Callitachykinin I and II, Two Novel Myotropic Peptides Isolated From the Blowfly, *Calliphora vomitoria*, That Have Resemblances to Tachykinins

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LUNDQUIST, C. T., F. L. CLOTTENS, G. M. HOLMAN, R. NICHOLS, R. J. NACHMAN AND D. R. NÄSSEL. *Callitachykinin I and II, two novel myotropic peptides isolated from the blowfly, Calliphora vomitoria, that have resemblances to tachykinins.* PEPTIDES 15(5) 761–768, 1994.—Two peptides, related to the locust myotropic peptides locustatachykinin I-IV, were isolated from the blowfly *Calliphora vomitoria*. Whole, frozen flies were used for extraction with acidified methanol. A cockroach hindgut muscle contraction bioassay was used for monitoring fractions during subsequent purification steps. A series of eight different high performance liquid chromatography column systems was required to obtain optically pure peptides. Two peptides were isolated and their sequences determined by Edman degradation and confirmed by mass spectrometry and chemical synthesis as APTAFYGVR-NH_2 and GLGNNAFVGVR-NH_2. They were named callitachykinin I and II. The peptides have sequence similarities to the locustatachykinins and vertebrate tachykinins. Both callitachykinins were recognized by an antiserum to locustatachykinin I in enzyme-linked immunosorbent assay (ELISA) tests and callitachykinin II was additionally recognized by an antiserum to the vertebrate tachykinin kassinin, suggesting that immunolabeling of blowfly neurons with these antisera is due to neuronal callitachykinins.

Tachykinins   *Calliphora vomitoria*   Insect neuropeptide   Insect nervous system   Locustatachykinin

RECENTLY four myotropic neuropeptides with some sequence homology to vertebrate tachykinins were isolated from extracts of the brain and the corpora cardiaca–corpora allata complex of the locust *Locusta migratoria*. These peptides were named locustatachykinins I-IV [LomTK I-IV; (6,33,34)]. Together with eledoisin, a tachykinin isolated from the salivary glands of the cephalopod *Eledone* (11), urechistachykinin I and II, isolated from the echiuroid worm, *Urechis unicinctus* (19) and sialokinin I and II, isolated from the salivary glands of the mosquito *Aedes aegypti* (2), the LomTKs were to date the only identified invertebrate representatives of this large peptide family. In the locust, *Locusta migratoria*, these peptides stimulate the contraction of the oviduct and foregut (33,34). Additionally, locustatachykinins stimulate pheromonotropic activity in the pheromone glands of the moth *Bombyx mori* (13). In the blowfly several, clearly dif-

ferent, neuron populations contain material reacting with antisera raised against different tachykinins, including LomTK I (24,25,31,32). The distribution of the immunoreactive material indicates that native tachykinin-related neuropeptides may act as neurotransmitters or neuromodulators in interneuronal circuits in the blowfly central nervous system. LomTK-like immunoreactivity was, in addition, found in intrinsic endocrine cells of the blowfly midgut (25).

To establish some of the functional roles of the tachykinin-related peptides in the blowfly, it is necessary to determine the primary structures of the native neuropeptides. Here we describe the purification and isolation of two novel myotropic, tachykinin-related peptides from blowfly tissue extract. We employed the cockroach hindgut contraction bioassay, earlier used during the isolation of cockroach, locust, and cricket myotropic peptides (18),

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to monitor the blowfly peptides during purification. The two myotropic peptides isolated and characterized from *Calliphora vomitoria* extracts are also recognized in a LomTK ELISA. The novel peptides were named callitachykinin I and II.

