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TWO NOVEL TACHYKININ-RELATED PEPTIDES FROM THE NERVOUS SYSTEM OF THE CRAB *CANCER BOREALIS*

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Summary

Immunocytochemical and biochemical studies have indicated the presence of many neuroactive substances in the stomatogastric nervous system (STNS) of the crab *Cancer borealis*. In electrophysiological studies, many of these substances modulate the motor output of neural networks contained within this system. Previous work in the STNS suggested the presence of neuropeptides related to the invertebrate tachykinin-related peptide (TRP) family. Here we isolate and characterize two novel peptides from the *C. borealis* nervous system that show strong amino acid sequence identity to the invertebrate TRPs. The central nervous systems of 160 crabs were extracted in an acidified solvent, after which four reversed-phase HPLC column systems were used to obtain pure peptides. A cockroach hindgut muscle contraction bioassay and a radioimmunoassay (RIA) employing an antiserum to locustatachykinin I (LomTK I) were used to monitor all collected fractions. The amino acid sequences of the isolated peptides were determined by Edman degradation. Mass spectrometry and chemical synthesis confirmed the sequences to be APSGFLGMR-NH_2 and SGFLGMR-NH_2. APSGFLGMR-NH_2 is approximately 20-fold more abundant in the crab central nervous system than is SGFLGMR-NH_2. We have named these peptides *Cancer borealis* tachykinin-related peptide Ia and Ib (CabTRP Ia and Ib), respectively. Both peptides are myoactive in the cockroach hindgut muscle contraction bioassay, with CabTRP Ia being approximately 500 times more potent than CabTRP Ib. RIA performed on HPLC-separated *C. borealis* stomatogastric ganglion (STG) extract revealed that CabTRP Ia is the only detectable TRP-like moiety in this ganglion. Incubation of synthetic CabTRP Ia with the isolated STG excited the pyloric motor pattern. These effects were suppressed by the broad-spectrum tachykinin receptor antagonist Spantide I. Spantide I had no effect on the actions of the unrelated endogenous peptide proctolin in the STG. There was no consistent influence of CabTRP Ib on the pyloric rhythm. Given its amino acid sequence and minimal biological activity in the crab, CabTRP Ib may be a breakdown product of CabTRP Ia.

Key words: neuropeptide, neuromodulation, tachykinin-related peptide, Crustacea, stomatogastric ganglion, Spantide I, crab, *Cancer borealis*.

Introduction

Rhythmically active neural networks, in both vertebrate and invertebrate systems, are anatomically ‘hard wired’ but functionally flexible (Marder and Calabrese, 1996). One mechanism by which rhythmic motor activity is modulated is *via* the influence of neuroactive peptides (Dekin *et al.* 1985; Kuhlman *et al.* 1985; Harris-Warrick and Marder, 1991; Yamamoto *et al.* 1992; Grillner *et al.* 1995; Marder *et al.* 1995; Marder and Calabrese, 1996).

A prerequisite for fully understanding the neuromodulatory control of any neural network is a complete catalog of the neuroactive substances that influence such a system. One system whose chemical neuroanatomy has been studied extensively is the rhythmically active neural network located in the crustacean stomatogastric ganglion (STG), one of four ganglia that constitute the stomatogastric nervous system (STNS; Fig. 1) (Marder *et al.* 1994, 1995). The STNS controls the rhythmic movement of food through the foregut (Selverston and Moulins, 1987; Harris-Warrick *et al.* 1992*a*). The STG network receives modulatory influences directly within the STG neuropil from neurons that project to the ganglion from more anteriorly located ganglia (Coleman *et al.* 1992) as well as from circulating hormones (Turrigiano and Selverston, 1990; Christie *et al.* 1995). At present, in the crab *Cancer borealis*, more than a dozen neuroactive substances have been localized to the terminals of projection neurons in the STG neuropil (Marder *et al.* 1994; Christie, 1995). Similarly, more than a dozen neuroactive agents have been localized in this species to the two primary neurosecretory

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structures, namely the pericardial organs and the sinus glands (Christie *et al.* 1995). These neuroactive substances include small molecule transmitters and peptides. When superfused separately onto the isolated STNS, many of these substances elicit distinct pyloric motor patterns from the STG (Harris-Warrick *et al.* 1992*b*; Marder and Weimann, 1992; Weimann *et al.* 1993; Weimann and Marder, 1994).

Over the past decade, members of a peptide family that share a conserved carboxy (C)-terminal sequence, -FX_1GX_2R-NH_2, have been isolated from invertebrates (Schoofs *et al.* 1990*a*,*b*; Clottens *et al.* 1993; Ikeda *et al.* 1993; Lundquist *et al.* 1994; Muren and Nässel, 1996*b*, 1997). These peptides are hypothesized to be an ancient subgroup of the tachykinin peptide superfamily (Erspamer, 1981; Maggio, 1988; Schoofs *et al.* 1990*a*; Nachman *et al.* 1990). This is because single base pair substitutions in the predicted codons of the invertebrate peptides would produce the vertebrate tachykinin substance P (Nachman *et al.* 1990). Like the vertebrate tachykinins (which exhibit the carboxy-terminal sequence -F*X*GLM-NH_2), the invertebrate tachykinin-related peptides (TRPs) show broad tissue distribution and are purported to serve physiological functions similar to those of the vertebrate tachykinins. For example, in vertebrates, substance P regulates gastric and intestinal motility (Hershey *et al.* 1991; Otsuka and Yoshioka, 1993). In the cockroach *Leucophaea maderae*, the analogous series of nine native TRPs, LemTRP 1–9 (Muren and Nässel, 1996*b*, 1997), are potent regulators of hindgut muscle contraction (Muren, 1996; Muren and Nässel, 1996*b*, 1997). Similarly, in mammals, substance P regulates neurosecretion, and in locusts the TRP locustatachykinin I (LomTK I; Schoofs *et al.* 1990*a*) has been implicated in the regulation of adipokinetic hormone release (Nässel *et al.* 1995*b*).

In vertebrates, tachykinins act as neuromodulators throughout the CNS and peripheral nervous system (PNS) (Hershey *et al.* 1991; Otsuka and Yoshioka, 1993). In invertebrates, anatomical and biochemical studies suggest that TRPs may also serve neuromodulatory roles (Goldberg *et al.* 1988; Lundquist *et al.* 1994; Christie *et al.* 1995, 1997; Muren *et al.* 1995; Nässel *et al.* 1995*a*,*b*; Muren and Nässel, 1996*b*, 1997). Direct physiological demonstration of this function in invertebrate systems is, however, limited.

One invertebrate system where neuromodulation by TRPs has been demonstrated is the STNS of the crab *Cancer borealis* (Blitz *et al.* 1995). Here, superfusion of the locust TRPs LomTK I and II excites the pyloric rhythm in the isolated STG. However, this excitation is not as robust as that resulting from superfusion of comparable levels of other neuropeptides that have been identified in this system (e.g. Marder *et al.* 1986; Nusbaum and Marder, 1988, 1989; Weimann *et al.* 1993). It therefore appeared likely that the native crab TRPs were structurally similar but not identical to the LomTKs.

In the present paper, we purify and characterize *Cancer borealis* tachykinin-related peptide Ia and Ib (CabTRP Ia and Ib) using methodology developed for the isolation of TRPs from insect tissue (Muren, 1996; Muren and Nässel, 1996*a*). We show that these peptides are indeed similar but not identical in amino acid sequence to the previously isolated invertebrate TRPs. CabTRP Ia and Ib have identical amino acid sequences, with the exception that CabTRP Ib is truncated by two amino acids at the amino terminus. Moreover, we show that CabTRP Ia modulates the motor output of the pyloric network and that this modulation is suppressed by a broad-spectrum vertebrate tachykinin receptor antagonist. Some of this work has appeared previously in abstract form (Christie *et al.* 1996).

Materials and methods

Animals

Cancer borealis

Crabs, *Cancer borealis* Stimpson, were purchased from Commercial Lobster Company (Boston, Massachusetts, USA) and the Marine Biological Laboratory (Woods Hole, Massachusetts, USA). Animals were maintained in recirculating artificial seawater aquaria at 10–12 °C and fed frozen squid biweekly. Adult male animals (N=189) were used in this study. All animals were cold-anesthetized by packing in ice for approximately 45 min prior to dissection.

Leucophaea maderae

Cockroaches, *Leucophaea maderae* Fabricius, were from a colony maintained at Stockholm University (Stockholm, Sweden). Animals were raised at 25 °C with a 16 h:8 h light:dark cycle and were fed dog chow and water *ad libitum*. Adult animals (N=50) of both genders were used in this study. Cockroaches were cold-anesthetized by placing on ice for approximately 30 min prior to dissection.

Solutions and reagents

Crab physiological saline had the following composition (in mmol l^{-1}): NaCl, 440; $MgCl_2$, 26; $CaCl_2$, 13; KCl, 11; Trizma base, 10; maleic acid, 5; pH 7.4–7.6. Insect physiological saline had the following composition (in mmol l^{-1}): NaCl, 154; glucose, 22; Hepes, 12; KCl, 2.7; $CaCl_2$, 1.8; pH 6.8. For the isolation of tachykinin-related peptides (TRPs) from crab central nervous system (CNS), tissue extraction was performed using an acidified extraction mixture (Bennett's solution) described in Bennett *et al.* (1981): 1 % NaCl (w/v), 5 % formic acid (v/v), 1 % trifluoroacetic acid (TFA) (v/v) in 1 mol l^{-1} aqueous HCl. For the determination of TRP isoforms in the stomatogastric ganglion (STG), tissue extraction was performed using methanol:water:acetic acid (90:9:1).

