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Identification, physiological actions, and distribution of TPSGFLGMRamide: a novel tachykinin-related peptide from the midgut and stomatogastric nervous system of *Cancer* crabs

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Abstract

In most invertebrates, multiple species-specific isoforms of tachykinin-related peptide (TRP) are common. In contrast, only a single conserved TRP isoform, APSGFLGMRamide, has been documented in decapod crustaceans, leading to the hypothesis that it is the sole TRP present in this arthropod order. Previous studies of crustacean TRPs have focused on neuronal tissue, but the recent demonstration of TRPs in midgut epithelial cells in *Cancer* species led us to question whether other TRPs are present in the gut, as is the case in insects. Using direct tissue matrix assisted laser desorption/ionization Fourier transform mass spectrometry, in combination with sustained off-resonance irradiation collision-induced dissociation, we found that at least one additional TRP is present in *Cancer irroratus*, *Cancer borealis, Cancer magister,* and *Cancer productus*. The novel TRP isoform, TPSGFLGMRamide, was present not only in the midgut, but also in the stomatogastric nervous system (STNS). In addition, we identified an unprocessed TRP precursor APSGFLGMRG, which was detected in midgut tissues only. TRP immunohistochemistry, in combination with preadsorption studies, suggests that APSGFLGMRamide and TPSGFLGMRamide are co-localized in the stomatogastric ganglion (STG), which is contained within the STNS. Exogenous application of TPSGFLGMRamide to the STG elicited a pyloric motor pattern that was identical to that elicited by APSGFLGMRamide, whereas APSGFLGMRG did not alter the pyloric motor pattern.

Keywords: APSGFLGMRG, APSGFLGMRamide, *Cancer borealis* tachykinin-related peptide, TPSGFLGMRamide, matrix assisted laser desorption/ionizaton Fourier transform mass spectrometry, stomatogastric nervous system, sustained off-resonance irradiation collision-induced dissociation. *J. Neurochem.* (2007) **101**, 1351–1366.

The tachykinins are one of the largest and most diverse groups of peptides in the animal kingdom. In all vertebrates and in one invertebrate chordate, as well as in salivary tissue of some blood-feeding and venomous invertebrates (e.g., mosquitoes and octopi), members of the tachykinin family possess the carboxy (C)-teminal amino acid motif -F*X*GLMamide, where *X* represents a variable residue (Nachman *et al.* 1999; Nässel 1999; Vanden Broeck *et al.* 1999; Severini *et al.* 2002; Satake *et al.* 2004). In invertebrate neural and gut tissues, a family of peptides containing the C-terminal amino acid motif -FX_IGX_{II}Ramide (with *X*s again representing variable residues) has been identified;

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Abbreviations used: CabTRP Ia, *Cancer borealis* tachykinin-related peptide Ia; CabTRP Ib, *Cancer borealis* tachykinin-related peptide Ib; CabTRP II, *Cancer borealis* tachykinin-related peptide II; CaiTRP, *Cancer irroratus* tachykinin-related peptide; CoGs, commisural ganglias; DHB, dihydroxybenzoic acid; LP, lateral pyloric; MALDI-FTMS, matrix assisted laser desorption/ionizaton Fourier transform mass spectrometry; PD, pyloric dilator; PMC, posterior midgut caecum; POs, pericardial organs; PPG, poly(propylene glycol); PY, pyloric; SGs, sinus glands; SORI-CID, sustained off-resonance irradiation collision-induced dissociation; STG, stomatogastric ganglion; STNS, stomatogastric nervous systems; TRP, tachykinin-related peptide.

these peptides have collectively been termed tachykinin-related peptides or TRPs (Nachman *et al.* 1999; Nässel 1999; Vanden Broeck *et al.* 1999). Comparisons of sequence homology, tissue distribution, chemical/conformational requirements for receptor interaction and physiological function suggest a common evolutionary origin for the vertebrate- and invertebrate-type peptide subgroups (Nachman *et al.* 1999; Nässel 1999; Vanden Broeck *et al.* 1999; Severini *et al.* 2002).

