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Isolation and Immunocytochemical Characterization of Three Tachykinin-Related Peptides from the Mosquito, *Culex salinarius*

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Three myotropic peptides belonging to the Arg-amide insect tachykinin family were isolated from whole-body extracts of the mosquito, *Culex salinarius*. The peptides, APSGFMGMR-NH_2, APYGFTGMR-NH_2 and APSGFFGMR-NH_2 (designated culetachykinin I, II, and III) were isolated and purified on the basis of their ability to stimulate muscle contractions of isolated *Leucophaea maderae* hindgut. Biologically inactive methionine sulfoxides of two of the three peptides were isolated using an ELISA system based upon antiserum raised against APYGFTGMR-NH_2 and identified with mass spectrometry. Immunocytochemistry localized these peptides in cells in the brain, antennae, subesophageal, thoracic and abdominal ganglion, proventriculus and midgut. Nerve tracts containing these peptides were found in the median nerve of the brain, central body, nervi corpus cardiaci, cervical nerve, antennal lobe and on the surface of the midgut.

KEY WORDS: Peptide identification; insect tachykinins; myotropins; homology; neuromodulators.

INTRODUCTION

Peptides of the tachykinin family have been extensively studied in vertebrates and are widely distributed within the central and peripheral nervous systems where they have been found to evoke a variety of physiological responses. These actions include excitation of neurons, vasodilatation, stimulation of smooth muscle contractions, and induction of behavioral responses. Tachykinins are also potent secretagogues (1). Insect neuropeptides are currently being isolated and their structure determined in order to provide a new class of biocompatible insecticides using analogues of these compounds. The first tachykinin peptide isolated and identified from an invertebrate source was eledoisin from the cephalopod, *Eledone* (2) in 1962. More than 25 years elapsed before other tachykinin-related peptides were isolated from invertebrates. In 1988, tachykinin-like peptides were isolated from the central nervous system of the locust, *Locusta migratoria* based on their myotropic effect on the isolated hindgut of the cockroach, *Leucophaea maderae*. Four of these peptides, designated lo-

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custatachykinins, share a similar C-terminal pentamer (-Phe-Tyr-Gly-Val-Arg-NH_2). In one peptide (locustatachykinin IV), the tyrosine residue is replaced by a histidine. Although the two terminal residues differ from the typical C-terminal of vertebrate tachykinins (-Leu-Met-NH_2), the insect peptides were considered tachykinin-related based on the sequence-Phe-Tyr-Gly-, the small change from leucine to valine, and because of their myotropic effect on smooth muscles (3,4).

Two similar peptides (callitachykinins) have recently been identified from the blowfly, *Calliphora vomitoria*, using a combination of ELISA and bioassay (5). Tachykinin-related peptides have also been isolated and characterized from the echiuroid worm, *Urechis unicinctus* (6). Two peptides containing the C-terminal pentamer of the vertebrate peptide, physaelamin, designated sialokinin I and II, have recently been isolated from salivary glands of the yellow fever mosquito, *Aedes aegypti* (7). These two peptides function as vasodilators in the host and facilitate feeding by the mosquito.

In the present study, we describe the isolation, purification, and structural characterization of three new members of the insect Arg-tachykinin peptide family from a whole-body extract of *Culex salinarius* mosquitos. Antiserum raised against one of these peptides was used in an immunohistochemical analysis to establish that these peptides were of both neuronal and gut origin. In addition, this antiserum was used in an ELISA system which resulted in the discovery of the third peptide of this series as well as two biologically inactive oxidized forms, which were artifacts generated during purification. The three biologically active peptides were designated culetachykinin I-III.

EXPERIMENTAL PROCEDURE

Insect Procurement. Culex salinarius. were obtained from wild populations collected in the Anahuac National Wildlife Refuge in Chambers County, TX. Wild populations of these species were used because laboratory colonies of *C. salinarius* could not economically produce the number of mosquitoes required in a timely fashion for peptide extraction. Mosquitoes were collected by established methods (8) at appropriate times in late October of 1990 and early April 1992 when *C. salinarius* is the dominant mosquito species in the marshlands and rice fields (9). For the isolation of the peptides, mosquitoes were frozen on dry ice immediately after collection and stored at −72°C until used. A second group of mosquitoes was maintained on a sugar water solution until prepared for immunocytochemistry two days after collection. *Leucophaea maderae* cockroaches were reared at 27°C on a 12 h light-dark cycle and fed dry dog food and water *ad libitum*.

Bioassay. The purification of culetachykinins (CTKs) was monitored with a standard myotropic assay previously utilized successfully for myotropic peptide isolation from several insect species (10). Briefly, a hindgut was dissected from the abdomen of *L. maderae* and suspended in a saline bath where it was connected to an isotonic muscle transducer. Test fractions were dissolved in saline and were directly added to the saline in the test chamber which can be drained and rinsed after each treatment.

Extraction Procedure. The extraction procedure for isolation of CTK I and CTK II is described elsewhere (11). In brief, a total of 2,571 g of whole mosquitoes was homogenized in ice cold 15% trifluoroacetic acid (TFA) and centrifuged 10 min at 2000 X g. The supernatant was filtered through Whatman No. 2 filter paper and the filtrate applied to a series of Mega Bond Elut C-18 cartridges (Varian, Harbor City, CA). The retained material was eluted with 40% acetonitrile (ACN) and 80% ACN in 0.1% TFA. The 40% ACN fraction was prepared for refractionation on a single Mega Bond Elut cartridge by rotary evaporation followed by addition of 2% heptafluorobutyric acid (HFBA). Fractions were collected from 10% to 50% ACN (0.2% HFBA) in 5% ACN incremental increases. Based upon myotropic bioassay, the 30% and 35% ACN fractions were pooled for HPLC purification. Isolation of CTK III was identical to that used for *Musca* CRF-like peptide (12) with 5,630 g of whole mosquitoes used for the extraction of CTK III.

HPLC Purification. HPLC purification was performed on a Waters ALC 100 liquid chromatograph, equipped with two Waters 6000A solvent delivery systems; a Waters 680 automated gradient controller; a Waters 480 Lambda Max tunable absorbence detector (Waters/Millipore) and a Rheodyne 7125 septumless injector (Rheodyne, Cotati, CA). After each purification step, aliquots from each fraction were tested for bioactivity with the hingut bioassay (purification of CTK I, CTK II, CTK III) or for binding to the antiserum in the ELISA (purification of methionine oxides of CTK II and CTK III which were not active in the myotropic assay).

The following column sequence was used to purify CTK I and CTK II with solvent A: 0.1% TFA in water; and solvent B: 50% ACN in aqueous TFA.

(1) *DeltaPak C-18*, 100 Å, 15 μm, 100 × 25 mm preppak (Waters/Millipore). A linear gradient of 0–100% B was applied over 150 min; flow rate of 7.5 ml/min; detector set at 4.0 AUF at 214 nm; and fractions collected every 2 minutes.

(2) *DeltaPak C-4*, 100 Å, 15 μm, 100 × 8 mm (Waters/Millipore). Conditions were the same as for 1, except the flow rate was 1.5 ml/min.

(3) *NovaPak Phenyl*, 60 Å, 4 μm, 100 × 8 mm (Waters/Millipore). Conditions were the same as for 2, except the detector was set as 2.0 AUFS.

(4) *Ultremex C-1*, 80 Å, 5 μm, 250 × 4.6 mm (Phenomenex, Torrance, CA). Conditions were the same as for 2, except solvent B was 25% ACN in 0.1% aqueous TFA and the detector was set at 0.5 AUFS.

(5) *NovaPak C-8*, 60 Å, 4 μm, 100 × 8 mm (Waters/Millipore). Conditions were the same as for 2, except the linear solvent gradient from 0–100% B was completed over 80 min and the detector was set at 0.2 AUFS. Peaks were collected manually.

(6) *ProteinPak I-125*, 10 μm, 300 × 7.8 mm (Waters/Millipore) with following solvents: solvent A: 95% ACN in 0.01% aqueous TFA; solvent B: 50% ACN in 0.01% aqueous TFA. A gradient of 0–100% B was applied over 80 minutes after 8 minutes isocratic A; the flow rate was 1.5 ml/min; the detector was set at 0.02 AUFS at 214 nm; and peaks were collected manually.

Purification of CTK III and the oxidized analogs of CTK II and CTK III used the following column sequence with solvent A: 0.1% TFA in water and solvent B: 60% ACN in 0.1% aqueous TFA.