Cancer borealis tachykinin-related peptides Ia and Ib (CabTRP Ia and Ib) were synthesized at the Cancer Research Center, University of Pennsylvania School of Medicine (Philadelphia, Pennsylvania, USA). Locustatachykinin I (LomTK I), proctolin and Spantide I were obtained from Peninsula Laboratories (Belmont, California, USA). ^{125}I-labeled N-terminally extended (3 × glycine) callitachykinin II ([^{125}I]^{3}GlyCavTK II) was produced by Ferring (Malmö, Sweden). All HPLC solvents were filtered (0.45 μm; Millipore,

Bedford, Massachusetts, USA) and dissolved in HPLC-grade water (Milli-Q Plus 185; Millipore) containing 0.1 % TFA as ion-pairing agent (spectroscopy grade; Merck, Darmstadt, Germany). Acetonitrile (MeCN) was chromatography grade (Merck). HPLC solutions were degassed with helium prior to and during all chromatography steps.

Tissue extraction and prepurification

For the isolation of TRPs from *C. borealis*, neural tissue [including the fused thoracic ganglia, the supraoesophageal ganglion, the paired commissural ganglia (CoGs), the single oesophageal ganglion (OG) and the eyestalks] was rapidly dissected in chilled physiological saline and flash-frozen in liquid nitrogen. Frozen tissue was stored at temperatures colder than −70 °C until all tissue was collected. This tissue (approximately 45 g in total) was split into two roughly equal samples. Each sample was homogenized for approximately 1 min in 200 ml of Bennett's solution, using a Polytron homogenizer (Kinematica AG, Littau, Switzerland) running at full speed. The resulting homogenates were sonicated for several minutes using a Vibra cell sonication system (Sonics and Materials Inc., Danbury, Connecticut, USA). After sonication, the homogenates were centrifuged at 10 000 ***g*** for 30 min at 5 °C using a Beckman J-21C centrifuge (Beckman Instruments Inc., Palo Alto, California, USA). The resulting supernatant was collected and stored on ice while the pellets were resuspended in an additional 200 ml of Bennett's solution and re-extracted using the same homogenization, sonication and centrifugation protocol. The supernatant resulting from the re-extraction of the pellets was pooled with that obtained from the original extraction. Crude tissue extract was prepurified using Sep-Pak Vac 20cc C18 cartridges (eight cartridges used in total; Waters Chromatography Division, Millipore Corp., Milford, Massachusetts, USA) that had been activated with 50 ml of 100 % MeCN followed by 50 ml of 100 % water. Each cartridge was equilibrated with 50 ml of water containing 0.1 % TFA. Following equilibration, approximately 100 ml of extract was applied to each cartridge and the cartridges were subsequently washed with approximately 12 ml of 10 % MeCN containing 0.1 % TFA. Following this wash, each cartridge was eluted with approximately 15 ml of 40 % MeCN containing 0.1 % TFA.

Reversed-phase high-performance liquid chromatography

Reversed-phase high-performance liquid chromatography (HPLC) was performed on a Waters HPLC equipped with a model 600E controller and a model 486 ultraviolet detector (set at 214 nm). Separation of peptides was carried out as described in Muren and Nässel (1996*b*) on a series of four separate column systems, as outlined below.

Delta-Pak C18 (RCM, 25 mm×100 mm, 30 nm, 15 μm; Waters)

In the first step, the prepurified extract was diluted to a concentration of 10 % MeCN containing 0.1 % TFA (final volume approximately 480 ml). The diluted extract was subsequently loaded onto the Delta-Pak C18 column, and 10 % MeCN containing 0.1 % TFA was then pumped over the column for 1 min, followed by a linear gradient to 40 % MeCN containing 0.1 % TFA for 110 min (0.27 % min^{-1}). Immediately following this linear gradient, the column was run in isocratic mode (40 % MeCN containing 0.1 % TFA) for 7 min and then switched to a linear gradient to 80 % MeCN containing 0.1 % TFA for the next 10 min (4 % min^{-1}). The flow rate from the column was set at 7.5 ml min^{-1} with fractions collected automatically during the first 120 min of the run. For analysis of each fraction, 1 % was used in the bioassay and in the radioimmunoassay (0.5 % in each assay; see below). Fractions that contained bioactivity and/or immunoreactivity were lyophilized (Speed-Vac vacuum concentrator: Savant, Farmingdale, New York, USA) and subsequently reconstituted in 1 ml of 10 % MeCN containing 0.1 % TFA for processing on the second column system.

Vydac Diphenyl (219TP54, 4.6 mm×250 mm, 30 nm, 5 μm; The Separations Group, Hesperia, California, USA)

Processing on the second column system included elution with 10 % MeCN for 5 min and then a linear gradient to 30 % MeCN over 40 min (0.5 % min^{-1}), followed by isocratic conditions at 30 % MeCN for 5 min. The flow rate was set at 1 ml min^{-1} with fractions collected automatically every minute. Fractions were tested as above, and bioactive and/or immunoreactive fractions were dried using the Speed-Vac concentrator.

Supelcosil C8 (LC-8-D8, 4.6 mm×150 mm, 10 nm, 3 μm; Supelco, Bellfonte, Pennsylvania, USA)

The operating conditions for the third column system used in our study were 10 % MeCN containing 0.1 % TFA during the first minute after injection of the sample (reconstituted in 1 ml of 10 % MeCN containing 0.1 % TFA) and then a linear gradient to 40 % MeCN containing 0.1 % TFA over 60 min (0.5 % min^{-1}). The flow rate was set at 1 ml min^{-1} and detected peaks were collected manually. As with the first two column systems, fractions were assayed for both bioactivity and immunoreactivity. Positive fractions were dried and then reconstituted in 1 ml of 15 % MeCN with 0.1 % TFA. Reconstituted fractions were directly subjected to the fourth and final column system.

Partisil C18 (ODS 3, 4.6 mm×250 mm, 5 μm; Fisons Scientific Equipment, Leicestershire, UK)

Operating conditions for the final column system used in this study were isocratic mode for 2 min with 15 % MeCN containing 0.1 % TFA followed by a linear gradient to 35 % MeCN containing TFA run over 40 min (0.5 % min^{-1}). The flow rate was set at 1 ml min^{-1} and, as with the previous column system, peaks were collected manually. This column yielded satisfactorily pure peptides for mass determination and amino acid sequence analysis.

Bioassay and radioimmunoassay

To follow TRPs during our isolation procedure (see above),

we monitored both the bioactivity and the immunoreactivity present in each HPLC fraction. Between 0.5 % and 2 % of each fraction was drawn off for each type of analysis. We monitored bioactivity using the cockroach *L. maderae* hindgut muscle contraction bioassay. This assay is described in detail elsewhere (Holman *et al.* 1991) and has been successfully employed for the detection TRPs during their isolation from insect tissue (Schoofs *et al.* 1990*a*,*b*; Lundquist *et al.* 1994; Muren and Nässel, 1996*b*, 1997). In brief, the hindgut was dissected from the cockroach and suspended in a 5 ml glass chamber (filled with insect saline). The posterior end of the gut was affixed *via* a cotton thread loop to the chamber and the anterior end was attached to the lever arm of an isotonic tension transducer (Kent Scientific Corp., Litchfield, Connecticut, USA). Lyophilized HPLC fraction samples were reconstituted in 500 µl of insect saline and added to the saline present in the glass chamber for 1 min. During this time, the tonus of the hindgut as well as the frequency and amplitude of spontaneous hindgut contractions were recorded *via* either a Servogor 120 pen recorder (Goerz, Neudorf, Austria) or an EasyGraf TA 240 thermal printer (Gould, Valley View, Ohio, USA). After incubation with the reconstituted fraction sample for 1 min, the chamber was flushed with fresh saline and the gut was allowed to recover for at least 1 min before application of another fraction sample. This bioassay was also used to determine the relative bioactivities of synthetic CabTRP Ia and Ib.

To monitor TRP-like immunoreactivity in each HPLC fraction, a radioimmunoassay utilizing a LomTK I antiserum and [^{125}I]^{3}GlyCavTK II as a tracer was employed. This assay is described in detail in Muren and Nässel (1996*a*) and has been successfully used to monitor TRPs during their isolation from cockroach tissue (Muren and Nässel, 1996*b*, 1997). Here, the amount of TRP-like activity in each HPLC fraction sample was determined by comparison with serially diluted (1.2–300 fmol) synthetic LomTK I. This radioimmunoassay was also used to determine the relative cross-reactivities of synthetic CabTRP Ia and Ib with the LomTK I antiserum and for the determination of the TRP-like moieties present in the stomatogastric ganglion.

Mass spectrometry, amino acid sequence determination and peptide synthesis

The molecular mass of isolated peptides was determined by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) using a Kompact MALDI II system (Kratos, Manchester, UK) at the Department of Medical and Physiological Chemistry, Uppsala University (Uppsala, Sweden). Samples of each isolated peptide (5×10^{-12} to 10^{-11} mol) were reconstituted in 5 ml of 10 % MeCN containing 0.1 % TFA and applied together with an equal amount of α-cyano-4-hydroxycinnamic acid as a matrix. All spectra were externally calibrated. The amino acid sequence of isolated peptides was determined using an Applied Biosystems model 477A gas-phase protein sequencer equipped with an on-line model 120A analyzer for the identification of phenylthiohydantoin (PTH)-derivatized amino acids. This analysis was also carried out at the Department of Medical and Physiological Chemistry, Uppsala University.

Synthetic CabTRP Ia and Ib were produced with an Applied Biosystems model 433A synthesizer using the standard F-moc procedures (Atherton *et al.* 1978) at the Cancer Research Center of the University of Pennsylvania School of Medicine. Crude peptides were purified by reversed-phase HPLC on a C18 column using a 5 % to 90 % MeCN gradient containing 0.1 % TFA. The purity of the synthetic peptide was assessed by HPLC and confirmed using MALDI-MS.