For many invertebrate species, particularly insects, there is an extensive literature documenting the presence of large families of TRPs in neural and/or gut tissues (Schoofs *et al.* 1990a,b, 1993; Lundquist *et al.* 1994; Muren and Nässel 1996, 1997; Meola *et al.* 1998; Kawada *et al.* 1999, 2000; Veelaert *et al.* 1999; Siviter *et al.* 2000; Riehle *et al.* 2002; Takeuchi *et al.* 2003; Predel *et al.* 2005). For the most part, the TRPs thus far identified appear to be species-specific, though a few insect isoforms have been identified in multiple species (Muren and Nässel 1996, 1997; Meola *et al.* 1998; Predel *et al.* 2005). Somewhat surprisingly, only one TRP has been identified in decapod crustaceans: APSGFLGMR-amide. To date, this peptide, commonly referred to as *Cancer borealis* tachykinin-related peptide Ia or CabTRP Ia, has been found in authentic form in neural tissue from six species spanning four infraorders (Christie *et al.* 1997; Nieto *et al.* 1998; Huybrechts *et al.* 2003; Yasuda-Kamatani and Yasuda 2004; Messinger *et al.* 2005; Stemmler *et al.* 2005). The conservation of this peptide across infraorders, in combination with a recent cloning study (Yasuda-Kamatani and Yasuda 2004), has lead to the hypothesis that CabTRP Ia is the only TRP isoform present in decapod crustaceans (Yasuda-Kamatani and Yasuda 2004). It should be noted that a truncated version of APSGFLGMRamide, SGFLGMRamide or *Cancer borealis* tachykinin-related peptide Ib (CabTRP Ib), was isolated from the crab *C. borealis*, but it is postulated to be a degradation product of the former peptide (Christie *et al.* 1997).

Recently, we identified a population of TRP-immunopositive epithelial endocrine cells in the posterior portion of the midgut of several species of *Cancer* crab (Christie *et al.* 2007). Given that the midguts of insects are known to be rich sources of TRPs (Lundquist *et al.* 1994; Muren *et al.* 1995; Veenstra *et al.* 1995; Nässel *et al.* 1995; Muren and Nässel 1996; Winther *et al.* 1999; Meola *et al.* 1998; Pabla and Lange 1999; Veelaert *et al.* 1999; Winther *et al.* 1999; Siviter *et al.* 2000; Sliwowska *et al.* 2001; Lange, 2001; Winther and Nässel 2001; Skaer *et al.* 2002; Johard *et al.* 2003; Predel *et al.* 2005), and include some gut-specific isoforms (Muren and Nässel 1996, 1997; Veelaert *et al.* 1999; Winther *et al.* 1999; Predel *et al.* 2005), we became interested in identifying the TRPs present in the midgut cells of *Cancer* crabs and in comparing them to those present in neural tissues. In this study, we report the identification of a novel TRP isoform, TPSGFLGMRamide, in *Cancer* crabs. We identified this isoform using direct tissue matrix assisted laser desorption/ionization Fourier transform mass spectrometry (MALDI-FTMS), a high resolution MS technique that can be used, in conjunction with sustained off-resonance irradiation collision-induced dissociation (SORI-CID), to identify and sequence neuropeptides from crustacean tissue samples (Kutz *et al.* 2004; Stemmler *et al.* 2005). Moreover, we have assessed the ability of TPSGFLGMRamide to block staining by a substance P antibody in tissues that show TPR-like immunoreactivity, and have compared the physiological effects of bath-applied CabTRP Ia and TPSGFLGMRamide on the stomatogastric neural circuit, which is responsible for the movement of the pyloric region of the foregut. Some of these data have appeared previously in abstract form (Peguero *et al.* 2006).

Materials and methods

Animals

Jonah crabs *C. borealis* and Atlantic rock crabs *Cancer irroratus* were purchased from local seafood suppliers in Brunswick and Harpswell, ME, USA. Red rock crabs *Cancer productus* and Dungeness crabs *Cancer magister* were collected by hand or trap at multiple locations in the San Juan Archipelago and greater Puget Sound areas of Washington State, USA. California spiny lobsters *Panulirus interruptus* were purchased from Tomlinson Commercial Fishing (San Diego, CA, USA). Red swamp crayfish *Procambarus clarkii* were purchased from Carolina Biological Supply (Burlington, NC, USA). Animals of all species, except *P. clarkii,* were maintained in aerated natural seawater aquaria; *P. clarkii* were maintained in aerated tanks of aged tap water. The holding temperature for all crab species was 8–10°C, while that for *P. interruptus* was approximately 15°C and *P. clarkii* was 20°C.

Peptide standards

APSGFLGMRamide was a gift from Dr Michael Nusbaum (Department of Neuroscience, University of Pennsylvania School of Medicine, Philadelphia, PA, USA). TPSGFLGMRamide and APSGFLGMRG were custom synthesized by GenScript Corporation (Piscataway, NJ, USA).