METHOD

Animals and Tissue Extraction

Pupae of the blowfly *Calliphora vomitoria* were purchased from EKOL AB (Linköping, Sweden). After hatching the flies were collected, frozen, and stored at $-80°C$ until further processing. The rearing of the cockroach *Leucophaea maderae*, the hindgut preparation, and bioassay are described in detail elsewhere (5,15,18). A total of 3.6 kg blowflies (about 90,000 whole specimens) was used for extraction. Batches of 120 g frozen flies were extracted in 1200 ml methanol:water:acetic acid (90:9:1). The flies were homogenized in cold extraction solvent with a Polytron® homogenizer. The homogenate was centrifuged at $8000 \times g$ for 30 min at $4°C$. The supernatant was filtered through two nylon filter membranes (5 μm and 1.2 μm; Poretics Inc., Livermore, CA) placed on top of each other. The filtrate was saved. The pellet was resuspended in 1200 ml fresh extraction solvent, and centrifuged and filtered as above. The filtrates were mixed and diluted in 1% trifluoroacetic acid (TFA; Sigma, St. Louis, MO) in water to a final concentration of 4:1 of filtrate and 1% aqueous TFA and centrifuged (5500 $\times g$ for 30 min at $4°C$). After centrifugation, the supernatant was filtered through an activated and equilibrated Mega Bond Elut® C18 cartridge (60 ml, 10 g; Varian, Harbour City, CA). After an initial wash with 50 ml of a 4:1 mixture of extraction solvent and 1% aqueous TFA, the filtrate was passed through the column. The eluate was concentrated by rotary evaporation (removal of methanol) and the remainder was applied to an activated and equilibrated C18 Sep-Pak-Vac® cartridge (35 ml, 10 g; Waters/Millipore, Milford, MA). After the extract was bound to the cartridge it was washed with 100 ml 0.1% aqueous TFA in water. Subsequently, the extract was eluted with 50 ml each of 10%, 30%, 50%, and 80% acetonitrile (Merck, Darmstadt, Germany) containing 0.1% aqueous TFA. Tissue (60 g equivalents) was loaded onto the cartridge for each run for a total of 60 runs. Aliquots of the different eluates (representing 1 g of blowfly tissue) were tested for bioactivity in the *Leucophaea* hindgut contraction bioassay. The acetonitrile of the different fractions was removed by rotary evaporation and the remainder was freeze-dried until further use.

Purification and Isolation Procedures

Isolation was performed on a Waters ALC 100 high performance liquid chromatograph equipped with two pumps, a solvent programmer, a Rheodyne septumless injector, and a variable wavelength detector. The peptide material was sequentially eluted through eight different column systems. Aliquots, representing 1–5% of each fraction, were tested on the hindgut contraction bioassay and the fractions containing myotropic activity were further processed through the next column. The columns and the operating conditions were:

(a) Waters Delta Pak C18, 300 Å, 15 μm, 25 × 100 mm (Waters Assoc., Milford, MA). Solvent A: 0.1% TFA in water; solvent B: 50% acetonitrile in 0.1% aqueous TFA. Conditions: a linear gradient from 0–100% B over 150 min; flow rate 7.5 ml/min; 15-ml fractions were collected every 2 min; detector set at 2.0 absorption units full scale (AUFS) at 214 nm.

(b) Waters Delta Pak C4, 300 Å, 15 μm, 25 × 100 mm (Waters). Conditions as for (a).

(c) Vydac Phenyl, 300 Å, 5 μm, 10 × 250 mm (Phenomenex, Torrance, CA). Solvents and gradient as for (a); flow rate 2 ml/min; 4-ml fractions were collected every 2 min; detector set at 4.0 AUFS at 214 nm.

(d) Waters Nova Pak C8, 100 Å, 4 μm, 8 × 100 mm (Waters). Solvents and gradient as for (a); flow rate 1.5 ml/min; 3-ml fractions were collected every 2 min; detector set at 2.0 AUFS at 214 nm.

(e) Vydac Diphenyl, 100 Å, 4.6 × 250 mm (Phenomenex). Conditions as for (a).

(f) Spherex C18 3-ODS, 4.6 × 150 mm (Phenomenex). Solvents as in (a); flow rate 1.0 ml/min; peaks collected manually; detector set at 0.5 AUFS at 214 nm.

(g) I-125 Waters Protein Pak, 7.8 × 300 mm (Waters). Solvent A: 95% acetonitrile in 0.01% aqueous TFA; solvent B: 50% acetonitrile in 0.01% aqueous TFA. Conditions: 100% A for 8 min, then a linear gradient of 0–100% B over 80 min; flow rate 1.5 ml/min; peaks collected manually; detector set at 0.2 AUFS at 214 nm.

(h) The last purification step was performed on an Applied Biosystems Microbore HPLC, consisting of a 140B solvent delivery system and a 785A programmable absorbance detector. It was equipped with a Progel®-TSK C18 NPR column, 4.6 × 35 mm (Supelco, Bellefonte, PA). Solvents: same as for (a). Conditions: 100% A for 3 min, then a linear gradient of 0–100% B over 50 min; flow rate 100 μl/min; peaks were collected manually; detector set at 0.1 AUFS at 214 nm.

Enzymatic Degradation

Dried aliquots of 1–3% of each isolated peptide were taken up in 200 μl cockroach saline and incubated with one unit of immobilized aminopeptidase M gel suspension (Pierce, Rockford, IL). As a control, an identical set of peptides was taken up in saline only. The mixtures were shaken in a $37°C$ water bath for 2 h. The gel was removed from the suspension by centrifugation and the supernatant was transferred directly to the hindgut bioassay chamber.

