion, and the achetakinin contents of these two regions were 630±141 fmol (*N*=6) and 450±75 fmol (*N*=6), respectively.

Identification of immunoreactive material in HPLC fractions prepared from extracts of the retrocerebral complex

Not all the immunoreactive material identified in tissue extracts may be attributable to achetakinins. To investigate further the nature of the antigenic material, an extract of the retrocerebral complex, the richest source of achetakinin-like immunoreactivity, was fractionated by reverse-phase HPLC. Fig. 3A shows the ultraviolet absorbance profile

Table 2. *The titre of achetakinin-like immunoreactive material in tissues from 8- to 10-day-old adult virgin male crickets*

Tissue	Achetakinin-like immunoreactive material (fmol insect)
Head capsule	0±0 (5)
Brain	31±9 (9)
Retrocerebral complex	1800±510 (12)
Suboesophageal ganglion	20±11 (9)
Thoracic ganglia	41±6 (5)
Ventral abdominal ganglia	8±1 (5)
Malpighian tubules	0±0 (5)
Flight muscle	0±0 (5)

Data are expressed as the mean ± S.E.M., with the number of replicates given in parentheses.

from a separation of 50 retrocerebral complexes and indicates the retention times of achetakinins I–V run under identical conditions. Those fractions having the same retention times as achetakinins, together with all other fractions containing ultraviolet-absorbing material, were taken for RIA (Fig. 3B). Immunoreactivity was concentrated in just two fractions, one of which (fraction 18) co-eluted with AK-I and AK-II. Fraction 16 contained a relatively large amount of immunoreactive material, but did not correspond with the retention time of any of the identified achetakinins. Fraction 21, which had the same retention time as AK-IV, showed a small amount of immunoreactivity. AK-III and AK-V eluted in fractions 37 and 9, respectively, but neither of these fractions contained material recognised by the antiserum. Minimal amounts of immunoreactive material were also detected in fractions 17, 22, 25, 26 and 38, but in all other fractions, the amount of material present in samples of 100 μl was below the detection limits of the RIA (i.e. <5 fmol).

Release of achetakinin-like immunoreactive material from the retrocerebral complex after depolarization in high concentrations of potassium

Retrocerebral complexes were removed from adult male crickets (6–8 days old), and incubated individually in small drops (50 μl) of *Acheta* saline (Coast, 1988) for 2 h. This preincubation period was needed to reduce the spontaneous release of achetakinins from the glands, presumably the result of damage during the dissection procedure. After 2 h, each retrocerebral complex was transferred sequentially to 5 μl drops of normal saline,

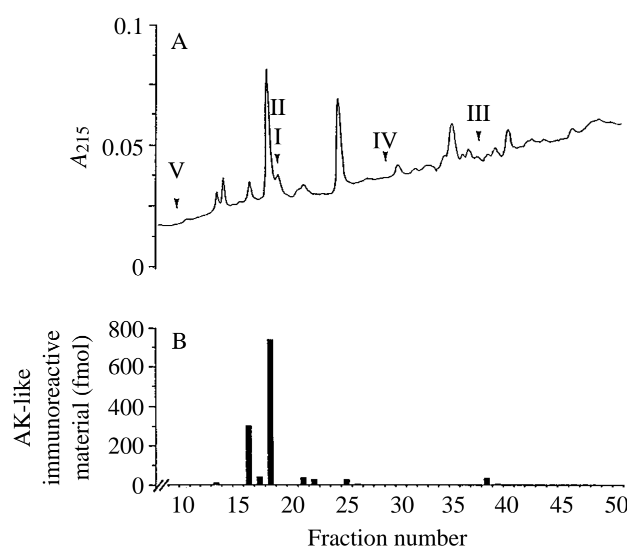

Fig. 3. The presence of achetakinin-like immunoreactive material in the retrocerebral complex. (A) The absorbance profile at 215 nm for an extract of 50 retrocerebral complexes separated by reverse-phase HPLC. The retention times of achetakinins I–V are indicated by the arrowheads. (B) The amount of achetakinin-like immunoreactive material in individual HPLC fractions determined by radioimmunoassay. Values are the means of duplicate determinations.