Distribution of CabTRP isoforms in the crab stomatogastric ganglion

To determine the distribution of CabTRP isoforms in the crab STG, tissue (both complete STNSs and individual STGs) was dissected and stored as described above for the isolation of peptides. Tissue pools were homogenized by hand for several minutes in 1.5 ml of methanol:water:acetic acid (90:9:1) using a glass homogenizer. The resulting homogenates were sonicated for 1 min using a Vibra cell sonication system. After sonication, the homogenates were centrifuged at 10 000 ***g*** for 30 min at 5 °C and the resulting supernatant was collected and stored on ice. The resulting pellets were resuspended in an additional 1.5 ml of extraction medium, re-extracted as just described and the supernatants obtained were pooled with the initial supernatant. Crude tissue extract was prepurified using Sep-Pak C18 cartridges (50 ml) activated and equilibrated as described above. After equilibration, tissue extract was applied to each cartridge, and the cartridges were subsequently washed with approximately 6 ml of 15 % MeCN containing 0.1 % TFA. Following this wash, each cartridge was eluted with 6 ml of 35 % MeCN containing 0.1 % TFA. The 35 % eluate from both the STNS extract and the STG extract was similarly dried using a Speed-Vac vacuum concentrator.

Dried extract was reconstituted with 1 ml of 15 % MeCN containing 0.1 % TFA. The STNS extract, which was used to determine the retention time of CabTRP Ia and Ib in a complex tissue extract, was spiked with 10^{-9} mol each of synthetic CabTRP Ia and Ib. Each of the reconstituted extracts was subjected to reversed-phase HPLC using the Vydac Dyphenyl column described above with the following operating conditions: 15 % MeCN containing 0.1 % TFA for 2 min, followed by a linear gradient to 35 % MeCN containing 0.1 % TFA for 40 min (0.5 % min^{-1}) at a flow rate of 1 ml min^{-1}. Fractions of HPLC eluate were collected automatically at 1 min intervals and dried as above. The TRP-like immunoreactivity present in each fraction was subsequently measured using the LomTK I RIA described earlier.

Immunocytochemistry

A series of preabsortion controls was carried out to confirm that the tachykinin-like (substance-P- and locustatachykinin-like) immunoreactivity previously reported in the crab STNS (Fig. 1; Goldberg *et al.* 1988; Blitz *et al.* 1995) could be blocked by CabTRP Ia. This immunocytochemistry was performed using techniques modified from those of Beltz and

Kravitz (1983), as described in Blitz *et al.* (1995). In brief, anti-substance P (clone NC1/34: Accurate Chemical and Scientific Corp., Westbury, New York, USA) or anti-locustatachykinin I (Nässel, 1993; Blitz *et al.* 1995) antibody was preincubated with CabTRP Ia peptide for 2 h at room temperature prior to application to the STNS. Whole mounts were viewed and photographed using either a Leica DMRB fluorescence microscope equipped with an Orthomat E camera system or a Leica TCS laser scanning confocal microscope equipped with a krypton/argon mixed-gas laser and standard filter cubes.

Electrophysiology

Electrophysiological experiments on the crab stomatogastric nervous system were performed using standard techniques (Blitz *et al.* 1995; Bartos and Nusbaum, 1997). Briefly, the STNS was pinned down in a silicone polymer (Sylgard 184; Dow-Corning)-lined Petri dish. The STNS consists of four interconnected ganglia plus their peripheral nerves (Fig. 1). These ganglia include the STG (containing approximately 25 neurons; Kilman and Marder, 1996), the OG (containing approximately 14 neurons) and the paired CoGs (each containing approximately 500 neurons; V. L. Kilman and E. Marder, unpublished observations). To facilitate access of substances to the STG neuropil, this ganglion was desheathed. Extracellular recordings of the motor nerves were accomplished by placing a Vaseline well around a section of each nerve of interest and placing one stainless-steel wire electrode in the well and a second wire electrode in the bath. In all experiments, the STG was isolated from the rest of the CNS, including the CoGs, by transecting the paired superior oesophageal nerves and inferior oesophageal nerves. The CoGs are the source of most of the modulatory inputs to the STG (Coleman *et al.* 1992). The entire preparation was superfused continuously with physiological saline (10–13 °C). Peptide application was accomplished by diluting the peptide in physiological saline immediately prior to its use. A Vaseline well was built around the STG so that peptide could be applied solely to this ganglion. All peptides were applied manually using a chilled 500 µl Hamilton syringe (Hamilton Co., Reno, Nevada, USA). During all peptide applications to the STG, the remainder of the preparation was continuously superfused with physiological saline.

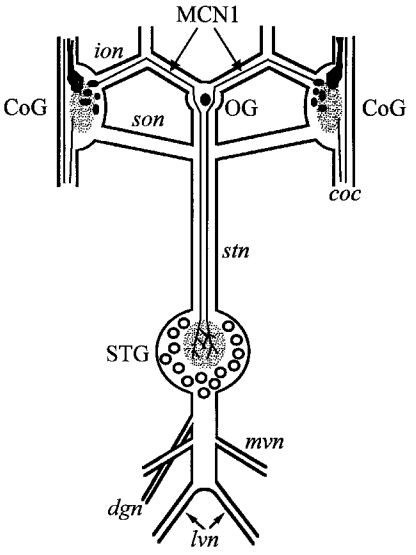

Fig. 1. Schematic representation of the stomatogastric nervous system (STNS) of the crab, including the distribution of substance-P/locustatachykinin-like immunoreactivity in neuronal structures of the STNS (Goldberg *et al.* 1988; Blitz *et al.* 1995). Tachykinin-like immunoreactivity is present in neuronal somata in the commissural ganglia (CoGs) and oesophageal ganglion (OG) (filled circles), in neuropil in the CoGs and stomatogastric ganglion (STG) (stippling) and in fibers in several nerves (solid lines). It is also present in a large, club-shaped structure in each CoG. All tachykinin-like immunoreactivity in the STG results from arborizations of the paired projection neuron MCN1 (Christie *et al.* 1993). *coc*, circumoesophageal connective; *dgn*, dorsal gastric nerve; *ion*, inferior oesophageal nerve; *lvn*, lateral ventricular nerve; *mvn*, medial ventricular nerve; *stn*, stomatogastric nerve; *son*, superior oesophageal nerve; MCN1, modulatory commissural neuron 1.

Statistical analyses and the production of figures

Kaleidagraph (version 3.0, Abelbeck Software, Reading, Pennsylvania, USA) and SigmaStat for Windows (version 1, Jandel Scientific, San Rafael, California, USA) software programs were used for the preparation of figures and for all statistical analyses.

Results

Purification of tachykinin-related peptides from the crab CNS

To begin the purification process, we applied prepurified extract of the CNS from 160 crabs to the first HPLC column system, a Delta-Pak C18 column, and eluted the applied material (see Materials and methods) to obtain the profile with the ultraviolet absorption characteristics shown in the top panel of Fig. 2A. Two sets of fractions from this initial column run had strong bioactivity (middle panel Fig. 2A), while numerous fractions showed significant immunoreactivity (bottom panel Fig. 2A). Ultimately, only one region (fractions 45–50) from this column system was shown to contain TRPs. As Fig. 2A shows, this region contained both bioactivity and immunoreactivity. The remainder of the Results section of this paper deals only with the material present in fractions 45–50, although all fractions showing bioactivity and/or immunoreactivity were processed further *via* HPLC and additional non-TRP peptides were isolated. It should be noted that the other major bioactive region shown in Fig. 2A is due to the presence of the pentapeptide proctolin (NH_2-RYLPT-OH), which has been previously identified in this system (Marder *et al.* 1986). The other major immunoreactive peaks are due to several novel non-tachykinin peptides whose sequences and actions are currently being investigated.

On the second column system (Vydac Diphenyl; Fig. 2B), we separated the material from fractions 45–50 of column system no. 1 into two immunoreactive regions, fraction 25 and