Amino Acid Sequence Analysis, Peptide Synthesis, and Mass Spectrometry

The amino acid sequence of the two isolated peptides was determined with an Applied Biosystems model 473A automated protein sequencer with on-line detection of PTH-amino acids using standard operating conditions. Approximately 100 pmol of each peptide was used for the analysis. The masses of the natural peptides were determined using laser desorption mass spectrometry on a Vestec model 2000 laser desorption time-of-flight mass spectrometer. Samples were run in a positive mode using α-cyano-4-hydroxycinnamic acid as the matrix. Mass accuracy is generally 0.05% or better. Calculated average masses were determined using the computer program PROCOMP version 1.2. This program was developed by P. C. Andrews, PhD, University of Michigan, for peptide data manipulations on IBM-compatible computers.

Based on the structural information obtained from the sequence analysis, the two peptides were synthesized by the solid-phase peptide synthesis on a Vega coupler 250 automated peptide synthesizer using Fmoc chemistry with diisopropylcarbodiimide as the coupling reagent and dimethylformamide as solvent. The structural identity of the synthetic peptides was confirmed by the presence of the molecular ions (MH⁺) in the mass spectra

taken on a VG-70-250 EHF spectrometer (VG Analytical, Manchester, UK) according to previously described procedures (28). The synthetic peptides were also analyzed by amino acid composition (Table 4) as described earlier (27).

To compare the retention times of the synthetic peptides with the respective natural peptides, a mixture of an equal amount of both synthetic and natural peptides were co-chromatographed on the following analytical HPLC columns:

(a) BioSep Sec-S2000, 7.8 × 300 mm (Phenomenex). Solvent A: 95% acetonitrile in 0.01% aqueous TFA; solvent B: 50% acetonitrile in 0.01% aqueous TFA. Conditions: 100% A for 8 min, then a linear gradient 0–100% B over 80 min; flow rate 1.5 ml/min; detector set at 0.1 AUFS at 220 nm.

(b) Vydac Diphenyl, 100 Å, 4.6 × 250 mm (Phenomenex). Solvent A: 0.1% aqueous TFA; solvent B: 60% acetonitrile in 0.1% aqueous TFA. Conditions: a linear gradient from 0–100% B over 120 min; flow rate 1.5 ml/min; detector set at 0.1 AUFS at 220 nm.

(c) Spherex C18 3-ODS, 4.6 × 150 mm (Phenomenex). Same solvents and conditions as (b); flow rate 1.0 ml/min.

(d) Deltabond C8, 5 μm, 4.6 × 250 mm (Keystone, Bellefonte, PA). Same solvents and conditions as (b).

(e) Progel®-TSK C18 NPR, 4.6 × 35 mm (Supelco). Same solvents and conditions as described above.

Threshold Concentrations

The quantity of synthetic peptide was calculated from the value obtained for the phenylalanine in the amino acid analysis. The quantity of natural peptide was calculated from a standard series of known quantities of synthetic peptide on a Vydac Diphenyl column with conditions described for the control of the synthetic peptides. Threshold concentrations were determined by adding known quantities of synthetic and natural peptide to the bioassay chamber containing the Leucophaea hindgut. The threshold concentration is defined as that concentration of peptide that is required to evoke an observable change in frequency, amplitude, or tonus of the spontaneous contractions within 1 min. For each peptide the respectively natural and synthetic peptides were tested on the same hindgut preparation. The threshold was determined from five different hindgut preparations.

Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) was used qualitatively to test whether the isolated peptides reacted with either of two different tachykinin antisera: one to the frog tachykinin kassinin (36) and one to locustatachykinin I [LomTK I; (25,29)]. The ELISA procedure, using these antisera, is described elsewhere (25). Briefly, the tests were performed as noncompetitive ELISAs with the LomTK I antiserum diluted 1:2000 and the kassinin antiserum diluted 1:500. One percent aliquots of the purified peptides were coated to the plate (polystyrene MaxiSorp microtiter plates; Nunc, Denmark) and assayed using both antisera. Furthermore, 5% aliquots of all remaining fractions from the first HPLC column (Delta Pak C18) were also checked in the ELISAs for immunoreactivity.

Finally, an ELISA with a dilution series of the antiserum was run on the synthetic peptides. In this case, the LomTK I antiserum was diluted from 1:1000 up to 1:100,000 with 10 pmol of the peptide coated to the plate. The kassinin antiserum was diluted from 1:500 up to 1:50,000 with 100 pmol peptide coated. Each test was performed in duplicate.