K^+-rich (80 mmol l^{-1} K^+ substituting for Na^+ in *Acheta* saline) Ca^{2+}-free saline, and K^+-rich saline containing 2 mmol l^{-1} Ca^{2+}. The glands were held in each saline for 5 min before being transferred on a fine glass rod to the next solution. Five glands were processed together, and the experiment was replicated five times. At the end of the experimental period, each of the solutions, containing material released from five complexes, was added to 50 μl of methanol and taken to dryness in a centrifugal evaporator. The material was resuspended in 40 μl of PBS for the determination of achetakinin-like immunoreactive material (Fig. 4). Small amounts (<10 fmol gland^{-1}) of material were released into normal saline and into K^+-rich, Ca^{2+}-free saline, whereas approximately 70 fmol gland^{-1} achetakinin-like immunoreactive material was released into K^+-rich saline containing 2 mmol l^{-1} Ca^{2+}.

Haemolymph titres of achetakinin-like immunoreactive material

Methanolic extracts prepared from 25 μl samples of pooled haemolymph from 2–3 adult male crickets (6–8 days old) were tested for achetakinin-like immunoreactivity in the RIA. The amount of material present was below that detectable with the method, indicating that the haemolymph titre was below 200 pmol l^{-1}. However, when the above procedure was repeated using acidic methanol (see Materials and methods) in the extraction procedure, achetakinin-like immunoreactivity was 72±13.0 fmol (N=6) in 25 μl, equivalent to a haemolymph titre of 2.8±0.5 nmol l^{-1}.

The use of methanol or acidic methanol for the extraction of achetakinins in cricket

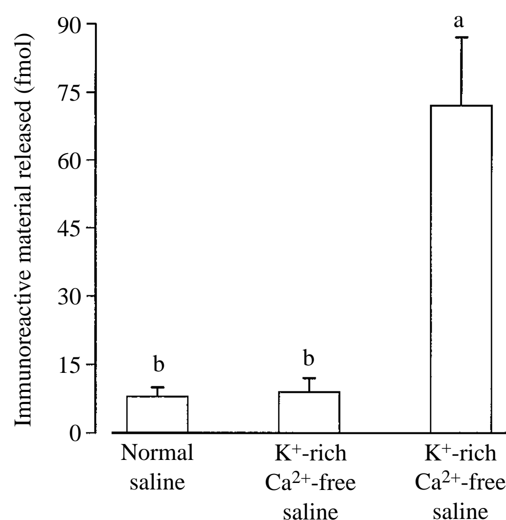

Fig. 4. The Ca^{2+}-dependent release of achetakinin-like immunoreactive material from retrocerebral complexes depolarised in K^+-rich (80 mmol l^{-1}) saline. Columns represent the mean values and bars +1 S.E.M. for five determinations. After a 2 h equilibration period, little immunoreactive material is released during 5 min incubations in normal saline or in K^+-rich, Ca^{2+}-free saline. On transfer to K^+-rich saline containing 2 mmol l^{-1} calcium, there is a 10-fold increase in the amount of material released over 5 min. Identical letters indicate no

Table 3. *The titre of achetakin-like immunoreactive material in haemolymph spiked with AK-I core peptide (500 fmol tube^{-1}) and extracted with either methanol or acidic methanol*

Sample	Achetakinin-like immunoreactive material (fmol)	
	Methanol	Acidic methanol
AK-I core	548.0±100.0 (5)	535.0±80.0^b (5)
Haemolymph	5.0±3.00^a (6)	72.0±13.0 (6)
AK-I core + haemolymph	8.0±6.0^a (6)	520.0±79.0^b (6)

Data are expressed as the mean ± S.E.M., with the number of replicates given in parentheses.
Identical superscript letters indicate no significant difference (P>0.05; ANOVA).

haemolymph was investigated further by measuring the amount of immunoreactive material in 25 μl samples of haemolymph spiked with 500 fmol of AK-I core peptide. In samples extracted in methanol, no (less than 5 fmol) immunoreactive material was detected in the RIA, whereas with acidic methanol all of the added AK-I core peptide was recovered (Table 3).