Tissue collection

For tissue collection, animals were anesthetized by packing in ice for 30–60 min, after which the dorsal carapace was removed and the foregut and midgut were dissected from the animal in chilled (approximately 10°C) physiological saline (composition of *Cancer* saline: 442 mmol/L NaCl, 11 mmol/L KCl, 13 mmol/L $CaCl_2$, 26 mmol/L $MgCl_2$, 12 mmol/L Trizma base, 1.2 mmol/L maleic acid; pH 7.4–7.5; composition of *Panulirus* saline: 479 mmol/L NaCl, 12.8 mmol/L KCl, 13.7 mmol/L $CaCl_2$, 10 mmol/L $MgSO_4$, 3.9 Na_2SO_4, 11 mmol/L Trizma base, 4.8 mmol/L maleic acid; pH 7.5–7.6; composition of *Procambarus* saline: 210 mmol/L NaCl, 5.4 mmol/L KCl, 13.5 mmol/L $CaCl_2$, 5 mmol/L $MgCl_2$, 10 mmol/L HEPES; pH 7.4). For experiments with *C. irroratus*, the eyestalks (containing the neuroendocrine sinus glands [SGs]) and the pericardial chamber (containing the pericardial organs

[POs], another neuroendocrine structure) were also isolated at this time. To obtain the stomatogastric ganglion (STG) and the commissural ganglia (CoGs), the foregut was flattened by making a longitudinal cut on its ventral side from the oesophagous to the pylorus, followed by a pair of medial cuts directed along the ossicles of the cardiac sac/gastric mill. After opening, the foregut was flattened and pinned, inside down, in a Sylgard 170-(KR Anderson, Santa Clara, California, USA) lined Pyrex dish containing chilled physiological saline. The STG and paired CoGs were then isolated from the foregut musculature via manual microdissection; tissue preparation for physiology as described below. To obtain the SG from *C. irroratus*, the carapace encasing an eyestalk was split both dorsally and ventrally and the two halves of the split shell were gently teased apart. The half of the eyestalk containing the optic ganglia was then pinned in a Sylgard 184-(KR Anderson) or wax-lined Pyrex dish filled with chilled physiological saline, and the SG, attached to the optic ganglion, was isolated. POs from *C. irroratus* were obtained by pinning the pericardial chamber in a Sylgard-lined Pyrex dish containing chilled physiological saline and dissecting the nerve roots forming this endocrine structure free from the surrounding musculature. To isolate the posterior midgut caecum (PMC), the posterior section of the midgut and the anterior region of the hindgut were pinned in saline and a small piece of the attached PMC was removed for analysis. Regardless of type, the isolated tissue was then pinned in a Sylgard 184-lined Petri dish filled with chilled physiological saline and processed for mass spectrometry or immunohistochemistry.

MALDI-FTMS

Sample preparation for MALDI-FTMS analysis

To prepare samples for direct tissue MALDI-FTMS, tissues were removed from the saline with fine forceps, rinsed sequentially in two 12 μL droplets of 0.75 mol/L fructose (Sigma–Aldrich, St Louis, MO, USA) and placed on a face of a ten-faceted probe tip, minimizing co-transfers of solution. A small piece of PMC, STG, CoG (sheath removed), SG, or PO was applied to the probe tip and the tissue was then sliced 10–20 times with a 0.1 mm needle, gathered together and covered with a 0.5 μL droplet of 1.0 mol/L 2,5-dihydroxybenzoic acid (DHB – Sigma–Aldrich, sublimed prior to use), prepared in 1 : 1 acetonitrile : water containing 2% phosphoric acid.

The spectra are representative of spectra measured from PMC and CoG tissues dissected from a minimum of three animals of each species. For *P. interruptus,* over 20 CoG tissue samples were examined. For *P. clarkii*, two CoGs from a single animal were assayed.

For some experiments, sections of PMC were extracted, delipidated, and concentrated using the following procedure. The PMC was removed from the animal in saline, washed in fructose and then placed in 50 μL of extraction solvent (64% methanol, 7% acetic acid, and 29% water). The tissue was homogenized with fine scissors and sonicated for 5 min. The homogenate was centrifuged at 2200 *g* for approximately 5 min in a mini centrifuge (Fisher Scientific, Pittsburgh, PA, USA), after which the extraction solvent was removed from the tissue and transferred to a clean tube. Water (20 μL) was added to the tissue remaining in the first tube and this mixture was sonicated and centrifuged at 2200 *g*, and the liquid was then combined with the original extraction solvent. The combined solvents were then delipidated by extracting three times with 20 μL of chloroform. The aqueous layer was transferred to a clean tube and dried using a SpeedVac (Thermo Electron, Waltham, MA, USA) at 25°C. The extract was then reconstituted in 5 μL of 0.1% trifluoroacetic acid in water.