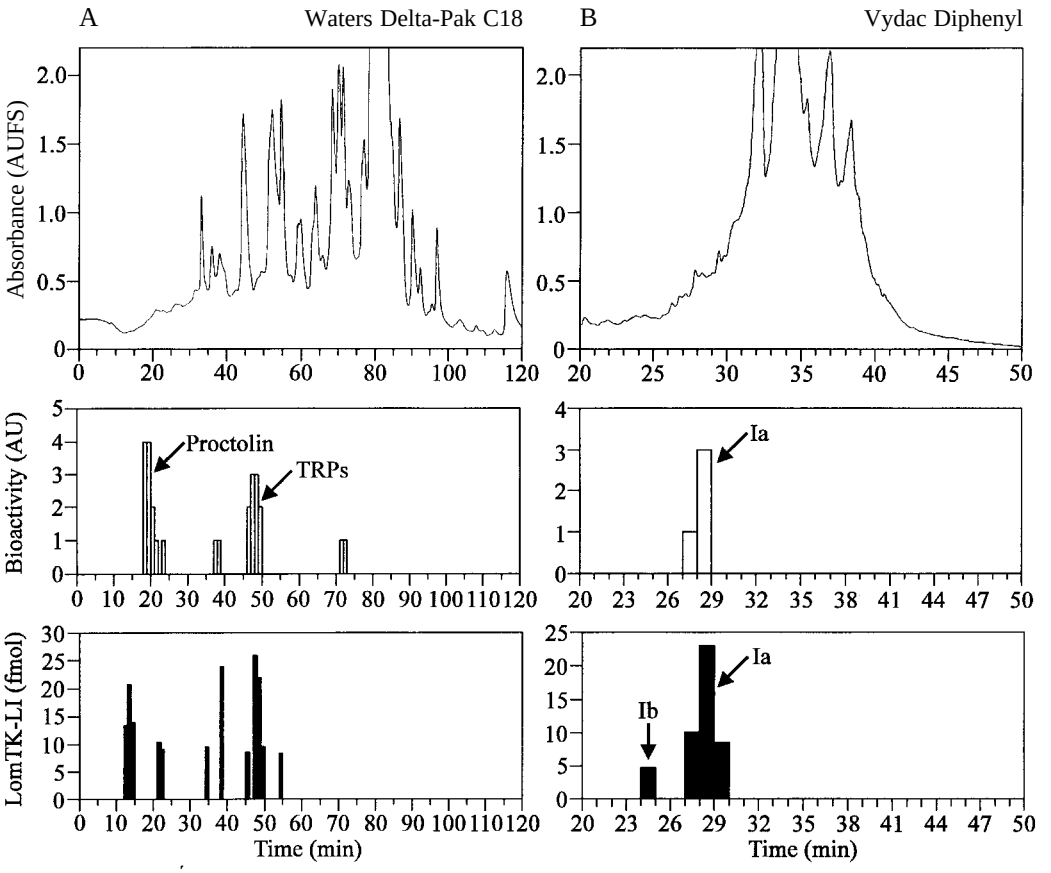

Fig. 2. Purification of tachykinin-related peptides from crab CNS: HPLC column runs 1 and 2. (A) HPLC separation of Sep-Pak-prepurified CNS extract on a Delta-Pak C18 column. Top, chromatogram indicating the ultraviolet absorbance (absorbance units full scale, AUFS: 214 nm) of separated material as it eluted from this column system. Middle, bioactivity of the fractions collected from this column system as tested on the isolated hindgut of cockroach *Leucophaea maderae* (arbitrary units, AU). Two regions of high bioactivity were detected in the collected fractions. The bioactivity in the early-eluting pool was ultimately determined to be due to the pentapeptide proctolin, while the later-eluting pool contained all of the isolated tachykinin-related peptides (TRPs) reported in this paper. Bottom, immunoreactivity of the same fractions as determined using the locustatachykinin I radioimmunoassay (fmol of locustatachykinin-like immunoreactivity, LomTK-LI). Multiple regions of LomTK-LI were detected in fractions resulting from this column run. Ultimately only fractions 45–50 were found to contain TRPs; however, all immunoreactive fractions were processed further using HPLC. (B) Fractions 45–50 from the first column system were pooled and separated on the Vydac Diphenyl column. Top, the ultraviolet chromatogram resulting from this column run. Middle, bioactivity of the fractions collected from this column run. Bottom, immunoreactivity in the same set of fractions. On this column system, the material that would ultimately be identified as *Cancer borealis* tachykinin-related peptide Ia (CabTRP Ia) was separated into fractions 28–30 and the material that would ultimately be identified as CabTRP Ib was separated into fraction 25. The pool containing CabTRP Ia (Ia; fractions 28–30) was both bioactive and immunoreactive, while the fraction containing CabTRP Ib (Ib; fraction 25) was only immunoreactive.

fractions 28–30. The latter set of fractions was also bioactive. These two regions were taken separately through the remaining two HPLC column systems (Supelcosil DB-8 C8 and Partisil ODS-3 C18; Figs 3–4; Table 1). Purification through all four HPLC column systems resulted in the isolation of two pure immunoreactive peptides, one of which was also bioactive.

The amount of each isolated peptide was estimated by peak integration and comparison with 10^{-10} mol of synthetic LomTK I applied to the Partisil ODS-3 C18 column (column system no. 4). We obtained approximately 4.4×10^{-9} mol of LomTK-equivalents of one peptide and 1.35×10^{-10} mol of LomTK-equivalents of the second peptide. Samples of the native peptides, representing approximately 10^{-11} mol of LomTK-equivalents of each substance, were subjected to MALDI-MS to obtain the molecular mass of each species (Table 2). Additional samples of each peptide, representing approximately 10^{-10} mol of LomTK-equivalents of each species, were subjected to amino acid sequence analysis using gas-phase Edman degradation. This analysis revealed that the peptides had the amino acid sequences APSGFLGMR and SGFLGMR. All previously identified TRPs are amidated (Table 3). In comparing the predicted molecular mass of each peptide (919.1 and 753.0 respectively) with the molecular mass determined by MALDI-MS (935.1 and 767.0 respectively), our

Table 1. *Elution times of* Cancer borealis *tachykinin-related peptides during purification*

Column	Retention time (min)	
	CabTRP Ia	CabTRP Ib
Waters Delta-Pak C18*	45–50	45–50
Vydac Diphenyl*	28–30	25
Supelcosil DB-8 C8†	31.0	29.3
Partisil ODS-3 C18†	28.5	27.9

*Fractions collected automatically.
†Fractions collected manually.

Table 2. *Mass spectrometry of isolated* Cancer borealis *tachykinin-related peptides*

Peptide	Calculated mass* for MH+ ($g\,mol^{-1}$)	Observed† molecular ion, MH+ ($g\,mol^{-1}$)	
		Native	Synthetic
CabTRP Ia	935.1	935.1	935.0
CabTRP Ib	769.0	767.0	767.0

All determinations are based on monoisotopic amino acids.

*The calculated molecular mass represents the theoretical value and includes an amidated carboxyterminus.

†The molecular mass determinations of the native and synthetic peptides were made by matrix-assisted laser desorption/ionization mass spectrometry (see Materials and methods).

peptides are also predicted to be amidated. Owing to the high degree of amino acid sequence identity between these peptides and members of the invertebrate tachykinin-related peptide family (Table 3), we named APSGFLGMR-NH_2 *Cancer borealis* tachykinin-related peptide Ia (CabTRP Ia) and SGFLGMR-NH_2 *Cancer borealis* tachykinin-related peptide Ib (CabTRP Ib).

Synthesis of peptides and comparison with native species

Synthetic CabTRP Ia and Ib were produced using standard F-moc chemistry in amidated forms and purified using HPLC. As with the native peptides, the molecular mass of each synthetic peptide was determined using MALDI-MS and was found to be essentially identical to the molecular mass determined for the corresponding native molecule (Table 2).

Because a sufficient amount of native CabTRP Ia remained after MALDI-MS and sequence analysis, we were able to make several additional confirmations of the sequence and amidation of this peptide. First, we compared the retention time of the native and synthetic peptide on the Partisil ODS-3 C18 column (column system no. 4). As shown in Fig. 5, when the native peptide was mixed with an equimolar concentration of the synthetic peptide, this mixture eluted as a single peak (top trace) with an identical retention time to that of the synthetic peptide run alone (lower trace). Similarly, we compared the effects of native and synthetic CabTRP Ia in the cockroach hindgut contraction bioassay. As is shown in Fig. 6, equal concentrations of native and synthetic peptide ($10^{-8}\,mol\,l^{-1}$) produce comparable effects on the hindgut (N=4 preparations). All of the native CabTRP Ib was used for mass spectrometry and sequence analysis. Thus, it was not possible to confirm the sequence and amidation of CabTRP Ib further than the

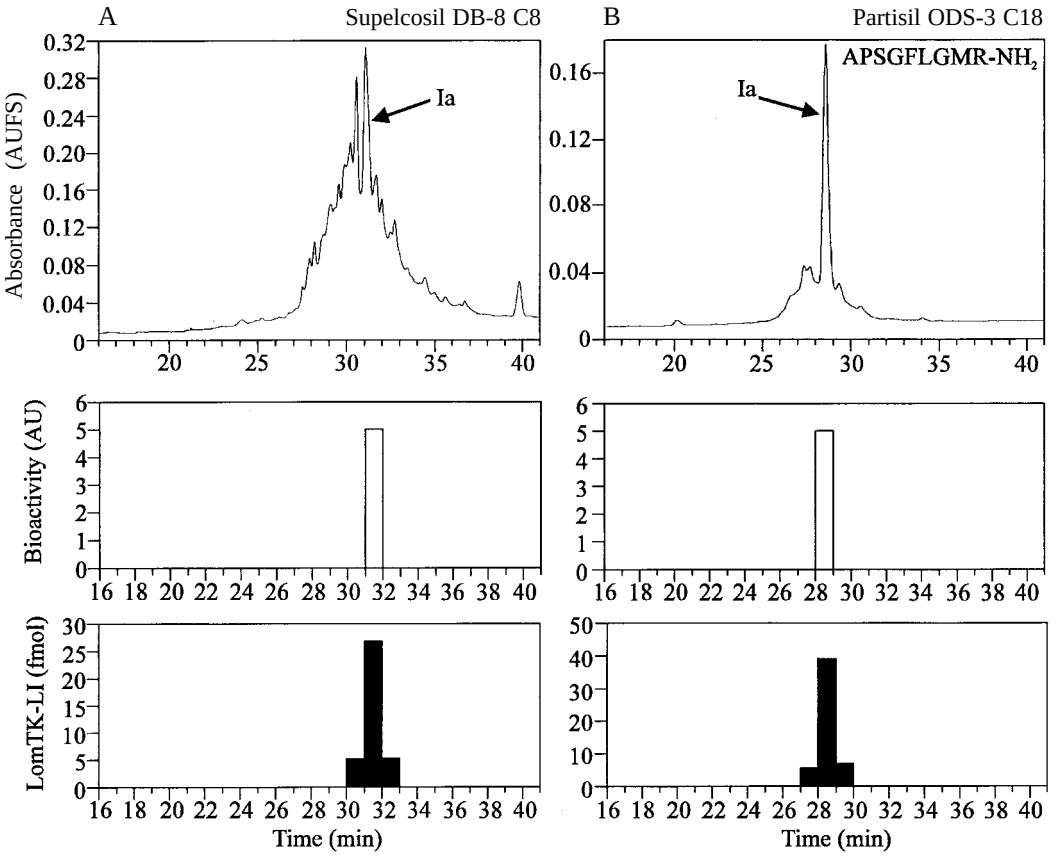

Fig. 3. Purification of *Cancer borealis* tachykinin-related peptide Ia (CabTRP Ia). (A) Fractions 28–30 from the Vydac Diphenyl column (see Fig. 2B) were pooled and separated on the Supelcosil DB-8 C8 column. Top, the ultraviolet chromatogram resulting from the elution of material from this column system (see Fig. 1 for explanation of units). Middle, bioactivity of the fractions collected from this column run. Bottom, immunoreactivity present in the fractions collected from this column system. On this system, the intense bioactivity and immunoreactivity eluted in the same peak (approximately 31 min, arrow on the ultraviolet trace). (B) The 31 min peak just described was further processed on the Partisil ODS-3 C18 column. Top, the ultraviolet chromatogram resulting from the elution of material from this column system. Middle, bioactivity of the fractions collected from this column run. Bottom, immunoreactivity present in the fractions collected from this column system. Peptide sufficiently pure for mass spectrometry and sequence analysis (arrow on ultraviolet trace) was obtained from this column run.

