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Identification of a New Tachykinin from the Midgut of the Desert Locust, *Schistocerca gregaria,* by ESI-Qq-oa-TOF Mass Spectrometry

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This paper reports the purification of a tachykinin isoform from the midgut of the desert locust, *Schistocerca gregaria*. One hundred locust midguts were extracted in an acidified methanolic solvent, after which three HPLC column systems were used to obtain a pure peptide. A tachykinin immunoassay was used to monitor all collected fractions. After each purification step the purity of the sample was monitored by MALDI-TOF mass spectrometry. The pure peptide was sequenced by ESI-Qq-oa-TOF mass spectrometry. Edman degradation-based automated microsequencing and chemical synthesis confirmed the sequences. The midgut peptide, GNTKKAVPGFYGTRamide (Scg-midgut-TK), belongs to the tachykinin family with identified members in all vertebrate phyla and some invertebrate phyla: arthropods, annelids and molluscs. Scg-midgut-TK is the first tachykinin purified from midguts of the desert locust *Schistocerca gregaria*. In comparison to locust brain tachykinins, the midgut tachykinin is N-terminally extended. Similar to neuropeptide γ, an N-terminally extended mammalian tachykinin, first isolated from rabbit intestine, the present identified locust intestinal tachykinin contains a putative dibasic cleavage site.

Tachykinins constitute a large and widespread family of peptides with a vast range of actions in the nervous system and other tissues of vertebrates, including the gastrointestinal tract (1). These peptides and their respective receptors have been implicated in a number of mammalian motor and sensory functions (2, 3). Substance P is the most well known tachykinin. It was isolated in 1931 and used for experimental purposes (4) but its sequence was determined 40 years later (5). In fact, the first tachykinin to be purified and sequenced, eledoisin, was derived from an invertebrate, the cephalopod *Eledone moshata* (6).

Antisera against substance P were used for immunocytochemical staining of insect nervous tissue, but the chemical structure of these peptides was not known (7). Finally, five isoforms of locustatachykinins (Lom-TKs) were isolated from brains of *Locusta migratoria* by screening chromatographic fractions in a cockroach hindgut contraction assay (8–11). Locustatachykinins have a well-conserved C-terminus, Phe-Xaa_1-Gly-Xaa_2-Arg-NH_2 (where Xaa_1 and Xaa_2 are variable). They have a Phe residue at position 5 from the C-terminus and the Gly residue at position 3 from the C-terminus is likely to form a type I β-turn supported by an intramolecular hydrogen bond between the Tyr or His carbonyl oxygen and the C-terminal amide proton (12). Since 1990, more brain tachykinins were isolated from animals belonging to other invertebrate classes and/or phyla: crustaceans (arthropods), mollusks and annelids (13, 14). Invertebrate tachykinins have a variety of actions in vitro: they are myostimulatory on insect visceral and skeletal muscle, they induce release of adipokinetic hormone from locust corpora cardiaca, depolarize identified locust neurons and induce pheromone biosynthesis in the moth, *Bombyx mori* (15). The myotropic and depolarizing activity can be blocked by spantide 1, a broad-spectrum substance P antagonist and by verapamil, a calcium-channel blocker (16). The recent localization of a tachykinin-receptor-like protein in the central nervous system of *Locusta migratoria* suggests the existence of additional roles for tachykinins (17). Immunocytochemical mapping indicated that in locusts, tachykinin immunoreactivity is present mostly in interneurons of the brain and in endocrine cells of the midgut (14).

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In this paper, we describe the purification and identification of a midgut tachykinin of *Schistocerca gregaria*, namely Scg-midgut-TK. The peptide was identified by one of the latest developments in mass spectrometry, i.e. nanoflow ElectroSpray Ionisation (ESI) double quadrupole (Qq) orthogonal acceleration (oa) Time of Flight (ESI-Qq-oa-TOF) mass spectrometry, which is sensitive in the lower femtomole range.