Mass spectrometric instrumentation

Samples were analyzed using a HiResMALDI Fourier transform mass spectrometer (IonSpec) with a Cryomagnetics (Oak Ridge, TN, USA) 4.7 Tesla actively-shielded superconducting magnet. Ions were generated using a pulsed nitrogen laser (337 nm) and were transported from the external ion source to the closed cylindrical cell using a quadrupole ion guide. The ion guide radio frequency potential and trapping delay time were optimized to transmit and trap ions of a selected mass range (optimized for *m/z* 1500 for the results presented here). A pulse of argon was introduced to the vacuum system during trapping to transiently elevate the system pressure for collisional cooling. All spectra were measured using ion accumulation techniques, where ions from seven successive laser shots were accumulated in the cell. A delay of 5–10 s preceded ion detection, which occurred with analyzer pressures of $1–2 \times 10^{-10}$ Torr. Transients from direct tissue spectra were apodized using a Blackman function and zero-filled prior to fast Fourier transformation while SORI-CID spectra were processed without apodization.

Exact mass measurements were made on internally calibrated spectra using the internal calibration on adjacent samples technique (O'Connor and Costello 2000), modified to include the accumulation of mass-selected calibrant ions (Stemmler *et al.* 2005). A mixture of poly(propylene glycol) 725 and 2000 (PPG; Sigma–Aldrich) was used as a calibrant. The calibrated masses reported in Table 1 reflect the average of three calibrations performed on the same sample.

For SORI-CID experiments, ions were selected using a correlated harmonic excitation fields isolation of precursor ions (de Koning *et al.* 1997), argon was used as the collision gas, the frequency offset was set to −1.8% of the reduced cyclotron frequency (Mirgorodskaya *et al.*, 2002) and the voltage amplitude was in the range of 6–8.5 V_{bp}. SORI-CID spectra were calibrated externally, with a one-point adjustment based upon a $[MH–NH_3]^+$ or $[MH–H_2O]^+$ fragment mass.

Mass spectrometric data analysis

Ion intensities were extracted from the MALDI-FT mass spectra using the Omega 8 software (IonSpec, Lake Forest, CA, USA) and percentages were calculated using Microsoft Excel. The intensity measurements were selected from spectra with signal-to-noise characteristics that would permit adequate intensity measurements (a software-derived scale factor of 250 or lower for *m/z* 934). We included 2–9 spectra for each animal, and used the mean values from three animals (four for the PMC from *C. borealis*) in calculating percentages and SE. Statistical analysis and graphing was performed using Prism 4 (GraphPad Software, San Diego, CA, USA). Statistical analysis involved two-way ANOVA testing, followed by Bonferroni *post hoc* testing when the ANOVA yielded a significant *p*-value.

Antibody adsorption studies

Previous immunohistochemical studies using a rat monoclonal antibody to substance P (clone NC1/34 HL; Cuello *et al.* 1979) have shown the presence of TRP-like immunoreactivity in the PMC and/or CoGs and STG of *C. borealis*, *C. magister* and *C. productus* (Christie *et al.* 1997, 2007; Messinger *et al.* 2005). In each case, the labeling was abolished when the antibody was adsorbed with APSGFLGMRamide. To determine if TPSGFLGMRamide also abolishes immunolabeling in the PMC, CoGs and STG, and hence is among the TRPs detected by this antibody, we conducted a set of adsorption studies using this peptide and tissues from *C. productus,* as well as the STG from *C. borealis*. Specifically, dissected PMCs, CoGs or STGs were pinned in a Sylgard 184 (World Precision Instruments, Inc., Sarasota, Florida, USA; catalog #SYLG184)-lined Petri dish and fixed for 12–24 h in a solution of 4% paraformaldehyde (EM grade; Electron Microscopy Sciences, Hatfield, PA, USA; catalog #15710) in 0.1 mol/L sodium phosphate (*P*) buffer (pH 7.4). After fixation, tissues were rinsed five times over approximately 5 h in a solution of *P* containing 0.3% Triton X-100 (*P-Triton*) and then incubated for approximately 24 h in either a 1 : 500 dilution of the substance P antibody (Abcam Incorporated, Cambridge, MA, USA; catalog # ab6338) that had been adsorbed with 10^{-5} mol/L TPSGFLGMRamide for 2 h at approximately 20°C or a 1 : 500 dilution of antibody that had been held at approximately 20°C for 2 h without peptide. Dilution of the antibody was carried out in *P-Triton*, with 10% normal donkey serum (NDS; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA; catalog #017-000-121) added to diminish non-specific binding. After incubation in the primary antibody, tissues were again rinsed five times over approximately 5 h in *P-Triton* and then incubated for 12–24 h in a 1 : 300 dilution of Alexa Fluor 594-labled donkey anti-rat IgG (Molecular Probes, Eugene, OR, USA; catalog #A-21209). As with the primary, dilution of secondary antibody was performed in *P-Triton* containing 10% NDS. After incubation in the secondary antibody, tissues were rinsed five times over approximately 5 h in *P* and mounted between a glass microscope slide and coverslip using Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA, USA; catalog #H-1000). Tissues were viewed and data collected using either a Bio-Rad Radiance 2000 laser scanning confocal microscope (Bio-Rad Microscience Ltd, Hemel Hempstead, UK) or a Nikon (Tokyo, Japan) Eclipse E600 epifluorescence microscope. Descriptions of the hardware and software used for imaging on these systems are extensively documented in previous publications (Christie *et al.* 2003; Messinger *et al.* 2005).