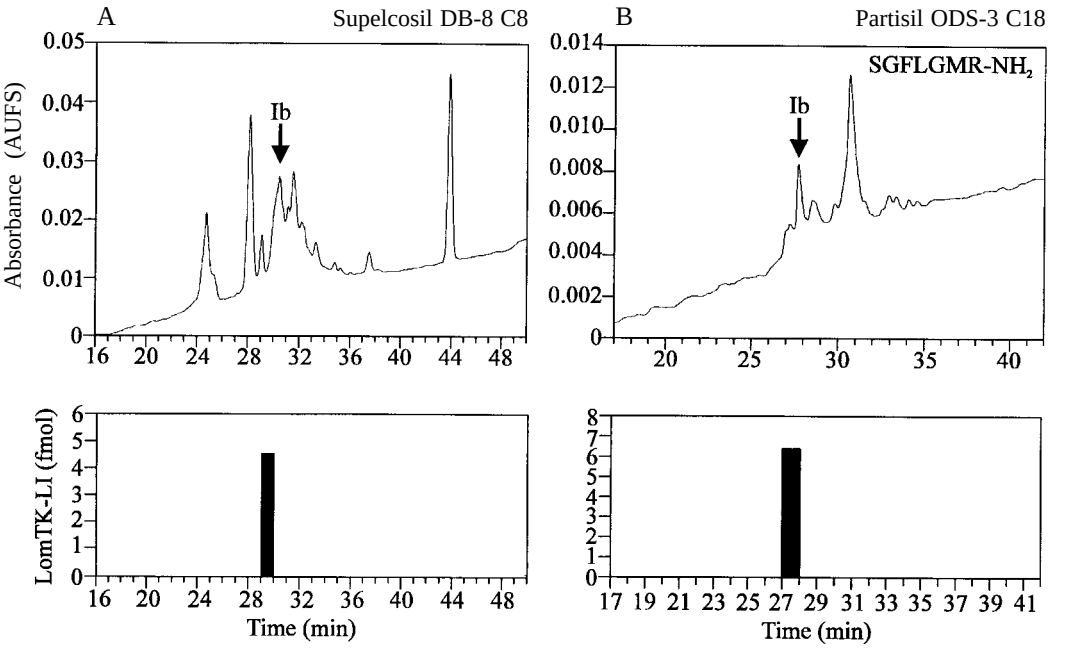

Fig. 4. Purification of *Cancer borealis* tachykinin-related peptide Ib (CabTRP Ib). Fraction 25 from the Vydac Diphenyl column (see Fig. 2B) was separated on the Supelcosil DB-8 C8 column. Top, the ultraviolet chromatogram resulting from the elution of material from this column system (see Fig. 1 for explanation of units). Bottom, immunoreactivity present in the fractions collected from this column system. On this system, the immunoreactivity eluted in one peak (elution time approximately 30 min; arrow on the ultraviolet trace). (B) The 30 min peak just described was further processed on the Partisil ODS-3 C18 column. Top, the ultraviolet chromatogram resulting from the elution of material from this column system. Bottom, immunoreactivity present in the fractions collected from this column system. Peptide sufficiently pure for mass spectrometry and sequence analysis (arrow on the ultraviolet trace) was obtained from this column run.

comparison of the molecular mass of the native and synthetic molecules.

Action of CabTRP Ia and Ib on the cockroach hindgut

During our isolation of CabTRP Ia and Ib, bioactive moieties were followed using the *Leucophaea maderae* hindgut muscle contraction bioassay. Application of each previously isolated invertebrate TRP increases both the tonus of the hindgut and the frequency and amplitude of the spontaneous hindgut contractions (Muren and Nässel, 1996*b*, 1997). During purification, CabTRP Ia was clearly myoactive (Figs 2, 3). CabTRP Ib, however, showed no myoactivity during its isolation (Fig. 2B). One possible explanation for this lack of myoactivity is that an insufficient amount of CabTRP Ib was used during the purification procedure. To examine quantitatively the myoactivity of CabTRP Ib and to compare it with that of CabTRP Ia, dose–response curves of tonus increase were prepared for each of these synthetic peptides (N=4 preparations for CabTRP Ia; N=3 preparations for CabTRP Ib). As shown in Fig. 7, CabTRP Ib can elicit contractile activity in the hindgut, although it is much less potent than CabTRP Ia. The EC_{50} of CabTRP Ia was approximately 10^{-8} mol l^{-1}, while that of CabTRP Ib was approximately 10^{-5} mol l^{-1}.

Distribution of CabTRP isoforms in the crab stomatogastric ganglion

Radioimmunoassay detection of CabTRP isoforms in an HPLC separated extract

While the CNS was used for the isolation of CabTRPs, our primary interest is to understand the actions of these peptides in a well-defined physiological context. We therefore focused on the stomatogastric ganglion (STG; Fig. 1). Most of the approximately 25 STG neurons make up a neural network that

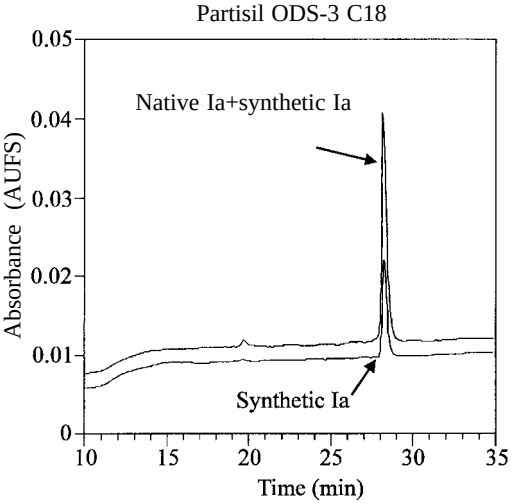

Fig. 5. Native and synthetic CabTRP Ia have identical retention times in HPLC. A mixture of 10^{-10} mol each of native and synthetic CabTRP Ia was run on the Partisil ODS-3 C18 column and found to elute as a single peak, monitored *via* ultraviolet detection at 214 nm (upper trace). When 10^{-10} mol of synthetic CabTRP Ia was run alone (lower trace) on the same column, using identical operating conditions, it too eluted as a single peak with the same retention time as the mixture. The two traces were superimposed with the aid of the Millennium Chromatography Manager software (see Fig. 1 for explanation of units).

Table 3. *Amino acid sequences of tachykinin-related peptides, including* Cancer borealis *tachykinin-related peptide Ia and Ib*

Organism	Peptide	Amino acid sequence
Cancer borealis (crab)	CabTRP Ia	APSGFLGMRamide
	CabTRP Ib	SGFLGMRamide
Leucophaea maderae (cockroach)	LemTRP 1	APSGFLGVRamide
	LemTRP 2	APEESPKRAPSGFLGVRamide
	LemTRP 3	NGERAPGSKKAPSGFLGTRamide
	LemTRP 4*	APSGFMGMRamide
	LemTRP 5	APAMGFQGVRamide
	LemTRP 6	APAAGFFGMRamide
	LemTRP 7	VPASGFFGMRamide
	LemTRP 8	GPSMGFHGMRamide
	LemTRP 9	APSMGFQGMRamide
Locusta migratoria (locust)	LomTK I	GPSGFYGVRamide
	LomTK II	APLSGFYGVRamide
	LomTK III	APQAGFYGVRamide
	LomTK IV	APSLGFHGVRamide
Calliphora vomitoria (blowfly)	CavTK I	APTAFYGVRamide
	CavTK II	GLGNNAFVGVRamide
Culex salinarius (mosquito)	CusTK I*	APSGFMGMRamide
	CusTK II	APYGFTGMRamide
	CusTK III	APSGFFGMRamide
Urechis unicinctus (echiuroid worm)	UruTK I	LAQSQFVGSRamide
	UruTK II	AAGMGFFGARamide

Amino acid alignment is done from the carboxy terminus.
Amino acids that are identical with those in CabTRP Ia are underlined.
*Peptides with identical amino acid sequences.
TRP, tachykinin-related peptide; TK, tachykinin.

controls the rhythmic movement of food through the gastric mill (chewing) and pylorus (filtering chewed food) of the foregut. Blitz *et al.* (1995) showed previously that the LomTK I antibody used in our RIA can also be used for immunocytochemical detection of TRPs in the STNS. To determine whether any of the CabTRP isoforms isolated in our study was responsible for the immunoreactivity reported in the STG, we employed HPLC and RIA techniques analogous to those described for the isolation of these peptides.

Critical to these experiments was the demonstration that both isoforms are readily recognized and are present in sufficient quantity to be detected in the LomTK I RIA. Relatively little of each peptide was likely to be present in an individual STG as there are only two TRP-like immunoreactive neurons that innervate this ganglion (Fig. 1; Christie *et al.* 1993). Moreover, there is only approximately 10^{-13} mol of proctolin or TNRNFLRF-NH_2 per STG (Marder *et al.* 1986, 1987; Weimann *et al.* 1993) and these peptides are each present in six or more neuronal inputs. To determine the cross-reactivity of our isolated peptides in this assay, synthetic CabTRP Ia and Ib were compared with synthetic LomTK I for their ability to compete with ^{125}I-labeled ^{3}GlyCavTK II for binding with the LomTK I antiserum (N=3 assays). We found that CabTRP Ia and Ib are both recognized in the RIA, but each is more than 500-fold less cross-reactive than LomTK I. In each assay, the EC_{50} value for LomTK I was approximately 2.0×10^{-11} mol l^{-1}, which is nearly identical to the EC_{50} value

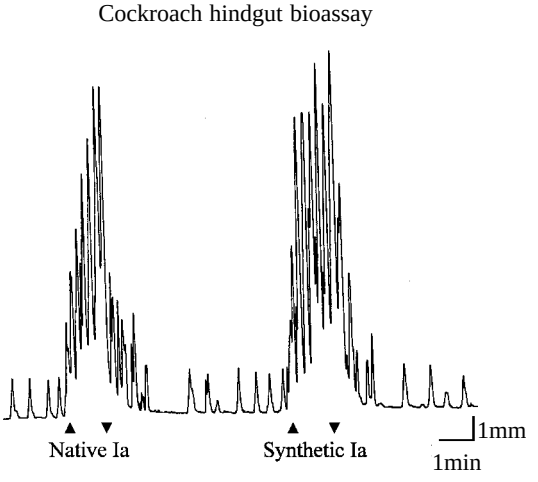

Fig. 6. Native and synthetic CabTRP Ia have similar effects on the cockroach hindgut. Left, application of 10^{-8} mol l^{-1} native CabTRP Ia (concentration estimated from peak integration of isolated material and comparison with locustatachykinin) to a *Leucopheae maderae* hindgut preparation at the time corresponding to the upward-pointing arrow. Application of this peptide increased the tonus of the hindgut as well as the number and amplitude of spontaneous hindgut contractions (N=4 preparations). Washout of the peptide (downward-pointing arrow) returned the preparation to control conditions. Right, application of 10^{-8} mol l^{-1} synthetic CabTRP Ia produced a qualitatively similar effect on the same preparation.