RESULTS

The different acetonitrile fractions that eluted from the Sep-Pak cartridges during the initial extraction were tested for myotropic bioactivity on the cockroach hindgut. Stimulatory effect was detected exclusively in the 30% acetonitrile fraction. The spontaneous activity was reestablished after rinsing the incubation chamber. The 10% and 50% fractions showed inhibitory effects on the spontaneous activity of the hindgut. The 30% fraction was taken for further purification. The subsequent separation and purification required eight different HPLC column systems. For each system, aliquots of 1–5% of the collected fractions were screened for myotropic activity on the cockroach hindgut. The 30% acetonitrile fraction, representing a total of 3.6 kg blowfly extract, was divided into eight sets and run separately on the first column. Myotropic bioactivity was detected in two regions with retention times of 58–61 and 62–65 min, respectively. The material eluting in these time ranges was further processed separately through all the other columns. The chromatograms of the first five preparative column systems yielded a multitude of UV absorbing peaks (data not shown). The elution times for the myotropic material in each column system are given in Table 1. The chromatograms of the last three column systems are shown in Fig. 1.

On the final column (Progel TSK C18), two pure peptides eluted at 13.5 and 15.0 min, respectively. To determine whether these peptides were N-terminally blocked or not, aliquots were treated with the peptide degradation enzyme aminopeptidase M for 2 h at 37°C. The bioactivity was measured in the hindgut bioassay. No activity could be found for either of the two peptides incubated with the enzyme. As a control, aliquots of the peptides were incubated in saline for 2 h at 37°C. No loss of the myotropic bioactivity was found in the control conditions.

Aliquots of both peptides, representing approximately 100 pmol, were sequentially degraded with the gas-phase sequencer. The estimation of the aliquots of both peptides was based on the absorption characteristics of 100 pmol of a presumed similar synthetic peptide (LomTK I) applied on the final column (Progel TSK C18), used for the purification of the callitachykinins.

TABLE 1

ELUTION TIMES OF MYOTROPIC MATERIAL
DURING PURIFICATION

Column	Retention Time (min)	
	Callitachykinin I	Callitachykinin II
Delta Pak C18	58–61	62–65
Delta Pak C4	56–57	58–59
Vydac Phenyl	50–51	52–55
Nova Pak C8	52–53	54–59
Vydac Diphenyl	38–39	40–45
Spherex C18	53.2	56.7
I-125 Protein Pak	39.6	43.4
Progel™ TSK C18	13.5	15.0

The table shows the retention times of the myotropic peptide material as it eluted on the different columns on HPLC. On the first column, the myotropic material eluted in two zones with retention times of 58–61 min and 62–65 min, respectively. The retention times of the myotropic material, during the different purification steps, can be followed by reading vertically. UV profiles from the three last column systems are illustrated in Fig. 1.
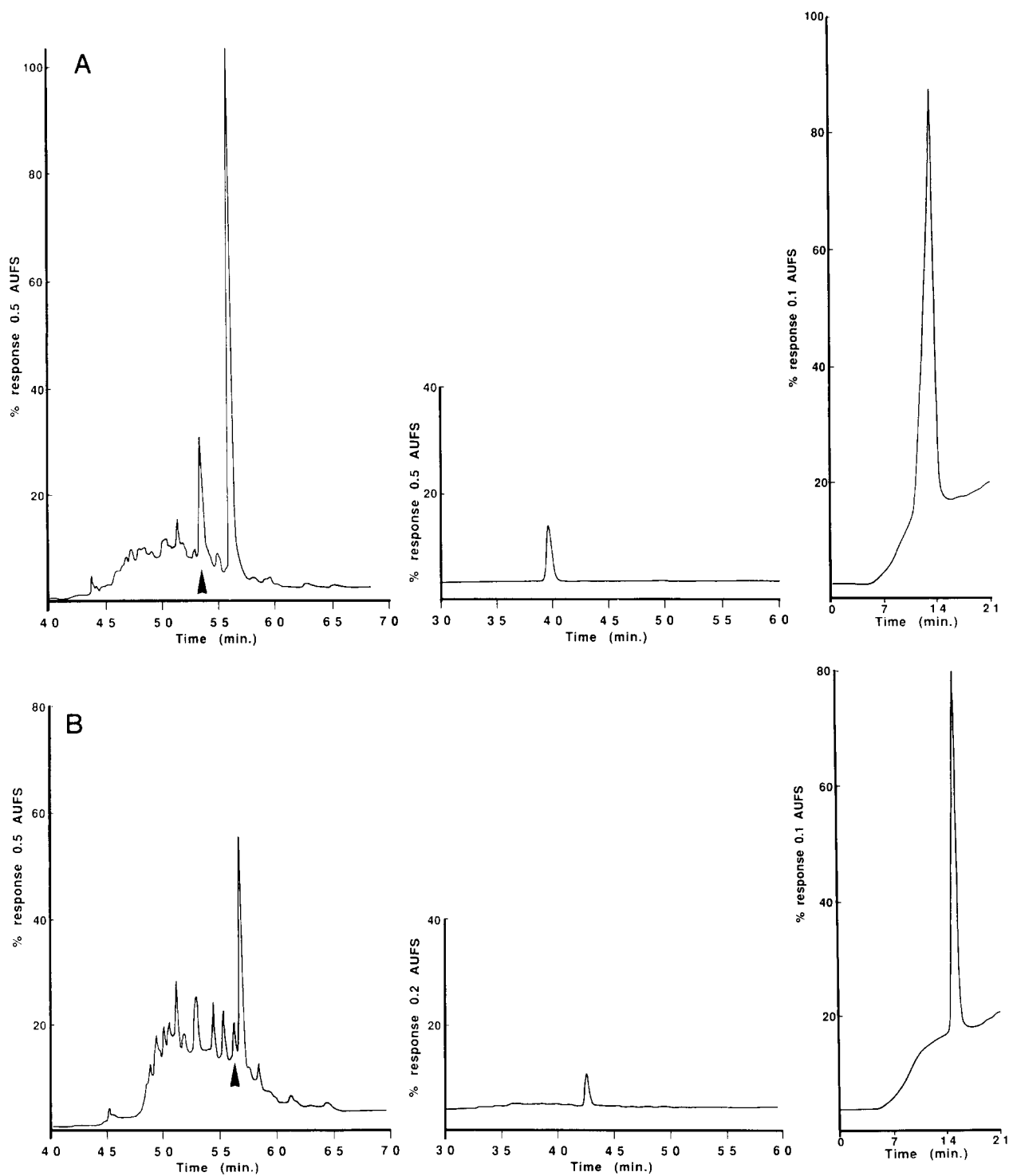
FIG. 1. Separation and purification of callitachykinin I (A) and II (B). The UV profiles represent the three last HPLC column systems: Spherex C18, I-125 Protein Pak, and Progel TSK C18 microbore. Peaks were collected manually and aliquots were tested in the hindgut contraction assay. The x-axes represent the retention times in minutes and the y-axes the percent UV response at different absorption units full scale (AUFS), as measured at 214 nm. Callitachykinin I eluted at 53.2 min (indicated by arrow) on the Spherex column and the material was pure on the two following columns (final column: 13.5 min). Callitachykinin II eluted at 56.7 min on the Spherex column and the material was pure on the two following columns (final column: 15.0 min).