(1) *DeltaPak C-18*, 300 Å, 15 μm, 100 × 25 mm preppak and 10 × 25 mm guardpak (Waters/Millipore). A linear gradient of 0–

100% B was applied over 180 min; the flow rate was 7.5 ml/min; the detector was set at 4.0 AUFS at 220 nm; and fractions were collected every 2 minutes.

(2) *DeltaPak C-4*, 300 Å, 15 μm, 100 × 25 mm preppak and 10 × 25 mm guardpak (Waters/Millipore). Conditions were the same as for 1.

(3) *Vydac Phenyl*, 300 Å, 5 μm, 250 × 10 mm (Phenomenex). Conditions were the same as for 1, except the flow rate was 2.0 ml/min and the detector was set at 1.0 AUFS.

(4) *Synchropak RP-1 C-1*, 300 Å, 6.5 μm, 250 × 10 mm (Phenomenex). Conditions were the same as for 3, except the gradient was applied over 120 minutes.

(5) *NovaPak C-8*, 60 Å, 4 μm, 100 × 8 mm (Waters/Millipore). Conditions were the same as for 4, except the detector was set at 0.5 AUFS.

(6a) *ProteinPak I-125*, 10 μm, 300 × 7.8 mm (Waters/Millipore) with following solvents: solvent A: 95% ACN in 0.01% aqueous TFA; solvent B: 50% ACN in 0.01% aqueous TFA. A gradient of 0–100% B was applied over 80 minutes after 4 minutes isocratic A; the flow rate was 1.5 ml/min; the detector was set at 0.2 AUFS at 214 nm; and peaks were collected manually.

(6b) *BioSep Sec-S2000*, 300 × 7.8 mm (Phenomenex). Conditions were the same as for the 6a, except the absorbence was measured at 220 nm.

Enzymatic Degradation. To determine if the peptides were blocked at the N-terminus, an aliquot of 10 g equivalents of initial extract of each isolated peptide was dried, redissolved in 50 μl cockroach saline (composition in mM: NaCl, 154.0; Glucose, 22.2; HEPES, 11.9; KCl, 2.7; $CaCl_2$, 1.8; pH 7.0), and incubated with one unit of immobilized aminopeptidase M gel suspension (Pierce, Rockford, IL). An identical set of peptides was prepared without aminopeptidase M as a control. Mixtures were vortexed gently and incubated for 2 hrs in a 37°C water bath. The gel was removed by centrifugation and the supernatant was tested in the cockroach hindgut bioassay.

Mass Spectrometry. The masses of the natural peptides isolated from the second extract were determined using laser desorption mass spectrometry on a Vestec model 2000 laser desorption time-of-flight mass spectrometer as previously described (5).

Amino Acid Sequence Analysis, and Peptide Synthesis. The amino acid sequence of CTK I and II was determined on an Applied Biosystems 470A sequences equipped with a Model 120A PTH amino acid analyzer (Applied Biosystems Inc., Foster City, CA). An Applied Biosystems 473A automated protein sequencer with on-line detection of PTH-amino acids was used to determine the amino acid sequence of oxidized CTK II, CTK III and unoxidized CTK III. Operating conditions were the manufacturers recommended protocols.

Synthesis of CTK I and II was accomplished by solid phase peptide synthesis on a Milligen Biosearch 9600 automated peptide synthesizer using TBOC chemistry according to previously described procedures (13); and CTK III on a Vega coupler 250 automated peptide synthesizer using FMOC chemistry with diisopropylcarbodiimide as the coupling reagent and dimethylformamide as solvent under previously described conditions (14). All peptides were synthesized in the C-amidated form, based upon the other known Arg-tachykinins (see Table III).

The synthetic culetachykinins were purified on a NovaPak C-8 column with solvents as described above for the purification of CTK III. A linear gradient of 0–100% B was applied over 60 minutes after 2 minutes isocratic A; the flow rate was 1.5 ml/min. Each synthetic peptide was compared with its natural analog by HPLC-runs on the same column systems; by amino acid analysis; and by biological effects on the *Leucophaea* hindgut. The amino acid composition and the quantity of the synthetic peptides were determined by amino acid analysis as previously described (15). Reduced and oxidized forms of the peptides were compared by applying them together in equal amounts on a NovaPak C-8 column with the same conditions used to purify the synthetic peptides. The oxidized forms were prepared by adding 10 μl of 30% H_2O_2 to 25 nmol of reduced peptide in 1 ml of 20% ACN (0.1% TFA) and holding the samples for 30 minutes at room temperature.

Threshold Concentrations. Threshold concentrations required for biological activity were determined by adding known concentrations of the different peptides to a cockroach hindgut in the bioassay chamber. The threshold was determined from five different hindgut preparations and results are expressed as the mean ± standard deviation. The threshold concentration is defined as that concentration of peptide required to evoke an observable change in frequency, amplitude or tonus of the spontaneous contractions within one minute.

Antisera Production and Characterization. Antisera against CTK I (3350-7, 3351-7) and II were raised in four New Zealand White rabbits. The peptides were N-terminally conjugated to thyroglobulin (Sigma, St. Louis, MO) with 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride (Sigma)(16). After conjugation, an extra incubation of the thyroglobulin CTK II complex with 1 M hyroxylamine hydrochloride (Sigma) converted the O-aryl isourea back to tyrosine (17). The antisera was characterized by ELISA using MaxiSorp immunoplates (Nunc, Denmark) as described previously (18). Briefly, peptides coated on microtiter plates were incubated with primary antiserum, followed by alkaline-phosphatase conjugated goat anti-rabbit serum (Pierce). Color produced by addition of p-nitrophenylphosphate (Pierce) was measured spectrophotometrically at 410 nm. For competitive ELISA, the antiserum (diluted 1:2000) was pre-incubated with different concentrations of a competing peptide. The immunoplate was coated with 10 pmol CTK II. After incubation, the reaction was run as for the non-competitive ELISA. The HPLC purification of biologically inactive CTK-oxides was monitored with competitive ELISA. Immunoplates coated with 10 pmol CTK II and antiserum (diluted 1:2000) were preincubated with aliquots (10 to 60 g equivalents of whole body extract) of each HPLC fraction and each fraction was tested in duplicate.

Immunohistochemistry. Mosquito specimens were fixed in situ in aqueous Bouins (19) a minimum of 48 hr. Whole bodies were dehydrated in ethanol, cleared in toluene and embedded in low temperature (50–54°C) paraplast (Oxford Labware, St. Louis, MO). Specimens were sectioned on a Reichert-Jung rotary microtome at 5 μm and immunohistochemically processed as described previously (20). Localization of the antibody/antigen reaction was accomplished with avidin-biotin staining kits (Pierce). The reaction was visualized with diaminobenzidine tetrahydrochloride (DAB) from Sigma. Immunohistochemical reactions were obtained using antiserum 3352-7 (directed against CTK II) at an optimal dilution of 1:2000. The antiserum was diluted in phosphate buffered saline containing 0.5% bovine serum albumen (Sigma) and Triton X-100 (Merck). The serum specificity was tested by pre-incubation of the antiserum (1:1000) overnight at 4°C with synthetic CTK II at a concentration of 10 nmol/ml diluted serum. Histochemical results were visualized with an Olympus AH-2 compound microscope and photographed with Kodak Tmax-100 film.

RESULTS

Isolation and Structure Characterization. All the acetonitrile fractions from the first whole-body extract were tested for myotropic bioactivity using the cock-

Table I. Elution Profile of the Culetachykinins During HPLC Purification

Column	% Acetonitrile		Column	% Acetonitrile		
DeltaPak C-18	20.7 - 22.0%	22.0 - 22.7%	*DeltaPak C-18*	16.0 - 17.3%	24.7 - 25.3%	24.7 - 25.3%
DeltaPak C-4	17.3 - 18.7%	16.7 - 18.7%	*DeltaPak C-4*	15.3 - 16.7%	20.0 - 20.7%	23.1 - 25.1%
NovaPak Phenyl	19.3 - 20.0%	19.3 - 20.0%	*Vydac Phenyl*	14.7 - 16.0%	18.0 - 19.3%	19.3 - 19.8%
Ultremex C-1	6.7 - 7.0%	6.3 - 7.0%	*Synchropak C-1*	12.0 - 14.0%	9.0 - 11.0%	6.5 - 8.5%
NovaPak C-8	36.6%	25.8%	*NovaPak C-8*			21.0%
ProteinPak I-125	76.5%	76.5%	*ProteinPak I-125*			78.0%
			BioSep Sec-S2000	75.5%	76.6%	
	CTK-II	**CTK-I**		**CTK-II (ox)**	**CTK-III (ox)**	**CTK-III**

The percentages are for relative proportions of acetonitrile at the pump when peptides eluted from the different columns. The elution profile for each peptide during different purification steps can be followed by reading the table vertically.
The left half of the table gives the elution profile of culetachykinins from the first extract, and the right half gives the elution profile of culetachykinins from the second extract.