Effect of starvation and feeding on the amounts of immunoreactive material in haemolymph and in the retrocerebral complex

Adult male crickets, 8–10 days old, were allocated into two groups. A control group of 11 insects was allowed continuous access to food, whilst the remaining group of 25 insects was starved for 48 h without access to water. After 48 h, haemolymph and retrocerebral complexes were removed from the controls and from 13 of the starved insects. The other 12 starved insects were given access to moistened food for 1.5 h, after which haemolymph and retrocerebral complexes were removed. Haemolymph samples were extracted immediately in acidic methanol (see above) for measurement in the RIA.

The mean body mass of fed crickets was 508±12 mg (N=11), which fell to 389±16 mg (N=13) after 48 h of starvation, but recovered to 499±15 mg (N=12) within 30 min of the insects having access to food. Haemolymph volume fell by 50 % during the period of starvation (from 46.2±2.0 μl, N=7, to 23.2±2.5 μl, N=7) and remained at a reduced level in re-fed insects (23.0±1.8 μl, N=10). The amount of achetakinin-like material in the retrocerebral complex was not significantly changed after 48 h of starvation, but the haemolymph titre increased 10-fold (Fig. 5). When the insects were re-fed, the amount of immunoreactive material in the retrocerebral complex decreased. The haemolymph titre also fell, although it remained sevenfold higher than in the controls.

Discussion

The presence of a tyrosine residue in the core of AK-I enables this pentapeptide to be radioiodinated to obtain a probe with high specific activity. This probe has been used in conjunction with an antiserum raised against an N-terminal extended analogue of the core peptide conjugated to keyhole limpet haemocyanin to develop an RIA with a detection limit of approximately 5 fmol 100 μl^{-1}. The antiserum used in this study had previously

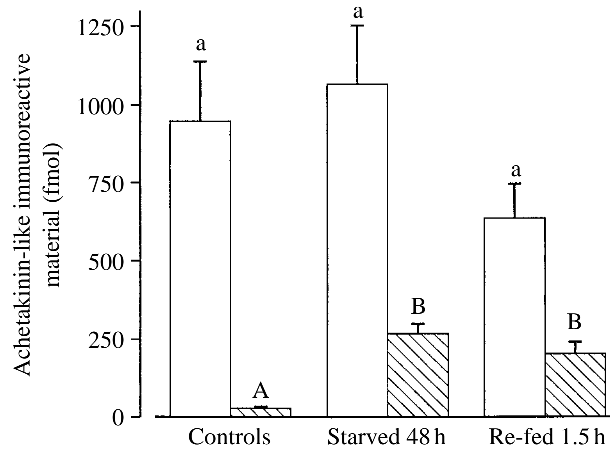

Fig. 5. The amount of achetakinin-like immunoreactive material in extracts of retrocerebral complexes (open columns) and haemolymph (hatched columns) from fed and starved crickets. Columns represent the mean values and bars +1 S.E.M. for 11–13 determinations. Starvation for 48 h promotes a dramatic increase in the amount of achetakinin-like immunoreactive material in the haemolymph, but only a small (not significant) change in tissue titre. The haemolymph titre falls when starved insects are given access to moistened food for 1.5 h, but remains significantly above control levels. Identical letters indicate no significant difference (P>0.05) between means.