TABLE 2

AUTOMATED EDMAN SEQUENCING: AMINO ACID YIELDS OF
NATURAL CALLITACHYKININ I AND II* AT EACH STEP

Cycle Number	Amino Acid Residue Assignment	Amount (pmol)
Callitachykinin I		
1	A (Ala)	143
2	P (Pro)	75
3	T (Thr)	41
4	A (Ala)	83
5	F (Phe)	75
6	Y (Tyr)	75
7	G (Gly)	68
8	V (Val)	79
9	R (Arg)	38
Callitachykinin II		
1	G (Gly)	17.5
2	L (Leu)	13.6
3	G (Gly)	14.1
4	N (Asn)	11.8
5	N (Asn)	10.1
6	A (Ala)	7.1
7	F (Phe)	4.8
8	V (Val)	1.7
9	G (Gly)	5.9
10	V (Val)	1.2
11	R (Arg)	1.7

* Twenty percent of the sample (approximately 20 pmol) was used for sequence analysis of callitachykinin II.

HPLC analysis of the PTH-amino acid generated in each cycle yielded the following primary sequences: 1—(peptide eluting at 13.5 min on Progel TSK C18 column) Ala-Pro-Thr-Ala-Phe-Tyr-Gly-Val-Arg, and 2—(peptide eluting at 15.0 min) Gly-Leu-Gly-Asn-Asn-Ala-Phe-Val-Gly-Val-Arg. No other amino acids were detected during sequencing. The amino acid yields of the sequencing are listed in Table 2. The peptides were assumed to be C-terminally amidated because locustatachykinins and ure-chistachykinins are C-terminally admidated. The amino acid sequences were confirmed by mass spectrometry (Table 3). The

TABLE 3

MASS SPECTROMETRY OF NATURAL AND SYNTHETIC
CALLITACHYKININ I AND II

Peptide	Calculated Mass for (MH+)* (g/mol)	Observed Molecular Ion (MH+)† Natural	Observed Molecular Ion (MH+)† Synthetic
Callitachykinin I	981.1	982.4	980.8
Callitachykinin II	1103.3	1104.4	1103.2

* The calculated molecular mass represents the theoretical value and includes an amidated C-terminus (NH₂). The molecular weights are based on monoisotopic amino acids.