Table II. Threshold Concentrations Required for Biological Activity of Natural and Synthetic Culetachykinins in the Cockroach Hindgut Bioassay

Peptide		Threshold concentration X ± S.D. (n=5)
Culetachykinin I	*natural peptide*	$1.26 \pm 0.24 \times 10^{-9}$ M
	synthetic peptide	$1.53 \pm 0.27 \times 10^{-9}$ M
Culetachykinin II	*natural peptide*	$2.53 \pm 0.55 \times 10^{-9}$ M
	synthetic peptide	$2.70 \pm 0.74 \times 10^{-9}$ M
Culetachykinin III	*natural peptide*	NT (*)
	synthetic peptide	$6.07 \pm 1.34 \times 10^{-11}$ M

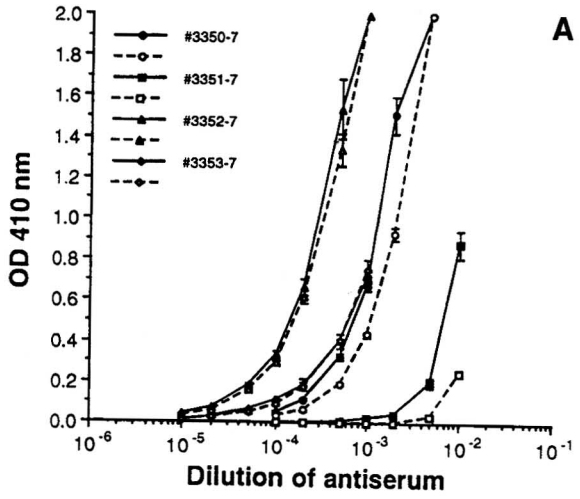
(*)Not tested. All natural product recovered was dedicated to structural identification.

roach hindgut assay, and the most significant reactivity was found in fractions 25–30% ACN and 30–35% ACN. These fractions were pooled and used for further purification in six different column systems (Table I). An aliquot of 5 to 10 g equivalents of initial extract from each HPLC fraction was screened for myotropic bioactivity. A total of 2,571 g of mosquito extract was initially separated by eight individual runs on the first column. Myotropic activity was found in a broad region of elution at 20.7–22.7% ACN (Table I). In addition, the subfraction eluting between 22.0–22.7% ACN showed a depolarizing effect on isolated Malpighian tubules (insect excretory organs) and was purified separately (11). Enzymatic degradations of the two isolated fractions resulted in a complete loss of biological activity in the cockroach hindgut bioassay, indicating that the peptides were not N-terminally blocked. No loss of biological activity was noticed in the control mixtures. Aliquots of both peptides were sequenced and revealed the following sequences: Ala-Pro-Ser-Gly-Phe-Met-Gly-Met-Arg (culetachykinin I) and Ala-Pro-Tyr-Gly-Phe-Thr-Gly-Met-Arg (culetachykinin II).

The amino acid compositions of synthetic CTK I and II were compared with the sequences of natural peptides. Threshold concentrations needed to evoke a biological effect in the cockroach hindgut assay were determined for natural and synthetic peptides (Table II). Threshold concentrations of each natural peptide and its synthetic analog were identical for both culetachykinins. A total of 10.5 nmol CTK I and 5.9 nmol CTK II were recovered from 2,571 g of whole body extract, representing about 1,200,000 mosquitoes. The amounts of recovered peptides are based upon the amino acid composition of the natural product. Therefore, each mosquito contains about 8.8 fmol CTK I and 4.9 fmol CTK II.

Antisera were prepared against synthetic peptides. Two antisera were prepared for each identified peptide: antisera 3350-7 and 3351-7 against CTK I, and antisera 3352-7 and 3353-7 against CTK II. The titer of the four antisera as well as their possible cross-reactivities were spectrophotometrically analyzed using 100 pmol/well of CTK I or CTK II. Antisera dilutions ranged from 1:100 to 1:100,000 (Fig. 1A). Tests indicated that all antisera showed a nearly 100% cross reaction with CTK I and CTK II respectively. Because all antisera had an identical specificity for the two culetachykinins, we chose the one with the highest titer (3352-7) for immunohistochemistry studies and for further screening of a second extract of mosquitoes to detect similar peptides. The antiserum did not cross-react with other known peptides from *C. salinarius* (Fig. 1B). Only a small cross-reactivity was observed with locustatachykinins (data not shown).

When the second extract was screened with competitive ELISA, immunopositive reactivity was found only in the 25% ACN fraction of solid-phase purification. This fraction, containing 5,600 g mosquito equivalents, was processed through the DeltaPak C-18 column with twelve consecutive runs. Duplicate aliquots (20 g



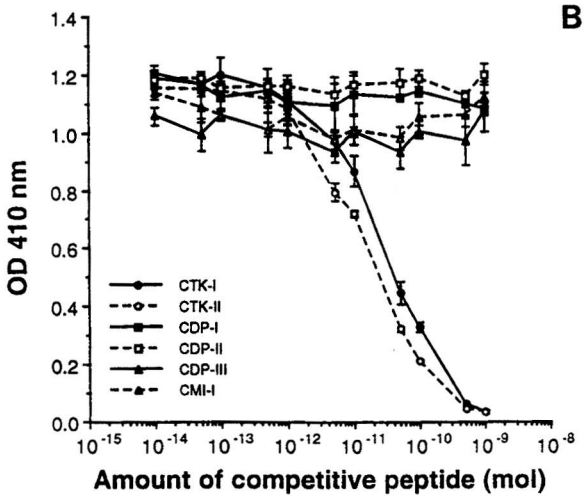


Fig. 1. A. Comparative ELISA with antisera 3350-7 and 3351-7, both directed against CTK I, and antisera 3352-7 and 3353-7, both directed against CTK II. The antigen was coated in a concentration of 100 pmol/well. Solid symbols represent a reaction of the antisera with CTK I and open symbols represent a reaction of the antisera with CTK II. **B.** Competitive ELISA with anti-culetachykinin serum #3352-7, diluted 1:2000 and pre-incubated with different peptides from *C. salinarius*. CTK: culetachykinin; CDP: culekinin depolarizing peptide; CMI: culex myoinhibiting peptide.

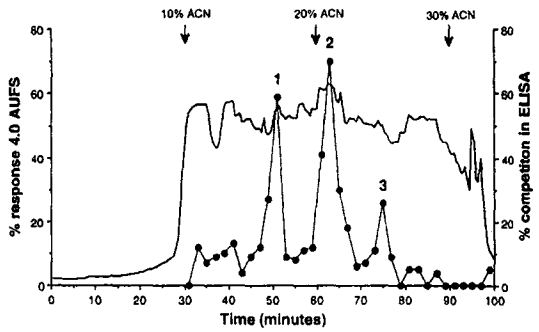


Fig. 2. Fractionation of the 10–25% ACN fraction of the second whole-body extract on a DeltaPak C-18 Column (solid line). An aliquot of 20 g equivalent was tested in a competitive ELISA with an antiserum (3352-7) against culetachykinin II. The percentage of competition of the aliquot with CTK II is indicated by the solid circles. Region 1 contained the oxidized form of CTK II. Region 2 contained the reduced forms of CTK I and II. Region 3 contained the oxidized and reduced form of CTK III.

equivalents of initial extract) of each fraction from the twelve runs were tested in the ELISA system and results of one of the 12 fractionations are shown in Fig. 2. Three definite regions (peaks 1–3) showed immunoreactivity with the antiserum: 16.0–17.3% ACN; 20.0–22.0% ACN; and 24.7–25.3% ACN. The second region (20.0–22.0% ACN) contained the two previously identified CTKs (see above). The two other regions (peaks 1 and 3) were further purified with the five different column systems shown on the right-hand side of Table I. Purification of the immunopositive (but biologically inactive) peptide in the short retention time region (16.0–17.3% ACN) of the C-18 column yielded a pure material with a sequence identical to CTK II. Laser desorption mass spectrometry of that peptide showed a mass (MH^+) of 1016.5 whereas the calculated average mass of CTK II was 998.2. This result strongly suggested that the methionine in the 8-position was oxidized to methionine-sulfoxide, a biologically inactive analog.