been characterized by Clottens *et al.* (1993) in indirect and competitive enzyme-linked immunosorbent assays. They showed that the antiserum contained populations of antibodies recognising two distinct regions of the core peptide that encompass the Phe-Tyr and Pro-Trp residues. Results obtained with the RIA are largely in agreement with these findings. This was surprising, because the presence of iodine on Tyr^2 might have been expected to prevent recognition of the Phe-Tyr region. Indeed, we have found that when Tyr^2 of the AK-I core peptide is iodinated, the ligand is not recognised by receptors on Malpighian tubule plasma membranes (J. S. Chung, C. H. Wheeler, G. J. Goldsworthy and G. M. Coast, unpublished observations). Consistent with the presence of antibodies recognising two sites within the C-terminal pentamer, binding affinity is highest for AK-I and AK-I core, which contain both sites, whereas AK-II and AK-IV, in which Ser or Asn replaces Tyr at position 2, suffer a 10-fold reduction in affinity for the antiserum. AK-III, in which Ser replaces both Tyr^2 and Pro^3, is not recognised at all, demonstrating that no antibodies are directed against the C-terminal sequence Trp-Gly-NH_2. There is some evidence for antibodies with very low affinity recognising AK-V, which contains neither Phe-Tyr nor Pro-Trp. It is possible that these antibodies are directed against Ala-Phe, a sequence represented in the analogue conjugated to keyhole limpet haemocyanin. The RIA was standardised using the core peptide of AK-I. To the extent that AK-II and AK-IV (and to a very limited extent AK-V) are also recognised by the antiserum, the amount of achetakinin-like immunoreactive material will be underestimated by an amount dependent on the relative quantities of these peptides. Other classes of myoactive

peptides that were tested in the RIA, namely leucopyrokinin and *Locusta* tachykinin-IV, were not recognized by the antiserum.

Achetakinin-like immunoreactivity is widespread in the central nervous system, but by far the highest amount is present within the retrocerebral complex. The corpora cardiaca (plus hypocerebral ganglion) and the corpora allata contain similar amounts of achetakinin-like material. In crickets, both the corpora cardiaca and the nervi corpora allati-II have been described as neurohaemal organs (Weber and Gaude, 1971) and therefore represent potential sites for the release of achetakinins into the haemolymph. The distribution of achetakinins is consistent with the localization of leucokinins (LK-I) in adult *Leucophaea maderae* (Nassel *et al.* 1992; Muren *et al.* 1993). The major immunoreactive fraction from a reverse-phase HPLC separation of cricket retrocerebral complexes co-elutes with AK-I and AK-II, but significant immunoreactivity is found in a fraction that does not correspond to any of the identified achetakinins. The antigenic material within this fraction has yet to be characterised, but could represent a degradation product.

The presence of large quantities of achetakinins in identified neurohaemal structures is consistent with their possible role as circulating neurohormones. In support of this, a calcium-dependent release of achetakinin immunoreactive material was demonstrated in retrocerebral complexes depolarised in a high concentration of potassium. Over a 5 min period, the amount of immunoreactive material released was equivalent to approximately 5 % of the total store. Muren *et al.* (1993) have also described a calcium-dependent release of LK-I immunoreactive material from retrocerebral complexes of *Leucophaea*, which amounted to approximately 2 % of the total store in 120 min. However, it is most likely that the bulk of this material is released over a much shorter period (Maddrell and Gee, 1974) comparable with that used here.

Achetakinins have a number of actions in crickets, including myotropic (P. Blake, personal communication) and diuretic activity (Coast *et al.* 1990) *in vitro*, and effects on the concentrations of lipid in the haemolymph and levels of protein synthesis in the fat body *in vivo* (Goldsworthy *et al.* 1992). Potencies (EC_{50}) in these bioassays are very similar (10–100 $pmol\,l^{-1}$) and give no indication of their primary site of action. With a detection limit of approximately 5 fmol, and using 25 μl samples of haemolymph, the RIA can measure concentrations greater than 200 $pmol\,l^{-1}$, and it is therefore not sufficiently sensitive to measure haemolymph titres in the lower part of the physiological range, which might be expected to vary by an order of magnitude around the EC_{50}. However, in this study, the circulating titre was determined as 2.8 $nmol\,l^{-1}$. Similarly, Muren *et al.* (1993) found the titre of leucokinin immunoreactivity in haemolymph from *Leucophaea* to be in the nanomolar range. The presence of achetakinin/leucokinin-like material in the haemolymph suggests that these peptides are released *in vivo* and could therefore function as neurohormones. However, in both cases, the haemolymph titre is unexpectedly high, given the potency of these peptides, and would be sufficient to evoke maximal responses in all of the above-mentioned bioassays. There are several possible explanations for this. The high titre may be a reflection of a long half-life for achetakinins in the haemolymph. Our data suggest that achetakinins bind weakly to some (protein?) component in the haemolymph and can only be extracted in acidic methanol. It is possible