Physiology

For physiological recordings, the stomatogastric nervous system (STNS) was dissected and pinned out as described above, with the motor nerves and the nerves interconnecting the ganglia intact. In addition, the sheath over the STG was removed to provide superfused peptides with greater access to the neuropil within the ganglion, and a section of sheath around the stomatogastric nerve (*stn*) was removed so that action potential conduction could be reversibly blocked using isotonic (750 mmol/L) sucrose in a petroleum jelly well surrounding this desheathed area of nerve. This sucrose block eliminated all modulatory inputs to the STG, as the *stn* is the only nerve that carries inputs from the CoGs and the oesophageal ganglion to the STG. During recordings, the dish containing the STNS was constantly superfused with chilled (11–13°C) physiological saline. A petroleum jelly wall constructed across the dish allowed us to superfuse the STG with the peptide-containing saline while superfusing the remainder of the dish with normal saline. Synthetic APSGFLGMRamide, TPSGFLGMRamide or APSGFLGMRG was dissolved in deionized water, stored frozen as a 10^{-3} mol/L stock solution, and diluted to the required concentrations in physiological saline just before use.

Neuronal activity was recorded using standard electrophysiological techniques. Activity on the motor nerves was recorded extracellularly via A-M Systems Model 1700AC amplifiers (A-M Systems, Inc., Carlsborg, WA, USA) using stainless steel pin electrodes, which were isolated from the bath with petroleum jelly wells. Electrical activity was further processed with a Brownlee 410 instrumentation amplifier (Brownlee Precision Co., San Jose, CA, USA) and recorded directly onto a PC computer via a Micro 1401 board and Spike 2 (v5) software (Cambridge Electronic Design, Cambridge, UK). Data were processed using Spike 2 and further analyzed with Prism4 (GraphPad Software).

Results

Identification of APSGFLGMRamide and TPSGFLGMRamide from midgut tissue of *Cancer irroratus*

In the MALDI-FTMS analysis of a small piece of PMC freshly dissected from the crab *C. irroratus*, we observed strong signals corresponding to APSGFLGMRamide at *m/z* 934.49 as well as a prominent peak that appeared at *m/z* 964.50 (Fig. 1a). Both peaks were also present when a larger piece of PMC tissue was extracted and analyzed by MALDI-FTMS. We noted that, after exposure of the MALDI sample to air for 2 days, two new higher mass peaks appeared in the mass spectrum at *m/z* 950.49 and *m/z* 980.50, and two new lower mass peaks appeared at *m/z* 886.49 and 916.50. Exact mass measurements showed that the two higher mass peaks resulted from an addition of one oxygen atom at 15.995 Da, relative to the original peaks. This shift is consistent with oxidation of a methionine residue upon exposure to air; this hypothesis was substantiated by detection of the two lower mass peaks, which we attribute to the loss of $HSOCH_3$ (63.998 Da) from the two oxidized precursors. This evidence for methionine residues in both peptides suggested that the two peptides might be related.

Using internal calibration with PPG, the exact masses for the two peptides from *C. irroratus* were determined (see Table 1). The two masses differed by 30.0103 Da, a mass difference that supports an alanine to threonine substitution (30.0106 Da, calculated). To further characterize this unknown peptide, SORI-CID mass spectra were measured for both APSGFLGMRamide and the peak at *m/z* 964.50 following monoisotopic isolation of the $[M + H]^+$ ions. For APSGFLGMRamide at *m/z* 934.49, the SORI-CID spectrum

Table 1 Exact mass measurements for APSGFLGMRamide (CabTRP Ia), putative TPSGFLGMRamide, and putative APSGFLGMRG detected in the direct analysis of tissues using MALDI-FTMS