Two peptides were isolated and structurally characterized from the long retention time region (24.7–25.3% ACN): one was biologically inactive but ELISA-positive; the other was biologically active as well as ELISA-positive (designated CTK III). Both yielded the sequence Ala-Pro-Ser-Gly-Phe-Phe-Gly-Met-Arg but the MH^+ of the inactive peptide was 16 Daltons higher than that of the biologically active peptide, again indicating an oxidized methionine at position 8.

Synthetic CTK III (unoxidized) was used to construct a standard curve (peak height versus quantity injected) to estimate recovery of CTK III (both reduced and oxidized) from the extract. This was necessary because all of the natural product (both reduced and oxidized) was used for sequence and mass spectrometry. The amino acid composition of synthetic CTK III was compared with the sequence of the natural peptide and the threshold concentration of the synthetic peptide was determined (Table II). A total of 7.4 nmol of CTK III (reduced and oxidized) was recovered from the 5,600 g whole-body extract. Because 5,600 g represents about 2,630,000 mosquitoes, each mosquito would contain about 2.8 fmol of CTK III.

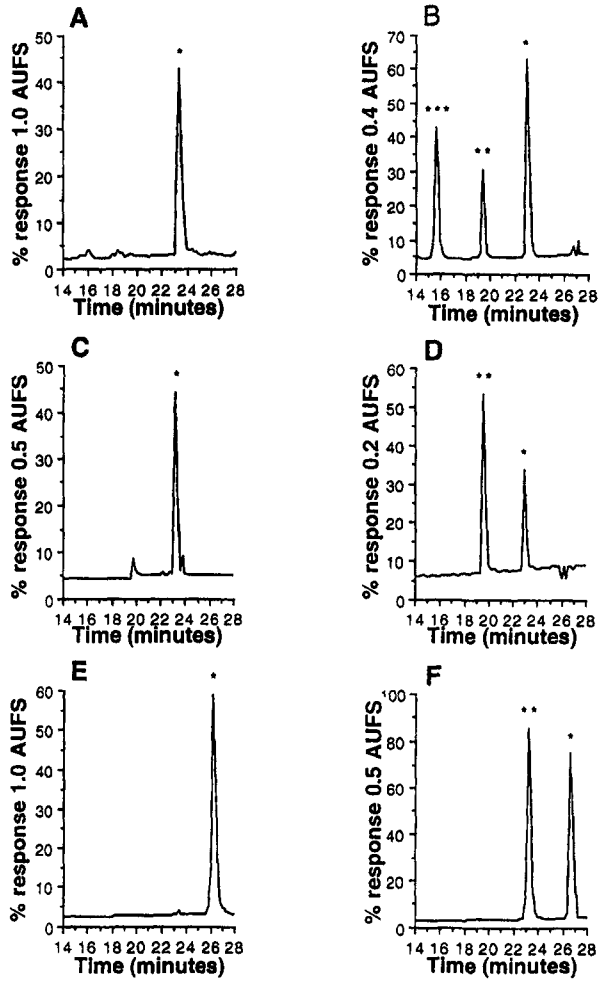


Fig. 3. Effect of the oxidation of the synthetic culetachykinins on a NovaPak C-8 column: * = reduced peptide; ** = one methionine oxidized; *** = two methionines oxidized. A, C and E represent the HPLC-pattern of 50 nmol of CTK I, CTK II and CTK III, respectively. B, D and F represent an equal mixture of oxidized and reduced forms of CTK I, CTK II and CTK III, respectively.

To evaluate the influence of oxidation on activity of the culetachykinins, a total of 50 nmol of each synthetic peptide was run separately on a NovaPak C-8 column. Each peak was collected and divided in half, each half containing about 25 nmol. One half was incubated with H_2O_2 for 30 minutes at room temperature, and then both halves were injected together on the same column system (Fig. 3). With each peptide, the more polar oxidized forms showed shorter retention times than reduced forms. Analysis of CTK I, which contains two methionine residues, revealed an extra peak in the chromatogram as expected. In the cockroach hindgut bioassay, the biological effect of oxidized and reduced forms of the peptides were compared at pharmacological concentrations (1×10^{-6} M). The reduced forms of CTK I, II, and III showed maximal biological activity as did the CTK I with one of its two methionine groups in the oxidized form (data not shown). The oxidized forms of CTK II and III, and CTK I with both methionines oxidized, were inactive. A comparative ELISA with the antiserum against CTK II did not show any difference between the oxidized and reduced forms of the different culetachykinins (Fig. 4).

Immunocytochemistry. Morphological terminology used in this report is based on that of Christophers (21). The CTK II antiserum reacted with neurons in the brain, subesophageal ganglion (SG), thoracic ganglia (TG) and abdominal ganglia (AG), as well as with endocrine cells in the wall of the digestive system. Because the antiserum against CTK II is unable to differentiate between CTK I, CTK II and CTK III, immunopositive labeled material in tissue sections was designated CTK immunopositive material.

The majority of the neurons containing CTK positive material was found in the brain and SG (Fig. 5). Controls tissues in which antiserum (1:1000 dilution) was pre-absorbed with 10 nmol/ml CTK II, or in which pre-immune rabbit serum was used as the primary antibody, failed to reveal CTK positive material (Fig. 6A,B). Four immunopositive cells (LNC) are located bilaterally in the anterior lateral regions of the protocerebrum (Fig. 6A). A cluster of 10 to 12 CTK positive neurons are located in each half of the medial protocerebrum bordering the pars intercerebralis (Fig. 6C,D). Some of these medial neurosecretory cells (MNC) terminate in the dorsal region of the medial neuropile of the protocerebrum (MNP) (Fig. 6C) while other neurites of these cells extend into the SG and enter the cervical nerve (CN) (Fig. 10B). CTK reactive fibers were found terminating in the fan-shaped body (FB) of the central complex (Fig. 6C). A large number of the neurites of the MNC enter the median nerve (Fig. 6D) that exits the brain as the nervi corporis cardiaci (NCC). The NCC enters the pharyngeal portion of the corpora cardiaca (CC) (Fig. 7A). This part of the retrocerebral system has been termed the neurohemal region of the CC in the mosquito by Burgess and Rempel (22). Fibers containing CTK positive material terminate in the wall of the aorta as well as adjacent to neurons of the hyocerebral ganglion (Fig. 7A). CTK positive fibers extending to the cardiacal/allatal complex terminate on the intrinsic neurosecretory cells (INC) of the CC lying adjacent to the cross trachea (Fig. 7B). These cells were originally termed the X-cells of the retrocerebral complex by Clements (23).

A cluster of five CTK positive neurons was found in the medial ventral region of each optic lobe (Fig. 7C).

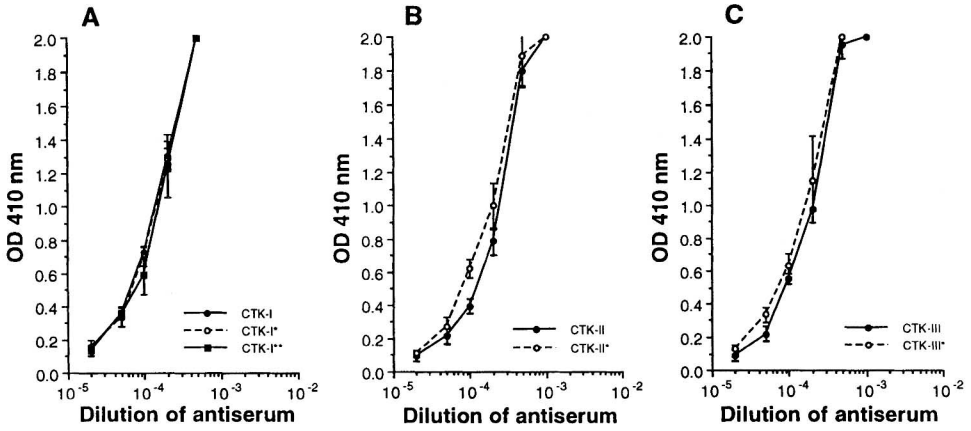


Fig. 4. A comparative ELISA of the oxidized and reduced forms of the culetachykinins with an antiserum (3352-7) against CTK II: * = one methionine oxidized; ** = two methionines oxidized.