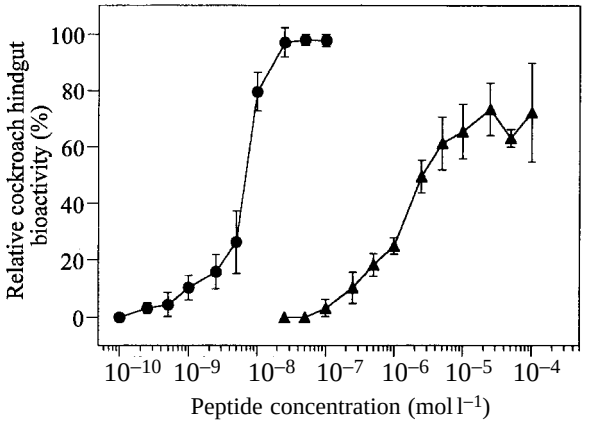

Fig. 7. Effects of CabTRP Ia and Ib on the tonus of the cockroach hindgut. Dose–response curves of the relative increase in tonus of the *L. maderae* hindgut were produced for both CabTRP Ia (filled circles) and CabTRP Ib (filled triangles). The relative activity is given as a percentage of the maximal tonus increase of individual hindguts in response to CabTRP Ia. As is evident from this graph, while both peptides induced a dose-dependent increase in the tonus of the hindgut, the EC_{50} of CabTRP Ia was approximately 500 times lower than that of CabTRP Ib. N=4 preparations for CabTRP Ia; N=3 for CabTRP Ib. Error bars indicate standard error of mean.

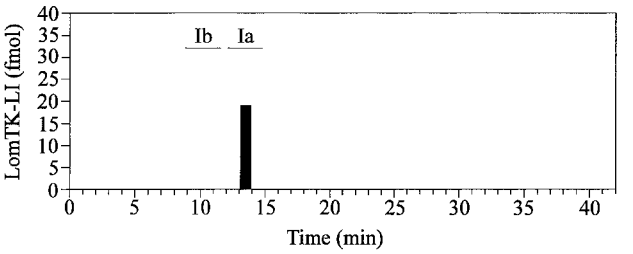

Fig. 8. CabTRP Ia is the only TRP-like moiety detected in the crab stomatogastric ganglion. To determine whether CabTRP Ia and/or Ib were present in the crab STG, tissue extract was prepared from 24 STGs and separated with reversed-phase HPLC on a Vydac Diphenyl column. Fractions were collected at 1 min intervals and assayed for TRP-like immunoreactivity using the LomTK I radioimmunoassay. One fraction was found to be TRP-immunopositive. The retention time of this fraction corresponds to that of synthetic CabTRP Ia.

of this peptide reported in an earlier study (Muren and Nässel, 1996*a*). The EC_{50} values we determined for CabTRP Ia and Ib were approximately 6.0×10^{-9} mol l^{-1} and approximately 10^{-8} mol l^{-1}, respectively. This suggests that the LomTK I RIA is 500–1000 times less sensitive to the CabTRPs than it is to LomTK I. The results of other cross-reactivity tests using *L. maderae* TRPs (LemTRPs) suggest that peptides containing a methionine residue in the X_2 position of the carboxy terminus (-FX_1GX_2R-NH$_2$) are significantly less immunoreactive in the LomTK I RIA than are TRPs which contain a valine residue in this position (Å. M. Winther, J. E. Muren, C. T. Lundquist and D. R. Nässel, unpublished observations). Given the cross-reactivity of the CabTRP isoforms in the LomTK RIA, we predict that approximately 10^{-12} mol of CabTRP Ia or Ib would be detected as approximately 10^{-15} mol of LomTK- like immunoreactivity in this assay.

Our original isolation of CabTRPs from the CNS utilized an initial extraction with the highly acidic Bennett's solution. This is optimal for preventing oxidation of peptides and can be used directly on HPLC. However, this extraction medium gives rise to a relatively high background level of non-specific immunoreactivity in the LomTK I RIA (J. E. Muren and D. R. Nässel, unpublished observations). Thus, for determination of CabTRP isoforms in the STG, the tissue extraction was performed using a solution of methanol, water and acetic acid (90:9:1). This solution produces a lower background level of TRP-immunoreactivity (J. E. Muren and D. R. Nässel, unpublished observations).

To determine the presence of CabTRP isoforms in STG extract, HPLC was performed using a Vydac Diphenyl column and a linear gradient MeCN from 15 % to 40 % over 40 min (approximately 0.6 % min^{-1}). This column was selected because the two CabTRP isoforms were readily separable on this column in the original isolation (Fig. 2B). Initially, we ran synthetic peptides (1 nmol each) on this column system and found both peptides were ultraviolet-detectable and readily separable (N=2 runs). Here, CabTRP Ia eluted from 12 to 14 min, and CabTRP Ib eluted earlier, from 9 to 11 min. It was possible that these peptides would have different elution profiles in a complex tissue extract than they did when run alone, so we confirmed the retention times of CabTRP Ia and Ib in Sep-Pak-purified tissue extract by spiking the extract with 10^{-9} mol of each synthetic peptide prior to its application to the system. In this experiment, the whole STNS, which includes the STG, was used. HPLC eluate was collected from this spiked run at 1 min intervals and the resulting fractions were measured in the LomTK RIA. Using this methodology, we found that CabTRP Ib was present in fractions 10–12 but most was concentrated in fraction 11, while CabTRP Ia eluted in fractions 13–15 and was most concentrated in fraction 14. These results showed that the elution profiles of these peptides did not change in the presence of tissue extract. The shift of retention time by 1 min resulted from the time necessary to pass through the automated fraction collector. As can be seen from Fig. 8, when non-spiked STG extract was run on this HPLC system, immunoreactive material with a retention time corresponding to that of CabTRP Ia was detected, but no immunoreactivity was evident with a retention time similar to that of CabTRP Ib. In fact, no immunoreactive fractions other than that corresponding to CabTRP Ia were seen in this extract. On the basis of the results of this experiment, in combination with the cross-reactivity tests, it appears that there is approximately 3.75×10^{-13} mol of CabTRP Ia per STG.

CabTRP Ia blocks substance-P-like and LomTK-like immunolabeling in the crab STG

As was discussed previously, tachykinin-like immunoreactivity is present in the crab STNS, including the STG. Specifically, neurons in this system are labeled by a rat

monoclonal antibody to substance P (Goldberg *et al.* 1988) and a rabbit polyclonal antiserum generated against LomTK I (Nässel, 1993; Blitz *et al.* 1995). Moreover, double-labeling experiments have demonstrated that these antibodies label an identical set of neuronal profiles in the STNS (Blitz *et al.* 1995). In fact, the only difference between the staining patterns produced by these two antibodies in the crab STNS is the presence of a set of non-neuronal, microglia-like cells in preparations stained with the LomTK I antiserum. Our results from the RIA experiments on purified extract from the STG suggest that CabTRP Ia is responsible for the TRP-related immunolabeling in the STG. This experiment did, however, utilize the same LomTK I antibody that was employed for mapping the distribution of tachykinin-like immunoreactivity in the STNS (Blitz *et al.* 1995). Thus, it was possible that the CabTRP Ia detected in the STG was due to the presence of the peptide in the microglia-like profiles and not in the neuronal structures. To confirm that CabTRP Ia is indeed responsible for the TRP-like immunolabling in the neuronal profiles of the STG, we conducted antibody preabsorption controls with this peptide for both anti-substance P and anti-LomTK I. When anti-substance P (1:300 dilution) was preincubated with CabTRP Ia (10^{-4} mol l^{-1}) and subsequently used for immunolabeling, no staining was detectable in the STG or any other region of the STNS (*N*=4 preparations). Similarly, when anti-LomTK I (1:300 dilution) was preincubated with CabTRP Ia (10^{-4} mol l^{-1}), there was a complete block of immunolabeling in all neuronal and non-neuronal profiles (*N*=4 preparations).