† The natural-occurring peptides were analyzed by laser desorption mass spectrometry and the synthetic peptide masses were determined by fast atom bombardment mass spectrometry. Positively charged molecular ions were analyzed. The observed weights correspond to the molecular weight plus one mass unit for hydrogen.

TABLE 4

AMINO ACID COMPOSITION ANALYSIS OF SYNTHETIC
CALLITACHYKININ I AND II

Callitachykinin I (APTAFYGVR)	Callitachykinin II (GLGNNAFVGVR)
Gly: 6.2 nmol = 1 × G	Asx: 5.7 nmol = 2 × N or D
Arg: 6.0 nmol = 1 × R	Gly: 9.7 nmol = 3 × G
Thr: 5.3 nmol = 1 × T	Arg: 2.9 nmol = 1 × R
Ala: 10.7 nmol = 2 × A	Ala: 3.0 nmol = 1 × A
Pro: 6.0 nmol = 1 × P	Val: 5.6 nmol = 2 × V
Tyr: 6.5 nmol = 1 × Y	Leu: 3.0 nmol = 1 × L
Val: 5.3 nmol = 1 × V	Phe: 2.9 nmol = 1 × F
Phe: 6.0 nmol = 1 × F	

two peptides are named callitachykinin I and II (referring to the genus name *Calliphora*) in accordance with other identified invertebrate tachykinin-like peptides.

Synthetic callitachykinin I and II were compared to the natural peptides. Co-chromatography of an equal mixture of natural and synthetic peptides on five different HPLC columns revealed that they both eluted in one single peak on each column system. This indicates that, for both callitachykinin I and II, the structures of the natural and amidated synthetic peptides are identical (including a C-terminal amidation of the natural peptides). The masses of the synthetic peptides, as revealed by mass spectrometry, are shown in Table 3. The calculated masses (MH+) of the peptides are based on the molecular weight of the monoisotopic forms of amino acids; callitachykinin I ($C_{46}H_{70}N_{13}O_{11}$) 981.1 g/mol, and callitachykinin II ($C_{48}H_{80}N_{17}O_{13}$) 1103.3 g/mol.

For both peptides the threshold concentration to cause an increase in the spontaneous contraction of the *Leucophaea* hindgut was determined (Table 5 and Fig. 2). Threshold concentrations for each natural peptide and their respective synthetic analogue were nearly identical. This also indicates that the natural peptides are C-terminally amidated, because the synthetic peptides were also. The amount of natural product obtained for each peptide was determined. For callitachykinin I a total of 3.499 nmol was recovered after purification out of a total of 90,000 flies. For callitachykinin II a total of 1.837 nmol was
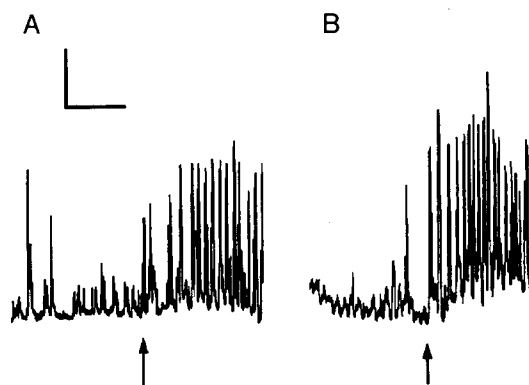

FIG. 2. Typical response of the isolated hindgut of the cockroach *Leucophaea maderae* to (A) 6.5 × 10⁻¹⁰ M of callitachykinin II natural product, and (B) 6.5 × 10⁻¹⁰ M of callitachykinin II synthetic product. The arrows indicate addition of peptide. Vertical calibration = 500 mmHg displacement; horisontal time mark = 1 min.

TABLE 5

THRESHOLD CONCENTRATIONS OF NATURAL
AND SYNTHETIC CALLITACHYKININ I AND II
IN THE *Leucophaea* HINDGUT ASSAY

Peptide	Threshold Concentration ($\bar{X} \pm SD$, $n = 5$)
Callitachykinin I	
Natural peptide	$1.19 \pm 0.41 \times 10^{-9}\ M$
Synthetic peptide	$1.27 \pm 1.04 \times 10^{-9}\ M$
Callitachykinin II	
Natural peptide	$3.60 \pm 2.68 \times 10^{-10}\ M$
Synthetic peptide	$3.72 \pm 2.73 \times 10^{-10}\ M$

recovered. Therefore, each fly contains 38.9 fmol or 38.2 pg of callitachykinin I and 20.4 fmol or 22.5 pg callitachykinin II.