Peptide Identity		APSGFLGMRa	TPSGFLGMRa[a]			APSGFLGMRG
Ion Identity		$[M + H]^+$	$[M + H]^+$	$[MH–C_2H_4O]^+$	$[MH–C_2H_4O–NH_3]^+$	$[M + H]^+$
Calculated *m/z*		934.492 73	964.503 30	920.477 08	903.450 53	992.498 21
Species	Tissue[b]	Measured *m/z*[c] (Error, ppm)	Measured *m/z*[c] (Error, ppm)	Measured *m/z*[c] (Error, ppm)	Measured *m/z*[c] (Error, ppm)	Measured *m/z*[c] (Error, ppm)
Cancer irroratus	PMC	934.492 99 (0.3)	964.504 23 (1.0)	920.478 42 (1.5)	903.451 26 (0.8)	992.498 84 (0.6)
	CoG	934.493 19 (0.5)	964.504 69 (1.4)	920.478 72 (1.8)	903.451 92 (0.6)	ND
	STG[d]	934.493 00 (0.3)	964.503 32 (0.02)	920.477 65 (0.6)	903.450 80 (0.3)	ND
	SG[d]	ND	ND	ND	ND	ND
	PO[d]	ND	ND	ND	ND	ND
Cancer borealis	PMC	934.493 98 (1.3)	964.504 69 (1.4)	920.477 95 (0.9)	903.450 40 (−0.1)	992.498 39 (0.2)
	CoG	934.493 62 (1.0)	964.504 98 (1.7)	920.478 14 (1.2)	903.451 12 (0.6)	ND
Cancer productus	PMC	934.492 84 (0.1)	964.504 63 (1.4)	920.478 75 (1.8)	903.451 77 (1.4)	992.498 15 (−0.1)
	CoG	934.493 18 (0.5)	964.505 36 (2.1)	920.478 99 (2.1)	ND	ND
Cancer magister	PMC	934.493 28 (0.6)	964.504 74 (1.5)	920.478 46 (1.5)	903.450 39 (−0.2)	992.498 75 (0.5)
	CoG	934.493 40 (0.7)	964.502 87 (−0.4)	920.476 64 (−0.5)	903.449 66 (−1.0)	ND
Panilirus interruptus	CoG	934.492 94 (0.2)	ND	ND	ND	ND
Procambarus clarkii	CoG[d]	934.494 41 (1.8)	ND	ND	ND	ND

[a]As described in the text, this peptide fragments under MALDI-FTMS conditions to give $[MH–C_2H_4O]^+$ and $[MH–C_2H_4O–NH_3]^+$ ions, which provide a mass fingerprint for this peptide; [b]PMC, posterior midgut caecum; CoG, commissural ganglia; STG, stomatogastric ganglion; SG, sinus gland; PO, pericardial organ. [c]Internal calibration with poly(propylene glycol) (PPG) 725 and 2000; ND = not detected, based upon the analysis of tissues from at least three animals, unless otherwise indicated; [d]Tissues from one animal were analyzed.