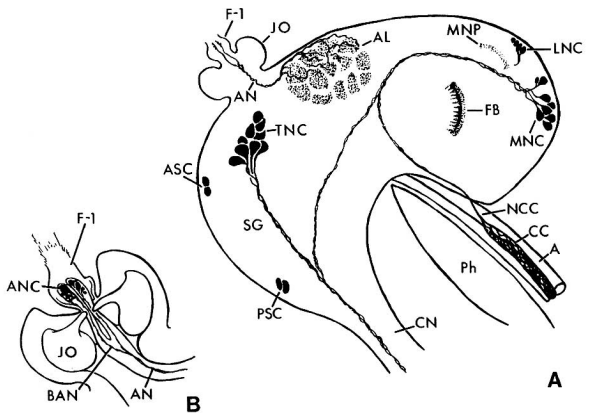


Fig. 5. A. Schematic diagram of a series of sagittal sections through the brain, subesophageal ganglion and antenna to show the relationship of clusters of neurons immunoreactive to antiserum recognizing all three CTKs and the pathway of their neurites. **B.** Enlargement of the pedicle and 1st flagellar segment (F-1) illustrating position of antennal neurosecretory cells (ANC) and bifercation of antennal nerve (BAN). A, aorta, AL, antennal lobe; AN, antennal nerve; ASC; anterior subesophageal cells; CC, neurohemal lobe of corpus cardiacum; CN, cervical nerve; FB, fan body; JO, Johnson's organ; LNC, lateral neurosecretory cells; MNC, medial neurosecretory cells; MNP, medial neuropil of protocerebrum; NCC, nervus corpus cardiacum; Ph, pharynx; PSC, posterior subesophageal cells; SG, subesophageal ganglion; TNC, tritocerebral neurosecretory cells.

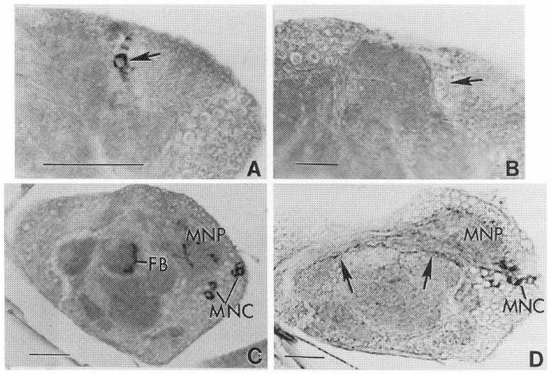


Fig. 6. Comparison of serial sections through brain of *C. salinarius* in which (**A**) lateral neurosecretory cells in the protocerebrum are labeled with CTK II antiserum (arrow), while those exposed to CTK II antiserum, preabsorbed with CTK II, (**B**) are not labeled (arrow). **C.** Midsagittal section through brain of *C. salinarius*. CTK immunoreactive neurons are present in the medial neurosecretory cell (MNC) group of the protocerebrum with immunoreactive fibers present in the fan-shaped body (FB) of the central complex and in the dorsal medial neuropil of the protocerebrum (MNP). **D.** Sagittal view of brain showing CTK immunoreactive axons in medial nerve (arrow) that sends CTK immunopositive fibers to the nervi corporis cardiaci and the circumesophageal nerve. Scale bars = 40 μm.

Due to the small number of cells in each cluster, it was not possible to trace the pathway of the neurites of these cells.

Two clusters of CTK positive neurons were located in the SG (Fig. 8 A,B). One cluster is in the anterior region of the SG near the origin of the labial nerves while the other cluster is in the posterior region of this ganglion. Each cluster contains two pairs of cells (Fig. 8B).

The largest concentration of CTK positive fibers was found terminating on the glomeruli of the antennal lobe of the deutocerebrum (Fig. 9 A,B). These fibers originate in neurons located in the third antennal segment and enter each antennal lobe via the paired antennal nerves (Fig. 9A). Relatively large amounts of CTK positive material were found in the antennal nerves and at the site of their terminals on the periphery of the antennal glomeruli (Fig. 9B).

A group of 6 to 7 CTK positive neurons was found in the anterior region of the paired tritocerebral lobes

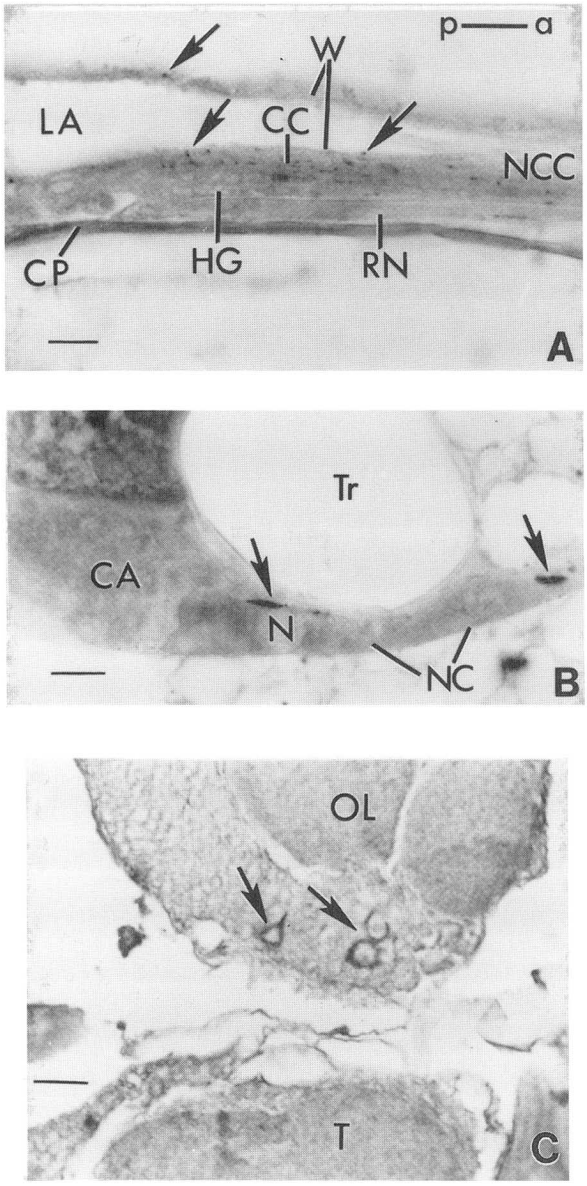


Fig. 7. Localization of CTK fibers in the retrocerebral nervous system. **A**. Immunoreactive CTK fibers enter the neurohemal region of the corpus cardiacum (CC) through the nervi corporis cardiaci (NCC) and terminate in the ventral and dorsal wall (W) of the aorta (arrows) and adjacent to neurons of the hypocerebral ganglion (HG). CP, cuticle of pharynx; LA, lumen of aorta; RN, recurrent nervi; p-a indicates posterior to anterior orientation of tissue. **B**. CTK fibers (arrows) are seen impinging upon the intrinsic neurosecretory cells (NC) of the corpus cardiacum that lie on the ventral surface of the cross trachea (Tr) located in the anterior region of the thorax. No CTK fibers were found in the corpus allatum (CA). N, nucleus of intrinsic neurosecretory cell of corpus cardiacum. **C**. A group of CTK reactive cells (arrow) was found in the medioventral region of the optic lobe (OL) at the level of the tritocerebral lobe (T). Scale bars = 20 μm.

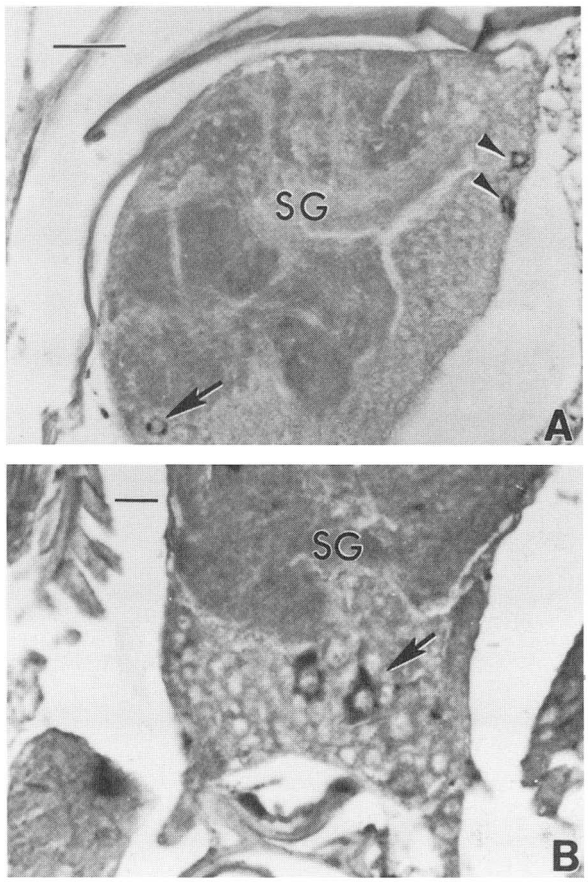


Fig. 8. A. Midsagittal section through subesophageal ganglion (SG) showing the anterior (arrow) and posterior group (arrow heads) of subesophageal CTK immunopositive cells. **B**. Transverse section through anterior pair of subesophageal cells containing CTK material (arrow) at level of exit of labial nerves to the mouthparts. Scale bars = 40 μm.