Aliquots of natural callitachykinin I and II were tested in ELISA with an antiserum to locustatachykinin I (LomTK I). Both peptides reacted with a maximum response. In the kassinin ELISA, only callitachykinin II gave a positive immunoreaction under the described conditions. Aliquots from all remaining fractions from the first three HPLC column systems (excluding fractions containing the bioactive material) were also tested in the LomTK I and kassinin ELISAs. No immunoreaction was detected. The synthetic callitachykinin I and II react very similar to LomTK I in the ELISA with the LomTK I antiserum. In our test system, only callitachykinin II was recognized by the kassinin antiserum (Fig. 3).

DISCUSSION

In the present study we have described the purification and isolation of two novel myotropic peptides from the blowfly *Calliphora vomitoria*. By a heterologous muscle contraction bioassay, using the hindgut of the cockroach *Leucophaea maderae* (16), myotropic peptide material was detected and followed through a series of different HPLC column systems until chromatographically pure peptides were obtained. The two peptides were named callitachykinin I and II. In addition to the bioassay, the isolated peptides were also analyzed in ELISA with antisera to the insect tachykinin-related peptide LomTK I and the frog tachykinin kassinin. Both isolated peptides were immunopositive in the LomTK ELISA and one of them in the kassinin ELISA.

The two callitachykinins have the C-terminus in common: A-F-X-G-V-R-amide (where X represents Y or V). The N-terminus, however, differs significantly (Table 6). The C-terminus portion, F-X-G-V-R-amide, is also identical to the locustatachykinins (33,34), indicating their relationship. The degree of homology between callitachykinin I and locustatachykinin I is 67%. The recently identified invertebrate peptides urechistachykinin I and II (19) also display some degree of sequence identity to the callitachykinins and the locustatachykinins by the F-X_1-G-X_2-R-amide in the C-terminus. As seen in Table 6, the similarities of the blowfly peptides to the vertebrate and cephalopod tachykinins and the mosquito sialokinins are less striking because the latter all have an F-X-G-L-M-amide C-terminal (X is either a Y, V, I, or an F). However, an interesting observation is that callitachykinin I shares the sequence F-Y-G with uperolein, scyliorhinin, and the sialokinins, and callitachykinin II the sequence F-V-G with kassinin and neurokinin A (Table 6). Further support for the relation between the locustatachykinins, callitachykinins, and vertebrate tachykinins is that in several positions single nucleotide substitutions in the codons would yield peptides with additional homologous amino acids. Several neuropeptides seem to have the position 4 (counted from the C-terminus) variable and the other C-terminal amino acids constant. This composition is also seen in the insect myokinins, like leucokinins, which have a C-terminal core of F-X-S-W-G-amide (16,17) and the allatostatins with the C-terminus Y-X-F-G-L-amide (7,8).

It could not be conclusively determined, at this stage of the investigation, whether the isolated peptides are neuropeptides or not, because an extract of whole flies was used for the isolation. However, the fact that the isolated peptides were recognized by the locustatachykinin and kassinin antisera in ELISA tests gives some clues about the distribution, because the same antisera recognize interneurons in the CNS and endocrine cells in the midgut of the blowfly in immunocytochemical experiments (24,25). Based on immunocytochemistry and immunochemistry, it was suggested that in the blowfly nervous system there are at least two tachykinin-related peptides, one resembling locustatachykinins and one sharing an epitope with kassinin (24,25). This could be confirmed in the present study in ELISAs of the natural and synthetic callitachykinins I and II. Immunocytochemical studies indicated that the two *Calliphora* peptides may be colocalized in the blowfly central nervous system (25), but cross-reactivity could not be ruled out. When region-specific antisera to callitachykinin I and II have been produced, it will be possible to more accurately determine the peptide localization
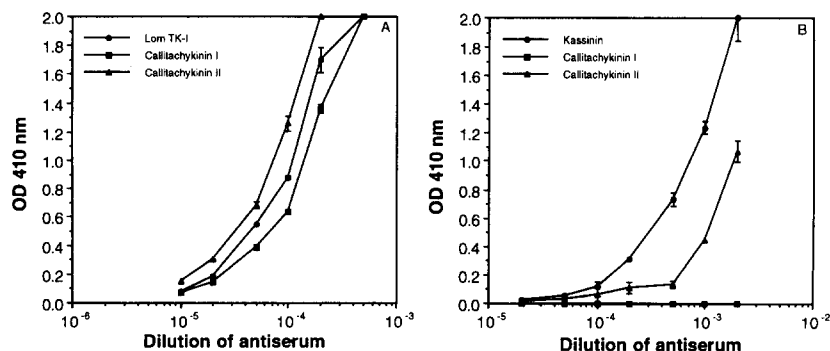

FIG. 3. ELISA tests on synthetic callitachykinin I and II. (A) Dilution series of LomTK I antiserum with 10 pmol peptide coated to the plate. (B) Dilution series of kassinin antiserum with 100 pmol peptide coated to the plate. Each test was performed in duplicate. The bars represent deviation from mean OD values.