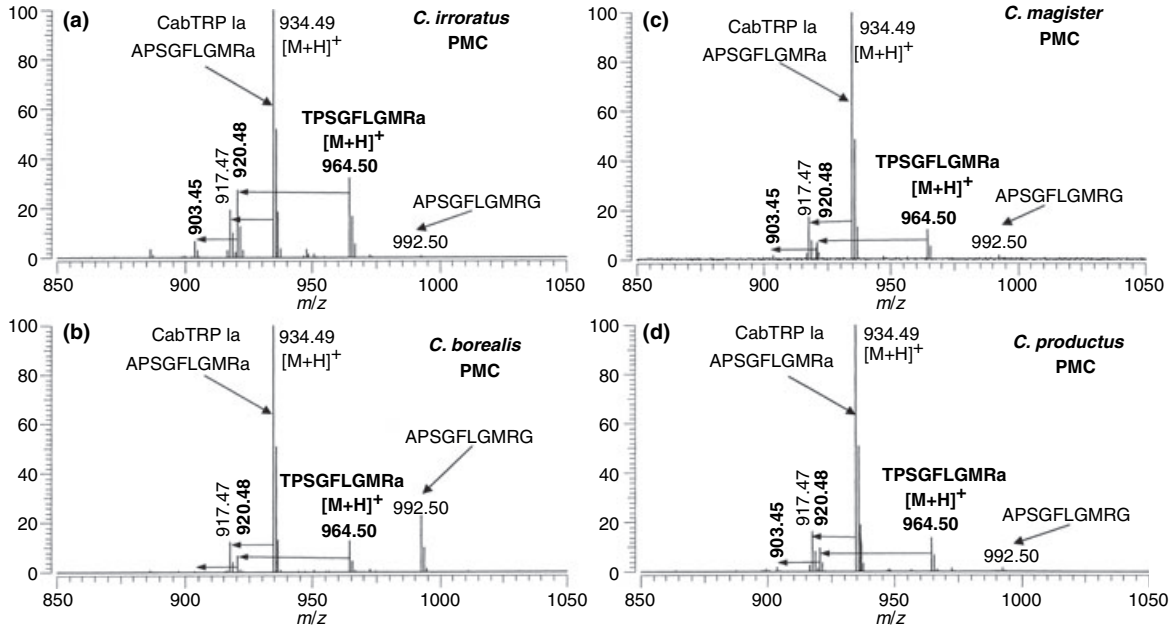

Fig. 1 Direct MALDI-FTMS spectra of small pieces of freshly dissected posterior midgut caecum from four *Cancer* crabs. (a) *C. irroratus,* (b) *Cancer borealis*, (c) *Cancer magister*, and (d) *Cancer productus*. All spectra were measured using DHB as the matrix and conditions optimized for accumulation of *m/z* 1500. Labeled peaks are those that correspond to the $[M + H]^+$ ions for APSGFLGMRamide, the TPSGFLGMRamide isoform, and APSGFLGMRG. MALDI-induced fragments at *m/z* 917.47 ($[MH–NH_3]^+$ for CabTRP 1a) and *m/z* 920.48 and 903.45 ($[MH–C_2H_4O]^+$ and $[MH–C_2H_4O–NH_3]^+$ for TPSGFLGMRamide) are also identified.

showed an abundant peak resulting from the loss of NH_3 at *m/z* 917.49 and lower intensity y- and b-type ions, as well as internal fragments consistent with the peptide sequence (see Supplementary materials). The neutral losses observed in the SORI-CID mass spectrum of the *m/z* 964.50 peptide were distinctly different, showing an abundant peak at *m/z* 920.48 (Fig. 2a). This fragment resulted from the loss of 44.0268 Da from the $[M + H]^+$ ion. Upon consideration of what could account for this loss, we excluded the loss of CO_2 (43.9898 Da) and NC_2H_6 (44.0500 Da), which gave errors of −40 and 25 ppm, respectively, for the measured mass. Instead, we concluded that the *m/z* 920.48 fragment resulted from a loss of C_2H_4O (44.0262 Da), which is in excellent agreement with the measured mass difference (−0.6 ppm error). This loss is consistent with the presence of a threonine residue in the sequence, with the C_2H_4O loss originating from the threonine side chain. The SORI-CID spectrum also showed an abundant peak at *m/z* 903.46, corresponding to the combined loss of C_2H_4O and NH_3. Both these peaks were also observed in the direct MALDI-FT mass spectrum (Fig. 1a). Although weak, the SORI-CID spectrum did show y-, b-, and internal fragment ions (Fig. 2b; exact masses reported in Supplementary materials). Based upon this sequence data, we identified the peptide as TPSGFLGMRamide. A series of y-type ions appeared at the same *m/z* values as those detected in the spectrum of APSGFLGMRamide. In contrast, all b-series ions were shifted in mass by 30.01 Da, reflecting the substitution of threonine for alanine, and peaks resulting from the additional loss of C_2H_4O were detected for many b-series, but not y-series, ions. These data, in conjunction with the abundant C_2H_4O loss, provided support for an N-terminus threonine residue. The SORI-CID mass spectrum also showed abundant internal fragments that were detected in both spectra, as expected based upon the overlap between the two sequences.

To further substantiate the identification of the *m/z* 964.50 peak, TPSGFLGMRamide was synthesized and characterized by SORI-CID. Ions detected in the direct analysis of the standard at *m/z* 964.50, 920.48, and 903.45 were the same as those fragment ions observed in the direct MALDI-FT mass spectra. Monoisotopic isolation of *m/z* 964.50 followed by SORI-CID produced a mass spectrum of the standard (Figs 2c and d) that showed excellent agreement with that of the PMC-derived neuropeptide (Figs 2a and b). In summary, the accurate mass measurements, SORI-CID experiments, and the close agreement between the synthetic standard and tissue-derived peptide spectrum support the identification of TPSGFLGMRamide as a new TRP in *C. irroratus*.