Effects of CabTRPs on the crab stomatogastric system

The TRP immunoreactivity within the STG results from the arborization of a paired projection neuron called modulatory commissural neuron 1 (MCN1; Christie *et al.* 1993; Coleman and Nusbaum, 1994; Christie, 1995). MCN1 projects to the STG from the commissural ganglion, *via* the inferior

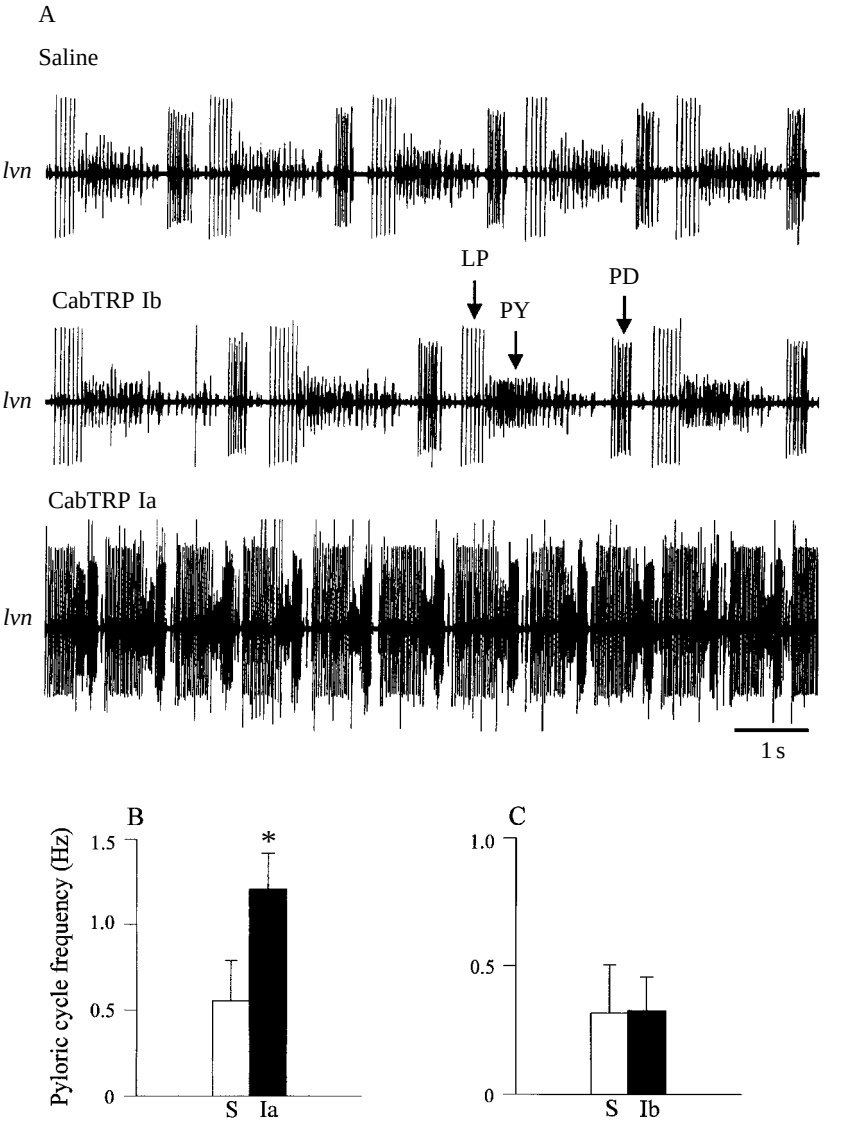

Fig. 9. Modulatory action of CabTRP Ia and Ib on the pyloric motor pattern. (A) Comparison of the effects of CabTRP Ia and Ib on the same isolated stomatogastric ganglion. The pyloric motor pattern is characterized by rhythmic alternating bursts of activity in the lateral pyloric (LP), pyloric (PY) and pyloric dilator (PD) neurons. These neurons are monitored simultaneously *via* an extracellular recording of the lateral ventricular nerve (*lvn*). As is evident in the top trace, there was a relatively slow pyloric rhythm occurring in physiological saline. Application of CabTRP Ib (10^{-6} mol l^{-1}) had little effect on the ongoing pyloric rhythm (middle trace). In contrast, application of CabTRP Ia (10^{-6} mol l^{-1}; bottom trace) increased both the pyloric cycle frequency and impulse activity in the LP neuron. (B,C) CabTRP Ia (10^{-6} mol l^{-1}), but not CabTRP Ib (10^{-6} mol l^{-1}), caused a statistically significant increase in the pyloric cycle frequency. Data are plotted as the mean pyloric cycle frequency + S.D. One pyloric cycle extends from the onset of one PD neuron burst to the onset of the subsequent PD burst. The cycle frequency is the inverse of the duration of a cycle. Means of the pyloric cycle frequency in saline (S) and in the presence of either CabTRP Ia (Ia; *N*=5 preparation) or CabTRP Ib (Ib; *N*=4 preparation) were compared using a paired Student's *t*-test. **P*<0.05. Note the different scales of pyloric cycle frequency in B and C.

oesophageal and stomatogastric nerves (Fig. 1; Coleman and Nusbaum, 1994). MCN1 activity influences the gastric mill and pyloric motor patterns, which are generated by neural network activity within the STG (Nusbaum *et al.* 1992; Coleman and Nusbaum, 1994; Coleman *et al.* 1995; Bartos and Nusbaum, 1997). Here, we examined the effects of the CabTRPs on the pyloric system. In several of these preparations, we also compared the modulation of this system by CabTRP Ia with that produced by superfusion of LomTK, the insect TRP whose influence on the STG was studied previously (Blitz *et al.* 1995).

Incubation of CabTRP Ia with the isolated STG routinely excited the pyloric motor pattern (Fig. 9). In five of five preparations with ongoing pyloric rhythms, CabTRP Ia (10^{-6} mol l^{-1}) significantly increased the pyloric rhythm cycle frequency (Fig. 9B). These increases in frequency ranged from 78 to 631 %, with the absolute cycle frequencies in CabTRP Ia ranging from 0.89 Hz to 1.42 Hz. In four of these preparations, LomTK II (10^{-6} mol l^{-1}) was also applied. In these preparations, LomTK II also increased the cycle frequency of the pyloric rhythm, but to a considerably lesser extent than did CabTRP Ia. Here, the frequency increases ranged from 22 to 111 %, with the absolute cycle frequencies ranging from 0.29 to 0.64 Hz (data not shown).

In addition to increasing the pyloric rhythm cycle frequency, CabTRP Ia (10^{-6} mol l^{-1}) produced statistically significant alterations in the impulse activity of several pyloric network neurons (Table 4). Specifically, there were increases in the number of spikes per burst in the lateral pyloric (LP), inferior cardiac (IC) and ventricular dilator (VD) neurons. Only one of these neurons, the IC neuron, has previously been shown to exhibit altered impulse activity in the presence of LomTK (Blitz *et al.* 1995). Here also, LomTK only elicited a statistically significant increase in the number of spike per burst in IC. This increase was, however, not as large as with CabTRP Ia.

Unlike CabTRP Ia, CabTRP Ib produced few consistent effects on the pyloric system. Incubation of CabTRP Ib (10^{-6} mol l^{-1}) with the STG produced no significant change in either the cycle frequency of the ongoing motor pattern (Fig. 9) or in the impulse activity of the IC and VD neurons (Table 4). The sole parameter in which there was a statistically significant change elicited by CabTRP Ib was the impulse activity of the LP neuron. Here, CabTRP Ib caused a modest increase in the number of spikes per burst, from 3.0 to 3.6 (Table 4).

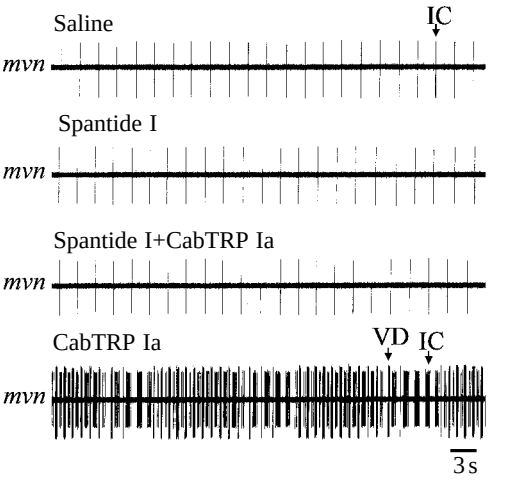

Fig. 10. The broad-spectrum vertebrate tachykinin-receptor antagonist Spantide I suppresses the CabTRP-Ia-mediated excitation of the pyloric rhythm. Here the pyloric rhythm is monitored *via* an extracellular recording of the medial ventricular nerve (*mvn*), in which is recorded the activity of the inferior cardiac (IC) and ventricular dilator (VD) neurons of the pyloric system. The pyloric rhythm was also monitored *via* a lateral ventricular nerve recording (not shown). In normal saline (top trace) and saline containing Spantide I (10^{-4} mol l^{-1}), pyloric-rhythm-timed activity was present in IC but not VD. Co-application of Spantide I (10^{-4} mol l^{-1}) and CabTRP Ia (10^{-7} mol l^{-1}) to the isolated stomatogastric ganglion produced no change in the ongoing motor pattern (third trace). In contrast, application of CabTRP Ia (10^{-7} mol l^{-1}) alone to the stomatogastric ganglion increased the pyloric cycle frequency (note the briefer duration between successive IC bursts; bottom trace). This peptide application also increased the impulse activity in IC and elicited rhythmic bursting in VD.

Suppression of CabTRP Ia actions by the vertebrate tachykinin receptor antagonist Spantide I

The NKD receptor is a *Drosophila* TRP receptor with homology to the vertebrate NK1–3 receptors (Monnier *et al.* 1992). When expressed *in vitro*, the NKD receptor is activated by LomTK and, like its vertebrate homologs, this activation can be blocked by the broad-spectrum antagonist Spantide I ([D-Arg^{1},D-TRP^{7,9},Leu^{11}]-substance P; Folkers *et al.* 1984) (Monnier *et al.* 1992). Because CabTRP Ia is structurally similar to the insect TRPs (Table 3), we investigated whether Spantide I could also suppress the actions of CabTRP Ia in the crab STG.

Table 4. *Effects of* Cancer borealis *tachykinin-related peptides Ia and Ib on impulse activity of pyloric neurons*

	Spikes per burst			Spikes per burst		
Neuron	Saline	10^{-6} mol l^{-1} CabTRP Ia	*N*	Saline	10^{-6} mol l^{-1} CabTRP Ib	*N*
LP	2.8±2.2	11.2±3.7**	4	3.0±3.1	3.6±3.0*	3
IC	0.8±0.9	2.7±1.4*	5	0.1±0.2	0.3±0.5	4
VD	0±0	1.8±1.2*	5	0±0	0±0	4

Values are means ± S.D.; *N*, number of preparations.
Statistically significant difference compared with the control value in saline based on a paired Student's *t*-test, $^{**}P \leqslant 0.01$, $^{*}P \leqslant 0.05$.
LP, lateral pyloric neuron; IC, inferior cardiac neuron; VD, ventricular dilator neuron.