TABLE 6

AMINO ACID SEQUENCES OF CALLITACHYKININ I AND II IN COMPARISON TO VARIOUS TACHYKININS AND TACHYKININ-RELATED PEPTIDES

Peptide Name*	Amino Acid Sequence†	Reference
Callitachykinin I	A-P-T-A-F-Y-G-V-R	This paper
Callitachykinin II	G-L-G-N-N-A-F-V-G-V-R	This paper
Locustatachykinin I	G-P-S-G-F-Y-G-V-R	33
Locustatachykinin II	A-P-L-S-G-F-Y-G-V-R	33
Locustatachykinin III	A-P-Q-A-G-F-Y-G-V-R	34
Locustatachykinin IV	A-P-S-L-G-F-H-G-V-R	34
Urechistachykinin I	L-A-QI-S-Q-F-V-G-S-R	19
Urechistachykinin II	A-A-G-M-G-F-F-G-A-R	19
Sialokinin I	N-T-G-D-K-F-Y-G-L-M	2
Sialokinin II	D-T-G-D-K-F-Y-G-L-M	2
Substance P	R-P-K-P-Q-Q-F-F-G-L-M	4
Callitachykinin I in comparison to similar peptides		
Callitachykinin I	A-P-T-A-F-Y-G-V-R	This paper
Locustatachykinin I	G-P-S-G-F-Y-G-V-R	33
Sialokinin I	N-T-G-D-K-F-Y-G-L-M	2
Uperolein	pE-P-D-P-N-A-F-Y-G-L-M	12
Scyliorhinin I	A-K-F-D-K-F-Y-G-L-M	5
Eledoisin	pE-P-S-K-D-A-F-I-G-L-M	11
Callitachykinin II in comparison to similar peptides		
Callitachykinin II	G-L-G-N-N-A-F-V-G-V-R	This paper
Uperolein	pE-P-D-P-N-A-F-Y-G-L-M	10
Kassinin	D-V-P-K-S-D-Q-F-V-G-L-M	1
Neurokinin A	H-K-T-D-S-F-V-G-L-M	21
Neurokinin B	D-M-H-D-F-V-G-L-M	20
Locustatachykinin I	G-P-S-G-F-Y-G-V-R	33
Urechistachykinin I	L-A-Q-S-Q-F-V-G-S-R	19

* Origin of peptides is as follows: eledoisin (cephalopod), kassinin and uperolein (amphibians), locustatachykinins and sialokinins (insects), neurokinins A and B, and substance P (mammals), scyliorhinin I (elasmobranch), urechistachykinins (echiuroid worm).

† All peptides are C-terminally amidated.

by immunocytochemistry and to corroborate possible colocalization.

The question whether the different peptides of the tachykinin family of each species have different functions and activate different receptor types or not is highly interesting. Studies of the mammalian nervous system have indicated the presence of sev-

eral forms of tachykinin receptors and a multiplicity in tachykinin signaling (14,22). In another dipteran insect, *Drosophila*, two different putative tachykinin receptors have been demonstrated by recombinant DNA technique (23,26), suggesting that insects also operate with complex tachykinin-like systems. The *Drosophila* tachykinin receptor (NKD) cloned by Monnier and coworkers (26) is a G-protein receptor complex displaying a 38% homology with a mammalian tachykinin receptor and responds specifically to locustatachykinin II when expressed.

Immunocytochemical studies of the blowfly and *Drosophila* have indicated that antisera to substance P recognize a population of neurons in the brain and ventral ganglia that is distinctly different from that detected with antisera to locustatachykinin I and kassinin (31,32). The recent discovery of the sialokinins I and II from salivary glands of the mosquito *Aedes aegypti* (2) confirms the existence of insect peptides with the C-terminus structure F-X-G-L-M-amide, characteristic of vertebrate tachykinins (Table 6). The neuronal localization of sialokinins has not yet been determined, but future work may answer the question whether the insect nervous system is utilizing two classes of tachykinin-like peptides: those related to locustatachykinins and callitachykinins, and others with a C-terminus, like the vertebrate tachykinins and the sialokinins.

In the blowfly, *C. vomitoria*, we now know the amino acid sequences of peptides representing three different families: FMRFamide-related peptides (8), allatostatins (9), and the tachykinins shown in the present investigation. At least 20 different peptide families have been indicated in various insects (18,29,35), and it is likely that members of several additional families will be isolated from *C. vomitoria* in the future.

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