MATERIALS AND METHODS

Animals, tissue extraction, and purification. *Schistocerca gregaria* was raised under laboratory conditions (18). Midguts (100) from 12–14 day adults were dissected and immediately placed in an ice-cold methanol/water/acetic acid (90:9:1) solution. The guts were sonicated and centrifuged for 30 min (10,000 *g;* 4°C). The supernatant was dried in siliconized round bottom flasks. Subsequently, it was dissolved in aqueous trifluoracetic acid (TFA) (0.1%) and prepurified on MegabondElute C18 cartridges (10 g/cartridge) (Varian, Harbor City, CA), which had been activated with CH_3CN/H_2O/TFA (80:19.9:0.1) and then rinsed with aqueous 0.1% TFA. The cartridges were eluted with 25 ml of 50% and 80% CH_3CN in 0.1% aqueous TFA. Columns and operating conditions for high performance liquid chromatography on a Gilson HPLC system with variable wavelength detector (214 nm) were: (1) Bondapak C18 column (25 × 100 mm) (Waters Associates, Milford, MA), solvent A: 0.1% TFA in water; solvent B: 50% CH_3CN in 0.1% aqueous TFA. Column conditions: 100% A followed by a linear gradient to 40% B in 65 min subsequently followed by a linear gradient to 100% B in 30 min; flow rate: 6 ml/min; detector range: 1 absorption unit full scale (Aufs); (2) Spherisorb 5S Phenyl column (4.6 × 250 mm) (Waters Associates, Milford, MA), solvent A: 0.1% TFA in water; solvent B: 100% CH_3CN in 0.1% aqueous TFA. Column conditions: 100% A for 20 min, followed by a linear gradient to 65% B in 65 min; flow rate: 1 ml/min; detector range: 0.2 Aufs; (3) Biosep-Sec-S-2000 size exclusion column (300 × 7.8 mm) (Phenomenex, Torrance, CA, USA), solvent A: 95% CH_3CN in 0.01% aqueous TFA; solvent B: 0.01% aqueous TFA. Column conditions: 100% A followed by a linear gradient to 100% B in 100 min; flow rate: 1 ml/min; detector range: 0.2 Aufs.

Mass spectrometry and peptide sequencing. A concentrated sample containing 1/40th (0.5–1 pmol/μl) of the active peak was subjected to MALDI-TOF analysis (19). One μl was mixed with 1 μl of a 50 mM solution of α-cyano-4-hydroxycinnamic acid in CH_3CN/EtOH/TFA (50:49.9:0.1) and applied on the multi sample target. This mixture was air-dried and the target was then introduced in the instrument, a VG Tofspec SE (Micromass, UK) equipped with a N_2-laser (337 nm). The samples were measured either in the linear (acceleration voltage 25 kV) or in the reflectron mode (acceleration voltage 20 kV, reflectron voltage 28.5 kV). In either case, the laser energy was reduced until an optimal resolution and signal/noise ratio was obtained. The results of 10 to 20 shots were averaged to obtain the final spectrum.

Nanoflow electrospray (ESI) double quadrupole (Qq) orthogonal acceleration (oa) Time of Flight (TOF) mass spectrometry was performed on a Q-Tof system (Micromass, UK). One μ l of acetonitril/water/formic acid (50:49:1, v,v,v) containing 1/40th of peptide sample was loaded in a gold coated capillary (Micromass type A nanoflow needle). This sample was sprayed at a flow rate of 30 nl/min giving extended analysis time in which we acquired an MS spectrum as well as several MS/MS spectra. During MS/MS or tandem mass spectrometry fragment ions are generated from a selected precursor ion by collision induced dissociation (CID) (20). Since not all peptide ions fragment with the same efficiency, the collision energy is typically varied between 20 and 35 V so that the parent ion is fragmented in a satisfying number of different daughter ions.

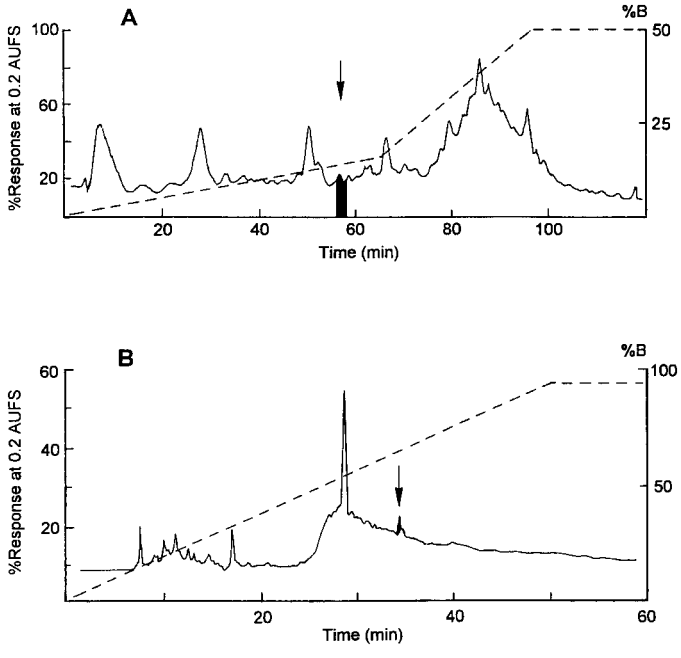

FIG. 1. Purification of midgut extract of *Schistocerca gregaria.* (A) First fractionation on a Bondapak C18 column. Fraction eluting at 56–58 min showed tachykinin immunoreactivity (shaded part). (B). Third fractionation on a Biosep-Sec-S-2000 size exclusion column. Tachykinin-like immunoreactive material eluted at 35 min (arrow). Dashed line shows the concentration of solvent B.