that any peptide that is bound is protected from degradation by proteases, but is not available for binding to membrane receptors. Alternatively, the antiserum may be recognising peptide fragments that are not biologically active. An endopeptidase that cleaves specifically on the C-terminal side of phenylalanine appears to play an important role in the degradation of adipokinetic peptides (Isaac, 1988). Cleavage of achetakinins at this point in the 'active core' would leave an inactive four-residue sequence that would still be recognised by antibodies directed towards the Pro-Trp region of the peptide.

In an attempt to investigate a possible regulatory role for achetakinins in the control of diuresis and/or gut emptying, haemolymph and tissue (retrocerebral complex) levels were measured in insects subjected to physiological stress. After 48 h of starvation without access to water, the titres of achetakinin-like immunoreactive material in haemolymph increase dramatically. Even with a 50 % reduction in haemolymph volume during the period of starvation, the increased titres represent the release of at least 500 fmol of peptide from neurohaemal structures, which is equivalent to the entire content of the corpora cardiaca or the corpora allata. However, the level of achetakinin-like immunoreactivity in the retrocerebral complex, the major site for storage, was maintained in starved insects, suggesting that starvation/dehydration stimulates peptide synthesis and the transport of material from sites of synthesis to this complex, if it is the site of release. Within 1.5 h of starved insects being given access to moistened diet, the haemolymph titres fall slightly but remain substantially above control levels. This fall cannot be explained by any increase in haemolymph volume, which is virtually unchanged, and must therefore represent a shift in the balance between peptide degradation and release.

On the basis of the current understanding of the control of water balance in insects, the increase in haemolymph achetakinin-like immunoreactive material in crickets starved for 48 h without access to water is not that expected of a hormone involved in the control of post-feeding diuresis. Rather, the data suggest that achetakinins could have a role in the control of metabolism, possibly in the mobilization of energy reserves during starvation. This would be consistent with their hyperlipaemic action and with their inhibition of protein synthesis *in vivo*, although both responses are generally held to be regulated by adipokinetic peptides (Goldsworthy *et al.* 1992). It may be that post-feeding diuresis is controlled by another diuretic peptide, for example *Acheta*-DP, which is a more effective stimulant of tubule secretion (Kay *et al.* 1991). However, even during periods of starvation and dehydration, primary urine production must be sustained to clear the haemolymph of toxic wastes. Thus, in crickets starved for 48 h, the rate of secretion of Malpighian tubule fluid does not fall to basal levels (Coast, 1988). This could be explained by the continued release of other diuretic factors such as the achetakinins. In this context, it is worth remembering that the rate at which water is lost from the body *via* the excretory system is ultimately determined by processes of reabsorption in the hindgut, which are controlled by other neuroendocrine factors (Phillips *et al.* 1986).

This work was supported by grants from the SERC under the Invertebrate Neuroscience Initiative, NATO, British Technology Group PLC and The Leverhulme Trust.

References

CLIFFORD, C. W., ROE, R. M. AND WOODRING, J. P. (1977). Rearing methods for obtaining house crickets, *Acheta domesticus*, of known age, sex and instar. *Ann. ent. Soc. Am.* **70**, 69–74.

CLOTTENS, F. L., MEOLA, S. M., COAST, G. M., HAYES, T. K., WRIGHT, M. S., NACHMAN, R. J. AND HOLMAN, G. M. (1993). Characterization of an antiserum against an achetakinin I-analog and its use for the localization of culekinin depolarizing peptide II in the mosquito, *Culex salinarius*. *Regul. Peptides* **49**, 145–157.

COAST, G. M. (1988). Fluid secretion by single isolated Malpighian tubules of the house cricket, *Acheta domesticus*, and their response to diuretic hormone. *Physiol. Ent.* **13**, 381–391.

COAST, G. M., HOLMAN, G. M. AND NACHMAN, R. J. (1990). The diuretic activity of a series of cephalomyotropic neuropeptides, the achetakinins, on isolated Malpighian tubules of the house cricket, *Acheta domesticus*. *J. Insect Physiol*. **36**, 481–488.