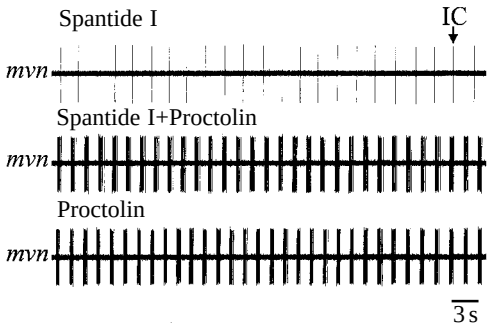

Fig. 11. Spantide I does not suppress the modulatory action of the neuropeptide proctolin on the pyloric rhythm. Here again the pyloric rhythm is monitored by an extracellular recording of the medial ventricular nerve (*mvn*), although the lateral ventricular nerve (*lvn*) was also recorded (not shown). In normal saline (see Fig. 10) and saline containing 10^{-4} mol l^{-1} Spantide I, the pyloric rhythm was cycling slowly, IC was weakly active and VD was silent. Co-application of Spantide I (10^{-4} mol l^{-1}) and proctolin (10^{-7} mol l^{-1}) to the isolated stomatogastric ganglion excited the pyloric rhythm. This included increasing IC neuron impulse activity (second trace) without activation of VD (Nusbaum and Marder, 1989). This response was indistinguishable from the response to application of proctolin (10^{-7} mol l^{-1}; bottom trace) alone.

As a control, we first examined whether Spantide I itself influenced the STG motor output. Application of Spantide I (10^{-4} mol l^{-1}) to the STG did not alter the pyloric rhythm (*N*=4 preparations; Fig. 10). Similarly, there was no change in the pyloric rhythm when Spantide I (10^{-4} mol l^{-1}) was co-applied with CabTRP Ia (10^{-7} mol l^{-1}) (*N*=4 preparations; Fig. 10). To ensure that this co-application result represented Spantide-mediated suppression of CabTRP-Ia-mediated excitation of the pyloric rhythm, we also applied CabTRP Ia (10^{-7} mol l^{-1}) alone to the STG both before and after the co-application. As is shown in Fig. 10, application of CabTRP Ia alone readily excited the pyloric rhythm (*N*=4 preparations). There was no apparent long-term effect of Spantide I because the effects of CabTRP Ia were identical both before and after each co-application experiment.

To test the specificity of Spantide I as an inhibitor of CabTRP-Ia-elicited effects in the STG, we examined whether Spantide I would also interfere with the ability of the pentapeptide proctolin to excite the pyloric rhythm. Proctolin was chosen because it is the peptide cotransmitter of CabTRP Ia in MCN1 (Christie *et al.* 1993; Christie, 1995). Proctolin superfusion causes a dose-dependent excitation of the pyloric rhythm in the crab STG (Marder *et al.* 1986; Nusbaum and Marder, 1989). In the same preparations where Spantide I reversibly suppressed the pyloric network response to CabTRP Ia (10^{-7} mol l^{-1}), proctolin (10^{-7} mol l^{-1}) continued to excite the pyloric rhythm effectively whether or not Spantide I (10^{-4} mol l^{-1}) was co-applied (*N*=4; Fig. 11).

Discussion

We report here the isolation and characterization of two novel neuropeptides, APSGFLGMR-NH_2 and SGFLGMR-NH_2, from the nervous system of the crab *Cancer borealis*. The amino acid sequence of these peptides includes the carboxy-terminal sequence -FLGMR-NH_2, which places them as members of the invertebrate tachykinin-related peptide (TRP) family. This family is characterized by the carboxy-terminal sequence -FX_1GX_2R-NH_2. Members of this peptide family have been isolated and characterized from several insect species and an echiuroid worm (Table 3; Schoofs *et al.* 1990*a*,*b*; Clottens *et al.* 1993; Ikeda *et al.* 1993; Lundquist *et al.* 1994; Muren and Nässel, 1996*b*, 1997). We have therefore named these peptides *Cancer borealis* tachykinin-related peptide Ia (CabTRP Ia) and Ib (CabTRP Ib). These peptides are the first members of this family to be isolated from a crustacean.

The nervous systems of several crustacean species exhibit tachykinin-like immunoreactivity (Fingerman *et al.* 1985; Goldberg *et al.* 1988; Sandeman *et al.* 1990; Blitz *et al.* 1995; Christie *et al.* 1995). On the basis of the distribution of this immunolabeling, it has been postulated that TRPs serve both as locally released neuromodulators and as circulating hormones (Christie *et al.* 1995). The crab stomatogastric ganglion (STG) is one region innervated by TRP-like immunoreactive fibers (Goldberg *et al.* 1988; Blitz *et al.* 1995; Christie, 1995; Christie *et al.* 1997). In fact, the LomTK I antiserum used here to isolate the CabTRPs reveals TRP-like immunoreactivity in this ganglion (Blitz *et al.* 1995). We have shown here that CabTRP Ia is likely to function as a local modulator of neural network activity. Specifically, we have demonstrated that CabTRP Ia is present in the STG and that it represents the major if not the sole isoform of TRP in this ganglion. From our experiments, we estimate there to be 3.75×10^{-13} mol of CabTRP Ia per STG. This value compares well with the estimated amounts of other peptides in the STG, specifically proctolin (2.5×10^{-13} mol per ganglion; Marder *et al.* 1986) and TNRNFLRF-NH_2 (2.25×10^{-13} mol per ganglion; Marder *et al.* 1987; Weimann *et al.* 1993). Moreover, application of CabTRP Ia to the isolated STG excited the pyloric rhythm. In contrast, application of CabTRP Ib to the isolated STG produced no consistent response from the pyloric system. Collectively, these findings strongly suggest a neuromodulatory role for CabTRP Ia in the crab STG.

We have also shown that the pyloric rhythm excitation by CabTRP Ia is suppressed by Spantide I, a peptide antagonist of the NK1–3 receptors of vertebrates (Leander *et al.* 1981; Maggi *et al.* 1993; Maggi, 1995). This inhibitory effect of Spantide I on the actions of CabTRP Ia in the STG suggests a structural similarity between portions of the crab TRP receptors and vertebrate tachykinin receptors. In *Drosophila melanogaster*, two receptor proteins similar in structure to the NK1–3 receptors of vertebrates have been identified (Li *et al.* 1991; Monnier *et al.* 1992). One of these receptors, the NKD receptor, exhibits 38 % amino acid sequence identity to the transmembrane domains of the NK3 receptor, 35 % identity to those of the NK1 receptor and 32 % identity to those of the NK2 receptor (Monnier *et al.* 1992). Moreover, NKD-transfected cell lines show an increase in inositol trisphosphate

levels upon stimulation with the locust TRP LomTK II (Monnier *et al.* 1992). As in the crab, this TRP-mediated action is blocked by Spantide I (Monnier *et al.* 1992). This work on the NKD receptor, in conjunction with our findings in crab, strengthens the hypothesis that the $FX_1GX_2R\text{-}NH_2$ peptide family in invertebrates truly represents an ancient subgroup of the tachykinin superfamily of peptides (Schoofs *et al.* 1990*a*,*b*, 1993; Nachman *et al.* 1990).

We isolated approximately 20 times more CabTRP Ia than CabTRP Ib from the crab nervous system. Given the difference in the amount recovered for each peptide, their amino acid sequences and their physiological actions on the STG, it is possible that CabTRP Ib represents a degradation product of CabTRP Ia. In *C. borealis*, the pentapeptide proctolin is known to be cleaved by an extracellular peptidase activity (Coleman *et al.* 1994). The major cleavage product produced from proctolin showed a marked decrease in bioactivity in the STG relative to proctolin, while no other products exhibited bioactivity (Coleman *et al.* 1994). There are, however, also instances in other systems where peptide cleavage activates or alters the physiological actions of the molecule (Turner *et al.* 1985; Thorsett and Wyvratt, 1987; Hall and Lloyd, 1990; Owens *et al.* 1992). It remains, of course, also possible that extracellular peptidase degradation is not responsible for the production of CabTRP Ib. Instead, the shorter form may be directly cleaved from a precursor protein. If so, then CabTRP Ib presumably serves a physiological role in some other system in *C. borealis*.

The amino acid sequence identity between CabTRP Ia and other members of the TRP family is not limited to the carboxy terminus. The amino-terminal sequence APSG- of CabTRP Ia is also highly conserved across TRPs. Of the 20 TRPs (excluding CabTRP Ia and Ib), three contain this sequence (Table 3). Four others have amino-terminal sequences that differ by only one of these four amino acids, while seven of the remaining peptides are identical at two of the four positions. The most highly conserved amino acid is the proline residue at position two. Fifteen of the 20 peptides show amino acid identity at this position. The functional relevance of this conserved amino-terminal sequence remains unclear. It is interesting to note that the amino-terminal sequence APSG- is conserved not only among TRPs, but is also found in a number of non-TRP peptides, including *Diploptera punctata* allatostatin 7 (Dip-AST 7, formerly known as allatostatin I; Donly *et al.* 1993).

In conclusion, we have isolated two TRPs, CabTRP Ia and Ib, from the *C. borealis* central nervous system and demonstrated the presence of CabTRP Ia in the STG. We have also shown that this peptide modulates the pyloric motor pattern within the STG and that this modulation can be suppressed by the broad-spectrum tachykinin receptor antagonist Spantide I. We can now proceed to analyze in detail the actions of the endogenous TRP on the STG neural network. Moreover, because modulatory commissural neuron 1 (MCN1) is the only local source of CabTRP Ia in the STG (Christie *et al.* 1993), we can determine how CabTRP Ia contributes to the action of MCN1 in the STG. MCN1 is also known to contain the peptide proctolin and the small molecule γ-aminobutyric acid (GABA) as cotransmitters (Christie *et al.* 1993; Christie, 1995). Thus, we can also begin to determine the relative contribution of each of these cotransmitters to the transmitter-mediated effects of MCN1 on the STG. This continuing investigation will provide a detailed understanding of the neuromodulatory actions of TRPs at the cellular and systems level.

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