N-terminal amino acid sequencing of 1/20th of the sample was performed on a Perkin Elmer/Applied Biosystems Procise 492 microsequencer running in the pulsed mode. The peptide was synthesized using Fmoc polyamide chemistry (Research Genetics, Huntsville, AL) and was used for verification of the sequence and for bioassay.

Myotropic bioassay. The myotropic bioassay was performed as previously described by (10). Threshold concentrations were determined by adding known quantities of the synthetic peptides to the bioassay chamber containing the isolated hindgut of *Leucophaea maderae* or the isolated oviduct of *Schistocerca gregaria.* The threshold concentration was defined as the minimum concentration of synthetic peptide required to evoke an observable change in amplitude of the visceral muscle contractions within one minute.

Immunoassay. A dot immunobinding assay (DIA) according to Salzet (21) was used to screen the fractions. An aliquot of each fraction (1/20) was spotted onto nitrocellulose membrane (0.45 μm pore size). Membranes were baked (30 min, 110°C), blocked with skimmed milk in 50 mM TBS (Tris buffered saline) to reduce background staining (1 h gentle agitation, room temperature), and then incubated overnight at 4°C with the Pev-tachykinin antiserum (diluted 1:2000 in TBS) (Nieto *et al.*, 1998). Membranes were washed several times and incubated with peroxidase-conjugated goat anti-rabbit IgG for 2 h. Following detection by enhanced chemiluminescence (Amersham Int., Rainham, UK) immunoreactive spots were visualized by a short exposure to a blue-light sensitive autoradiography film.

RESULTS AND DISCUSSION

Passage through one Megabond Elute column was used to prepurify the extract containing 100 midgut