GOLDSWORTHY, G. J., COAST, G. M., WHEELER, H., CUSINATO, O., KAY, I. AND KHAMBAY, B. (1992). The structural and functional activity of neuropeptides. In *Royal Entomological Society Symposium on Insect Molecular Science* (ed. J. M. Crampton and P. Eggleston), pp. 205–225. London, San Diego: Academic Press.

HAYES, T. K., HOLMAN, G. M., PANNABECKER, T. L., WRIGHT, M. S., STREY, A. A., NACHMAN, R. J., HOEL, D. F. AND BEYENBACH, K. W. (1994). Culekinin depolarizing peptide: a mosquito leucokinin-like peptide that influences insect Malpighian tubule ion transport. *Regul. Peptides* (in press).

HAYES, T. K., PANNABECKER, T. L., HINKLEY, D. J., HOLMAN, G. M., NACHMAN, R. J., PETZEL, D. H. AND BEYENBACH, K. W. (1989). Leucokinins, a new family of ion transport stimulators and inhibitors in insect Malpighian tubules. *Life Sci*. **44**, 1259–1266.

HOLMAN, G. M., NACHMAN, R. R. AND WRIGHT, M. S. (1990). A strategy for the isolation and structural characterization of certain insect myotropic peptides that modify the spontaneous contractions of the isolated cockroach hindgut. In *Chromatography and Isolation of Insect Hormones and Pheromones* (ed. A. R. MaCaffery and I. D. Wilson), pp. 195–204. New York: Plenum Press.

ISAAC, R. E. (1988). Neuropeptide-degrading endopeptidase activity of locust (*Schistocerca gregaria*) synaptic membranes. *Biochem. J*. **255**, 843–847.

KAY, I., COAST, G. M., CUSINATO, O., WHEELER, C. H., TOTTY, N. F. AND GOLDSWORTHY, G. J. (1991). Isolation and characterization of a diuretic peptide from *Acheta domesticus*: evidence for a family of insect diuretic peptides. *Biol. Chem. Hoppe-Seyler* **372**, 505–512.

KUNIYOSHI, H., NAGASAWA, H., ANDO, T., SUZUKI, A., NACHMAN, R. J. AND HOLMAN, G. M. (1992). Cross-reactivity between pheromone biosynthesis activating neuropeptide (PBAN) and myotropic pyrokinin insect peptides. *Biosci. Biotech. Biochem.* **56**, 167–168.

MADDRELL, S. H. P. AND GEE, J. D. (1974). Potassium-induced release of the diuretic hormone of *Rhodnius prolixus* and *Glossina austeni*: Ca^{2+} dependence, time course and localization of neurohaemal area. *J. exp. Biol*. **61**, 155–171.

MUREN, E. J., LUNDQUIST, T. C. AND NASSEL, D. R. (1993). Quantitative determination of myotropic neuropeptide in the nervous system of the cockroach *Leucophaea madera*e: distribution and release of leucokinins. *J. exp. Biol*. **179**, 289–300.

NACHMAN, R. J., COAST, G. M., HOLMAN, G. M. AND HADDON, W. F. (1992). A bifunctional heterodimeric insect neuropeptide analog. *Int. J. Peptide Protein Res*. **40**, 423–428.

NASSEL, D. R., CANTERA, R. AND KARLSSON, A. (1992). Neurons in the cockroach nervous system reacting with antisera to neuropeptide Leucokinin I. *J. comp. Neurol.* **322**, 45–67.

PHILLIPS, J. E., HANRAHAN, J., CHAMBERLIN, M. AND THOMSON, R. B. (1986). Mechanisms and control of reabsorption in insect hindgut. *Adv. Insect Physiol*. **19**, 329–422.

WEBER, W. AND GAUDE, H. (1971). Ultrastruktur des Neurohaemalorgans im Nervus corporis allati II von *Acheta domesticus*. *Z. Zelforsch. mikrosk. Anat.* **121**, 561–572.