(Fig. 10A). Axons of these cells enter the paired cervical nerves (Fig. 10B) in which they extend to the fused thoracic ganglia. In addition to extrinsic CTK positive neurites from the tritocerebrum, the thoracic ganglia also contain two pair of intrinsic neurons reactive to CTK (Fig. 11A). The CTK-reactive neurons of the thoracic ganglia are located in the ventral cortex of the two anterior ganglia, the proto and meso thoracic neuromeres, designated such because the three thoracic ganglia of the adult mosquito are fused. Due to their small number, the axons of these cells could not be traced beyond the neuropil of this ganglion. A pair of CTK positive neurons is also present at the medial anterior periphery of the first abdominal ganglion (Fig. 11B). The axons of these cells enter the interganglionic nerve originating from the thoracic ganglion.

Immunohistochemical survey of the digestive tract revealed that endocrine cells in the outer (cardiac) wall of the proventriculus (Pr) contain CTK positive material

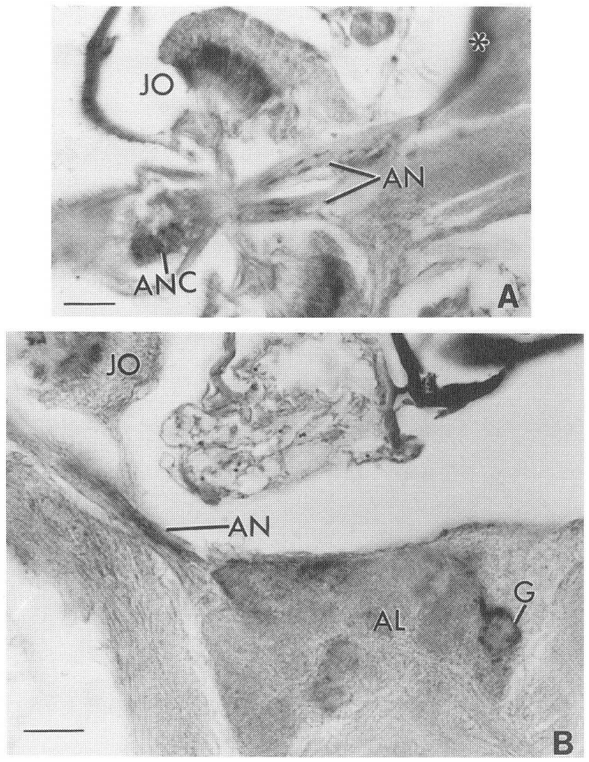


Fig. 9. Sagittal sections through the antenna and antennal lobe of *C. salinarius*. **A.** CTK immunoreactive fibers are seen arising from neurosecretory cells (ANC) in the third antennal segment and extending in twin antennal nerves (AN) through the Johnson's organ (JO) to the antennal lobe (asterisk). **B.** An antennal nerve (AN) containing large amount of CTK immunoreactive material is seen extending from the Johnson's organ (JO) to an antennal lobe (AL) where fibers of this nerve terminate around the periphery of individual glomeruli (G). Scale bars = 40 μm.

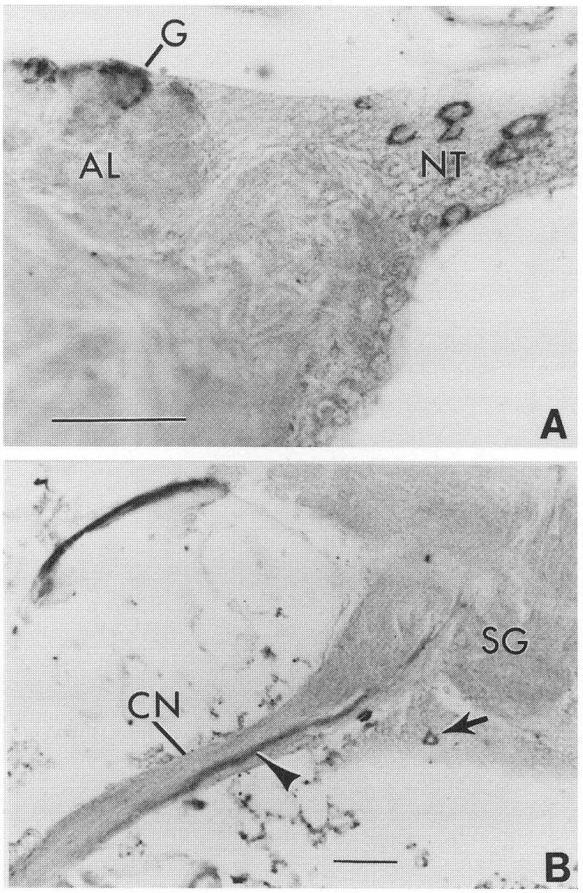


Fig. 10. A. Tangential section through the antennal and tritocerebral lobes showing the location of CTK immunoreactive neurons in the tritocerebrum (TNC) and the CTK reactive fibers from the antennal nerve terminating on the periphery of glomeruli (G) of the antennal lobe (AL). **B.** CTK immunoreactive fibers from the neurosecretory cells of the tritocerebrum extend through the subesphageal ganglion (SG) into the cervical nerve (CN) (arrow head) that extends to the fused thoracic ganglion. Also a posterior subesophageal cell (arrow) reacts immunopositive with the antiserum. Scale bars = 40 μm.

(Fig. 12A). In addition, numerous CTK positive endocrine cells were found in the anterior third of the midgut (Fig. 12B). While the soma of the CTK positive cells of the proventiculus and midgut are located in the basal region of the gut, their cytoplasm extends to the lumen of this organ into which their products are released (Fig. 12 A,B). In addition to CTK positive endocrine cells, the dorsal surface of the midgut is innervated by CTK positive fibers (Fig. 12C) that originate from CC.

DISCUSSION

This report began as an outgrowth of an earlier project (11) to isolate and characterize leucokinin-like neuropeptides from the mosquito, *C. salinarius*, and uses fractions of the same crude extract prepared in that study. In that study, purification of leucokinin-like peptides from *C. salinarius* was assessed by a dual bioassay: (a) depolarization of the isolated *A. aegypti* Malpighian tubule; and (b) stimulation of the contractions of the isolated cockroach hindgut. Tests of the C-18 fraction collected from 68–70 min (22.0–22.7% ACN) demonstrated a substantial amount of this dual activity, so that fraction was chosen for further purification. Additionally, the fractions, collected just before and immediately after 68–70 min did not contain depolarizing activity (see 6, Fig. 2), although the prior fraction did contain substantial myotropic activity. During bioassays of the fractions from the fourth column (C-1) in the series, two consecutive fractions that contain myotropic activity (no depolarizing activity) were clearly separated from the earlier eluting fractions that contained leucok-

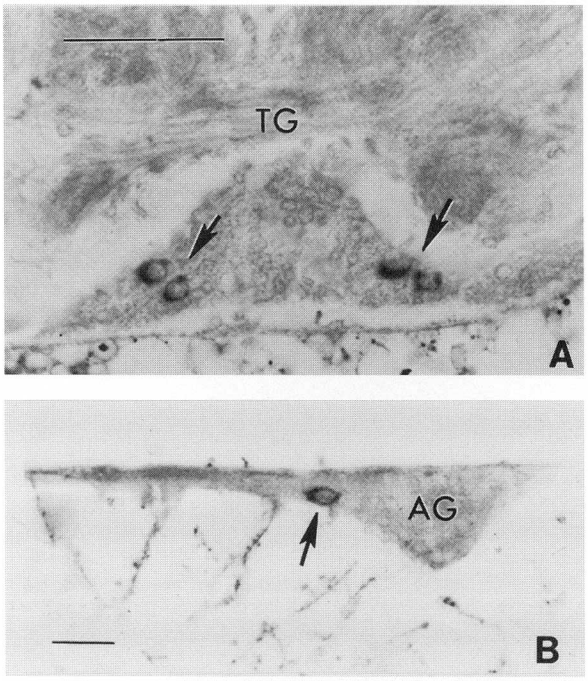


Fig. 11. Sagittal sections through the thorax and abdomen reveal the presence of immunoreactive CTK neurons in ganglia of these body segments. **A.** Two pairs of neurons (arrows) ventral to the prothoracic and mesothoracic neuropils of the thoracic ganglion (TG) contain CTK immunoreactive material. **B.** A CTK immunoreactive neuron (arrow) was found in the medial anterior region of the first abdominal ganglion (AG). Scale bars = 40 μm.