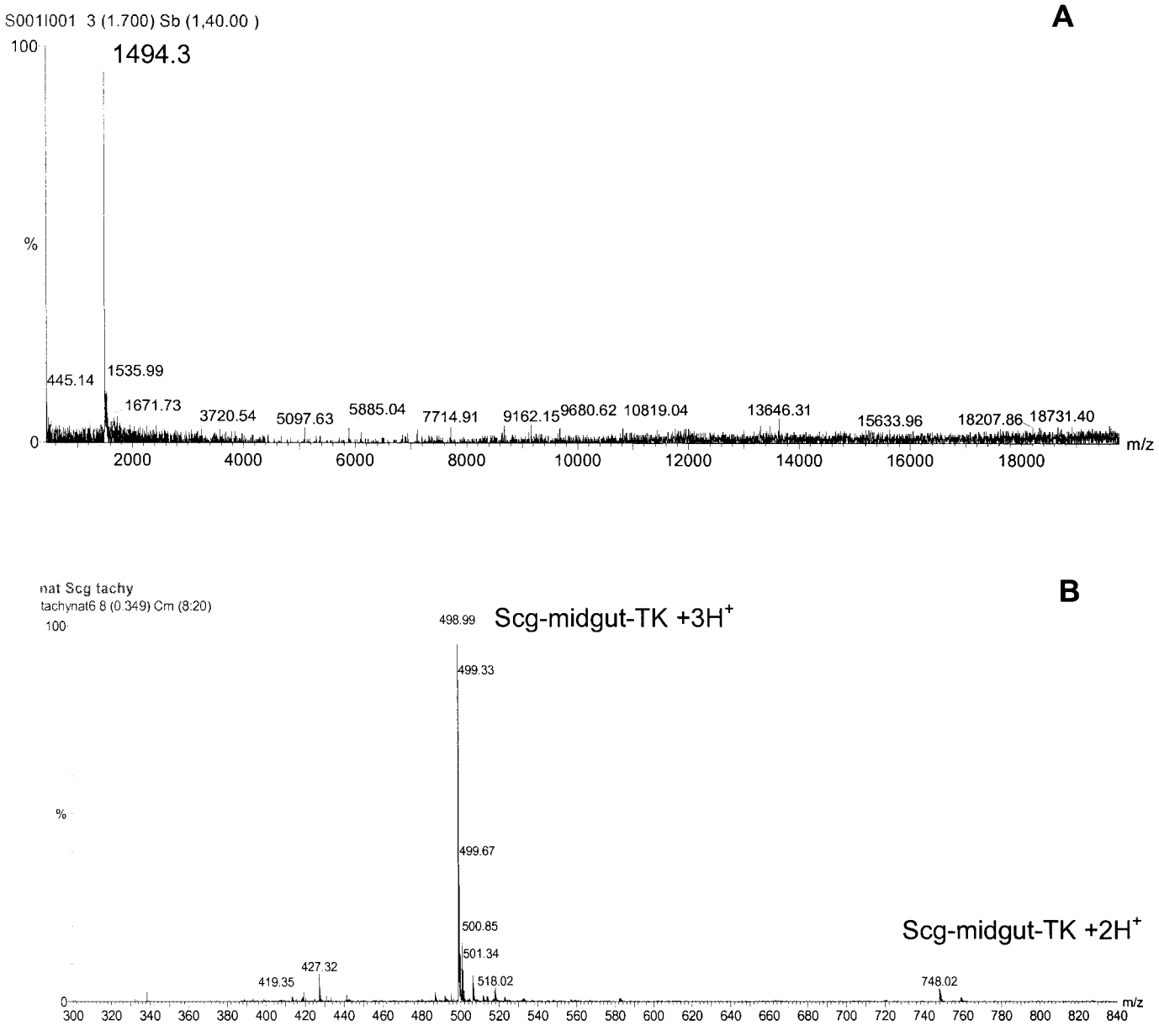

FIG. 2. (A) Maldi-TOF mass spectrum showing a single ion species of *m/z* 1494.3 Da. (B) Monoisotopic mass of the peptide. A triple charged ion species at 498.99 and a double charged ion species at 748.02 reveal a monoisotopic mass of 1494.06 Da ((499.02*3) – 3 or (748.02*2) – 2).

equivalents of *Schistocerca gregaria*. The material eluting in the 0–50% CH_3CN 0.1% TFA fraction showed tachykinin-like immunoreactivity. After each of the three HPLC purification steps all fractions were tested for tachykinin-like immunoreactivity. The fraction eluting at 56–58 min on a Bondapak C18 column showed strong tachykinin-like immunoreactivity (Fig. 1A). This fraction was further purified on the Spherisorb Phenyl column and tachykinin-like immunoreactivity eluted at 43 min. After final purification on the Biosep-Sec-S-2000 column the shape of the immunoreactive peak, eluting at 35 min, suggested homogeneity (Fig. 1B). This assumption was confirmed by MALDI-TOF-MS in the linear mode, showing a single charged ion at 1494.3 Da (Fig. 2A). The monoisotopic mass was determined by the first ESI-oa-TOF-MS analysis (Table 1). A clear triply charged ion at *m/z* 498.99 Da was evident, indicating a corresponding mass of 1493.97 Da (Fig. 2B). In a subsequent MS/MS experiment, this ion was selected to be fragmented in the collision cell of the Q-TOF device. The resulting mass spectral readout showed clear typical y″ and a number of b ions. Figure

TABLE 1

Predicted Fragment Ions, Monoisotopic Masses with 1 Positive Charge

a-ions	30.0	144.1	245.1	373.2	501.3	572.4	669.4	768.5	825.5	972.6	1135.6	1192.6	1293.7	—
b-ions	58.0	172.1	273.1	401.2	529.3	600.3	697.4	796.5	853.5	1000.6	1163.6	1220.6	1321.7	—
c-ions	73.0	187.1	228.1	416.2	544.3	615.4	712.4	811.5	868.5	1015.6	1178.6	1235.7	1336.7	—
i-ions	30.0	87.1	74.1	101.1	101.1	44.1	70.1	72.1	30.0	120.1	136.1	30.0	74.1	129.1
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	Gly	Asn	Thr	Lys	Lys	Ala	Pro	Val	Gly	Phe	Tyr	Gly	Thr	Arg
	14	13	12	11	10	9	8	7	6	5	4	3	2	1
x-ions	—	1463.8	1349.7	1248.7	1120.6	992.5	921.5	824.4	725.3	668.3	521.2	358.2	301.2	200.1
y″-ions	—	1437.8	1323.8	1222.7	1094.6	966.5	895.5	798.4	699.4	642.3	495.3	332.2	275.2	174.1
z-ions	—	1420.8	1306.7	1205.7	1077.6	949.5	878.5	781.4	682.3	625.3	478.2	315.2	258.2	157.1

Note. Underlined masses are found in the MS/MS spectrum.

3 shows several combined spectra where all *m/z* values are transformed into their singly charged values and all peaks are "de-isotoped" after treatment with the Maxent sequencing software (Micromass, UK). The amino acid sequence, GNTKKAVPGFYGTRamide, is determined by calculating the *m/z* or mass differences between two adjacent y-ion peaks sequentially from high to low mass in the mass spectrum. In the same way, the order of succeeding b-ions from low to high mass also corresponds to the same sequence, GNTKKA-VPGFYGTRamide. The calculated mass of 1493.79 Da is, within the limits of accuracy inherent in the technique used, in agreement with the measured molecular weight. The sequence was confirmed by Edman degradation-based sequencing. The advantage of sequencing by MS over Edman degradation-based sequencing is that, in addition to the amino acid sequence, other structural alterations including blocking groups can be identified. This is clearly illustrated by our MS/MS experiment, where the y-ion at *m/z* 172.13 (corresponding to Arg-amide) and the b ion at *m/z* 1477.9 (the mass difference with the parent at *m/z* 1495,07 corresponds to the loss of a C-terminal amide) are in full agreement with the presence of the blocking amide group at the C-terminus.

The identified peptide was designated *Schistocerca gregaria* tachykinin-1 (Scg-midgut-TK). The peptide is a stimulator of the spontaneous contractions of the hindgut of the cockroach *Leucophaea maderae:* threshold concentration of 10^{-9} M (Fig. 4). No myostimulatory

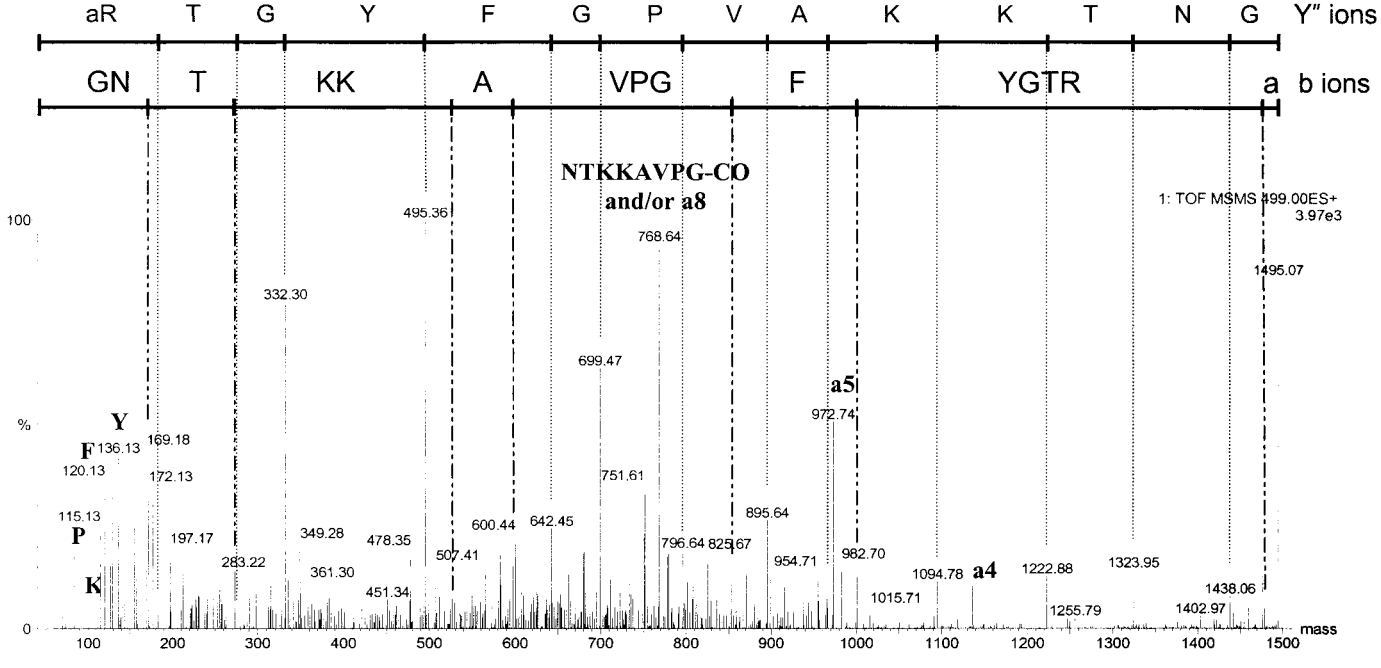

FIG. 3. The CID (collision induced dissociation) MS/MS spectrum of the triply charged ion at *m/z* 498.9 of the purified tachykinin-like peptide showing b and y″ sequence ions obtained on a Q-TOF instrument with nanoES (electrospray) source.