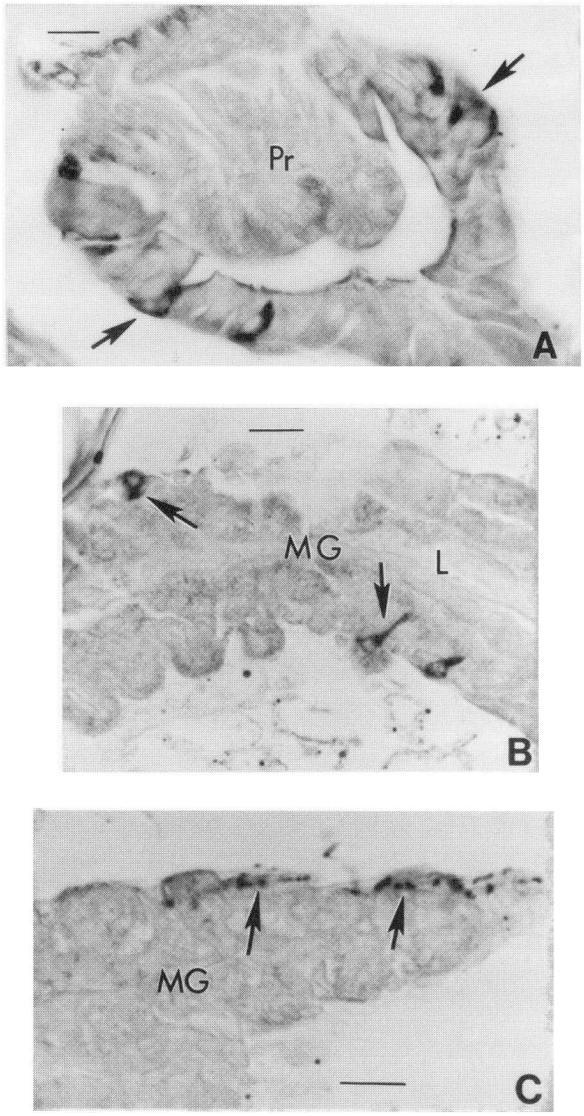


Fig. 12. Sections through the proventriculus and midgut contain CTK immunoreactive cells and neurites. **A.** Horizontal section through proventriculus (Pr) reveals CTK immunoreactive endocrine cells in the outer cardial wall of this organ (arrow). **B.** Endocrine cells containing CTK immunoreactive material (arrows) are found in the anterior third of the midgut (MG). The soma of these cells containing the nucleus lies in the basal region of the gut wall with cytoplasm of the CTK-reactive cells extending to the lumen (L) of the gut. **C.** A network of CTK immunopositive fibers (arrows) extend over the dorsal surface of the midgut (MG). Scale bars = A, 20 μm, B, 40 μm, C, 40 μm.

inin-like peptide activities. The leucokinin-like and "myotropic only" fractions were processed in parallel through the final two columns and yielded two unique peptides, NPFHSWG-NH_2 which was designated culekinin depolarizing peptide (11), and APSGFMGMR-NH_2 which was designated culetachykinin, based upon structural similarities to the locustatachykinins. Because the previous C-18 fraction also contained myotropic activity, we expanded our investigation of this fraction to determine if the same culetachykinin was present; it was not. However, the purification yielded another peptide, APYGFTGMR-NH_2, designated culetachykinin II (CTK II) (culetachykinin was consequently redesignated culetachykinin I [CTK I]).

Eighteen months later, polyclonal antibodies against CTK I and II were extracted with a competitive ELISA to determine if other members of this peptide family were present. Initially, the different fractions from the solid-phase extraction were evaluated and antigenic material was shown to be present in the 25% ACN fraction and in the 40% ACN solid-phase fraction. The 25% ACN fraction remained in the refrigerator for about 3 months before C-18 fractions were submitted to competitive ELISA analysis and three areas were positive for CTK-like antigen. Purification and structural characterization of the short-retention ELISA-positive antigen yielded the methionine oxide form of CTK II. The presence of this peptide in a much earlier fraction than the parent indicates that the peptide was oxidized

prior to fractionation on C-18. Whether the oxidation occurred in the insects or was an artifact produced during extraction cannot be determined from this result. However, the isolation and subsequent structural characterizations of CTK III (APSGFFGMR-NH_2) and its oxidized analog from the same C-18 fraction strongly suggest that oxidation occurs during purification when acetonitrile-TFA fractions are held for months, even under refrigeration. Although it was almost certainly present in the extract, the oxidation product of CTK I was not isolated because that peptide contains two methionines. Oxidation of both methionines would make the peptide substantially more polar than singly oxidized CTK II, and it would have been lost during HPLC-loading. Oxidized CTK III that was present in the extract before HPLC-fractionation would have eluted with the central ELISA-positive area that also contained large amounts of unoxidized CTK I and II. When processed through a C-4 column, CTK I and II were eluted in the expected fractions based upon ELISA and myotropic activities. No other ELISA-positive fractions were found. At this point we decided additional purification would not yield sufficient results and further purification was abandoned.

Our results indicated that oxidation of culetachykinins eliminated the bioactivity but had no influence on their interactions with an antiserum. This hypothesis was tested by comparing the oxidized and reduced forms of the culetachykinins in the hindgut bioassay and in a comparative ELISA. As can been seen in Fig. 5, the antiserum does not differentiate between oxidized and reduced forms of the peptides. However, the myotropic activity of the oxidized forms of CTK II and CTK III was completely abolished, even at pharmacological concentrations. Only the partially oxidized form of CTK I, where only one methionine is oxidized (presumably the methionine following the phenylalanine) still showed a maximal biological response. However, when both methionines were oxidized, no biological activity could be observed.

A previous study of the neuroendocrine system of *Aedes sollicitans* indicated that the neurons immunoreactive to the CTK II antiserum have a neurosecretory character (24), and therefore these peptides can be categorized as neuropeptides. The antiserum cross-reacts with all three culetachykinins, indicating it reacts with a shared epitope. Most likely the epitope that is recognized is the C-terminal Gly-Met-Arg-amide. The other common epitopes (Ala-Pro- and Gly-Phe-) are also present in locustatachykinins II, III, and IV, but are only weakly recognized by the antiserum. Because the antiserum only recognizes an epitope shared by all three culetachykinins, it will be impossible to determine if they are colocalized in the same neurons or if the localization of each culetachykinin is restricted to certain groups of neurons. Similar results were obtained in head ganglia sections of the blowfly, *C. vomitoria*, with an antiserum against locustatachykinin I that was able to recognize both blowfly callitachykinins (25,5). The localization of the culetachykinins in *C. salinarius* differs from the localization of culekinin depolarizing peptide II (18). A recent study (26) using antiserum raised against callitachykinin II found that this antiserum labeled all the neurons and midgut endocrine cells in *C. vomitoria* that reacted to the locustatachykinin I antisera.

Immunocytochemical analysis of whole body sections of *C. salinarius* with antiserum raised against CTK II revealed the presence of culetachykinins in neurons of the brain, subesophageal ganglion, thoracic ganglion and first abdominal ganglion. Culetachykinins were also found in neurons of the third antennal segment and endocrine cells of the proventiculus and midgut. Neurons reacting to CTK II antiserum were found in the medial and lateral cells of the pars intercerebralis in *C. salinarius* whereas in *C. vomitoria*, several cell clusters reactive to locustatachykinin I or callitachykinin II were found in the protocerebrum but not in the pars intercerebralis region (25,26). In addition, while CTK II-reactive nerve fibers of *C. salinarius* enter the neurohemal lobe of the corpus cardiacum and terminate in the wall of the aorta, as well as on the intrinsic cells of the corpus cardiacum, no immunoreactive axons were found in the corpora cardiaca or terminating on the wall of the aorta in *C. vomitora*, using either locustatachykinin I or callitachykinin II antisera (25,26). Thus, unlike the blowfly, *C. vomitoria*, in which most if not all of the tachykinin-like neurons appear to be interneurons, the CTK II-reactive neurons of the central nervous system of *C. salinarius* have a neurohumoral as well as a interneuronal function. CTK II fibers were also found terminating on the neurons of the hypocerebral ganglion, which lies adjacent to the neurohemal lobe of the corpus cardiacum, indicating a neurotransmitter function. The importance of any peptide effecting the cells of the hypocerebral ganglion is that this ganglion regulates digestion in insects (27).