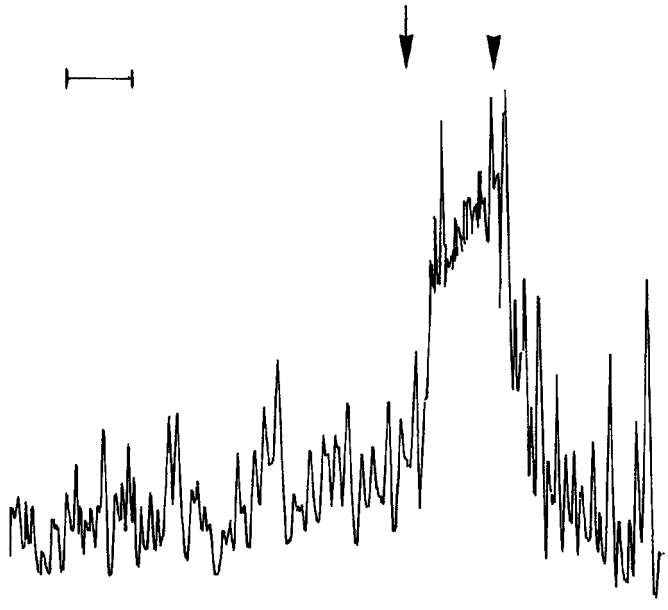

FIG. 4. Myotropic assay on the hindgut of *Leucophaea maderae.* Response of the hindgut to 10^{-9} M of Scg-TK-1. Arrowhead is application of synthetic Scg-TK-1, arrow rinse with saline.

effect on the oviduct of both *Schistocerca gregaria* and *Locusta migratoria* was found.

To date more than 20 members comprise the family of the insect brain tachykinin-related peptides, which is characterized by the C-terminal pentapeptide sequence FX_1GX_2Ramide (X_1 is Y, H, Q, L, M, F and X_2 usually is V, M, T) (15). Additional invertebrate tachykinins have been identified from other invertebrate species such as crustaceans (13, 22), mollusks (23) and echiuroid worms (24).

The presently identified Scg-midgut-TK displays structural features that differ from the locust brain tachykinins. Compared to the brain tachykinins (nona- and decapeptides) it is N-terminally extended. N-terminally extended tachykinins have also been found in the mammalian intestine (neuropeptide γ or DAGHGQISHKRHKTDSFVGLMamide) (25) and the cockroach intestine (Lem-TRP 2 or APEESPKRAPSGFLGVRamide and Lem-TRP 3 or NGERAPGSKKAPSGFLGTRamide) (26). Like these extended intestinal tachykinins, Scg-midgut-TK contains a putative dibasic cleavage site (KK), which could render a nonapeptide, when cleaved. In the cockroach intestine, the concentration of the isolated deduced nonapeptide is only half the amount of its precursor, Lem-TRP 2 (26). The deduced peptides of the Scg-midgut-TK and the cockroach Lem-TRP 3 were not detected. It is likely that the Scg-midgut-TK is not further processed. In fact, Scg-midgut-TK displays a high degree of sequence similarities with the cockroach Lem-TRP 3, which is also confined to the midgut (15). Both also have the Thr residue at position 2 from the C-terminus instead of the typical Val or Met in the insect brain tachykinins.

Immunochemical data have indicated that neural processes on the surface of the midgut in locusts were devoid of tachykinin-like immunoreactivity (15). Endocrine cells in the ampullae of the Malpighian tubules in the posterior part of the midgut, however, contain tachykinin-like immunoreactivity (27). Therefore, Scg-TK is likely to be specifically expressed by these endocrine cells of the midgut. The differential expression of brain and midgut tachykinins suggests different functions. The ampullae endocrine cells also contain the CRF-like locust diuretic hormone (27). Montuenga *et al.* (27) suggested that Lom-DH may regulate fluid secretions while the released tachykinins may increase spontaneous contractions of muscles in the Malpighian tubules, thus providing blood flow toward the intestine.

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