As reported thus far for other insects, the glomeruli of the antennal lobes of *C. salinarius* are well innervated by tachykinin-like fibers. However, unlike *C. vomitoria* (25) and the locust, *Locusta migratoria* (28), the antennal lobe is not innervated in *C. salinarius* by tritocerebral neurosecretory cells, but by CTK II positive cells located in the first flagellar segment. Large amounts of CTK II can be traced from the flagellar cells to the an-

Table III. Amino Acid Sequence of Culetachykinins in Comparison With Various Tachykinin-Related Peptides Isolated From Invertebrates

Peptide name (*)	amino acid sequence	reference
Culetachykinin I	Ala-Pro-Ser-Gly-Phe-Met-Gly-Met-Arg-NH_2	(this paper)
Culetachykinin II	Ala-Pro-Tyr-Gly-Phe-Thr-Gly-Met-Arg-NH_2	(this paper)
Culetachykinin III	Ala-Pro-Ser-Gly-Phe-Phe-Gly-Met-Arg-NH_2	(this paper)
Locustatachykinin I	Gly-Pro-Ser-Gly-Phe-Tyr-Gly-Val-Arg-NH_2	(29)
Locustatachykinin II	Ala-Pro-Leu-Ser-Gly-Phe-Tyr-Gly-Val-Arg-NH_2	(29)
Locustatachykinin III	Ala-Pro-Gln-Ala-Gly-Phe-Tyr-Gly-Val-Arg-NH_2	(28)
Locustatachykinin IV	Ala-Pro-Ser-Leu-Gly-Phe-His-Gly-Val-Arg-NH_2	(28)
Locustatachykinin V	xxx-Pro-Ser-Trp-Phe-Tyr-Gly-Val-Arg-NH_2	(30)
Callitachykinin I	Ala-Pro-Thr-Ala-Phe-Tyr-Gly-Val-Arg-NH_2	(17)
Callitachykinin II	Gly-Leu-Gly-Asn-Asn-Ala-Phe-Val-Gly-Val-Arg-NH_2	(17)
Urechistachykinin I	Leu-Arg-Gln-Ser-Gln-Phe-Val-Gly-Ser-Arg-NH_2	(14)
Urechistachykinin II	Ala-Ala-Gly-Met-Gly-Phe-Phe-Gly-Ala-Arg-NH_2	(14)
Sialokinin I	Asn-Thr-Gly-Asp-Lys-Phe-Tyr-Gly-Leu-Met-NH_2	(4)
Sialokinin II	Asp-Thr-Gly-Asp-Lys-Phe-Tyr-Gly-Leu-Met-NH_2	(4)
Eledoisin	pGlu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2	(9)

(*) Origin of peptides are as follows: culetachykinin, locustatachykinin, callitachykinin, and sialokinin (insects); urechistachykinin (echiuroid worm); and eledoisin (cephalopod).
Identities between sequences are underlined, allowing a shift of one residue at the N-terminus for locustatachykinin II, III, and IV.

tennal lobe via the relatively large antennal nerves. This high degree of regulation of the antennal lobe is understandable because the antenna is the major source of sensory information in mosquitoes. For example, the Johnson's organ of the male mosquito is responsible for receiving sound produced by the female in flight, and the male yellow fever mosquitoes have sensory hairs on their antennal flagellae that respond to volatile chemicals associated with plant nectar, whereas the same type of hairs on females respond to oviposition site attractants (29,30).

C. salinarius, like *C. vomitoria*, contains ventral pairs of tachykinin positive cells in each of the thoracic neuromeres, but only one pair of CTK II cells was found in the anterior region of the first abdominal ganglion of *C. salinarius*, unlike *C. vomitoria* in which several pairs of tachykinin reactive neurons were found in the fused abdominal ganglion (25).

The gut wall of *C. salinarius* contains endocrine cells reactive to tachykinin antiserum as was found in *C. vomitoria and L. migratoria* (25,28). In addition to CTK II-reactive endocrine cells, the midgut of *C. salinarius* is also innervated by tachykinin-reactive fibers as was found to also occur in *L. migratoria*. Tachykinin-reactive fibers were not found on the midgut of *C. vomitoria*. While endocrine cells are also present in the wall of the cardial region of the proventriculus of *C. salinarius*, these cells were not found in the proventriculus of *C. vomitoria* or *L. migratoria*. This region of the proventriculus produces the peritrophic membrane that surrounds the blood meal in the adult mosquito and the food bolus in the larva of these insects. The presence of CTK-containing endocrine cells in the proventriculus of *C. salinarius* adults, and their absence in *C. vomitoria* may be due to the former being a blood-feeding adult while the latter is not. A study (31) describing localization of locustatachykinin II in the midgut of *A. aegypti* did not report the presence of tachykinin-reactive cells in the proventriculus. However, that study did find reactive endocrine cells in both the anterior and posterior region of the midgut, while CTK-reactive endocrine cells were detected only in the anterior region of the midgut of *C. salinarius*. The plentiful supply of tachykinin reactive neurons and endocrine cells in *C. salinarius* indicates that this compound plays an important role in several vital functions of these insects.

The general structure of the three peptides isolated in this study is A-P-X-G-F-X-G-M-R-amide. Based upon the C-terminal pentamer, the culetachykinins can be classified in the same peptide family as five locustatachykinins (3,4), two callitachykinins (5), and two urechistachykinins (6). The general structure of this pentamer is F-X-G-X-R-amide (Table III). Culetachykinins are closely related to locustatachykinin II, III, and IV, because they all share the N-terminal Ala-Pro-sequence, the medial Gly-Phe-sequence, and the C-terminal Gly-X-Arg-amide. The similarity of this peptide family to two other invertebrate tachykinin-like peptides is less

striking. Both the two sialokinins from the mosquito *A. aegypti* (7) and eledoisin from the cephalopod *Eledone* (2) have the same C-terminal pentamer as the vertebrate tachykinins, F-X-G-L-M-amide. However, differences in the two last amino acids can be based upon single nucleotide substitutions.

This structural characteristic might divide the tachykinin peptide family in two super groups: The Met-tachykinins and the Arg-tachykinins. The Met-tachykinins group contains all tachykinin-like peptides ending with Met-amide, as is the case with all the vertebrate tachykinins known so far. The Arg-tachykinins group contains those tachykinin-like peptides ending with Arg-amide which are found in most invertebrate tachykinins. The second residue from the C-terminus is subject to some degree of variation in both super groups. In the Met-amide super group leucine is sometimes replaced by methionine. Examples are hylambatin (32) and enterohylambatin (33). The variability of this residue in the Arg-tachykinins is even greater (Table III). Arg-tachykinins and Met-tachykinins are structurally and biologically related. Members of both super groups have a biological influence on the contractions of smooth muscles. Different Met-tachykinins have been evaluated in the hindgut bioassay and potency seems to be related to the amino acid residue following phenylalanine. Kassinin, neurokinin A, and neurokinin B (F-V-G-L-M-amide) are not active, while eledoisin and phyllomedusin (F-I-G-L-M-amide) had potencies of 8.4×10^{-8} M and 9.1×10^{-7} M, respectively. The most active Met-tachykinins are physaelamin, uperolein (F-Y-G-L-M-amide), and substance P (F-F-G-L-M-amide) with potencies, 7×10^{-9} M; 3.5×10^{-8} M; and 8×10^{-9} M respectively (34). It has also been shown that locustatachykinin II reacts with a tachykinin receptor in Drosophila. This receptor shows 38% sequence homology with mammalian tachykinin NK3 receptors. Furthermore, the effect of locustatachykinin II is specifically blocked by spantide, a broad spectrum substance P antagonist (35). However, the question whether the Arg-tachykinins are truly related to vertebrate tachykinins might be resolved when more is known about their receptors and biological interactions.

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