Identification of both APSGFLGMRamide and TPSGFLFMRamide in the commissural and stomatogastric ganglia, but not sinus gland or pericardial organ, of *Cancer irroratus*

Previous studies have shown that the STNS is one portion of the crustacean nervous system that exhibits TRP-like immuno-

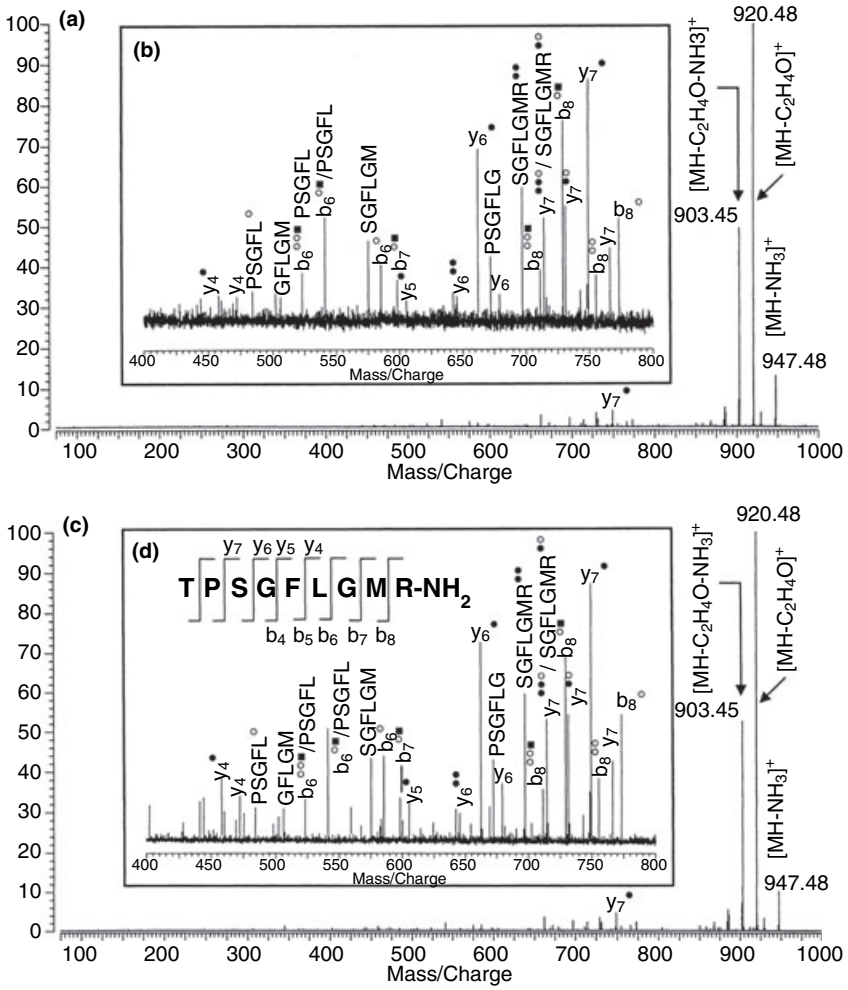

Fig. 2 SORI-CID MALDI-FTMS spectra of *m/z* 964.50, identified as TPSGFLGMRamide. (a) SORI-CID of the *m/z* 964.50 peak detected in a single tissue extract of the PMC from *Cancer irroratus*. The *m/z* 964.50 peak was dissociated following monoisotopic ion isolation. Ar was used as the collision gas with an excitation amplitude of 7.5 V_{bp}, $n = 10$; (b) Expansion of the region from *m/z* 400 to 800. (c) SORI-CID of the *m/z* 964.50, $[M + H]^+$ peak, from a synthetic standard of TPSGFLGMRamide. The *m/z* 964.50 peak was dissociated following monoisotopic ion isolation. Ar was used as the collision gas with an excitation amplitude of 7.5 V_{bp}, $n = 10$; (d) Expansion of the region from *m/z* 400 to 800. Ions that have lost NH_3 are shown with a filled circle, ions that have lost H_2O are shown with an open circle, ions that have lost C_2H_4O are shown with a filled square, and ions that have lost CH_2O are shown with an open square.

reactivity (Goldberg *et al.* 1988; Messinger *et al.* 2005). Within the crab STNS, the STG and paired CoGs contain extensive TRP-like labeling. For the CoGs, this includes staining in somata, synaptic neuropil, and a large neuroendocrine/paracrine plexus, the anterior commissural organ (Goldberg *et al.* 1988; Blitz *et al.* 1995; Christie *et al.* 1997; Messinger *et al.* 2005). Given the richness and diversity of TRP-immunopositive structures in the CoGs, we have used it and the STG to assess whether or not TPSGFLGMRamide, like APSGFLGMRamide, is also a neural isoform of TRP. We also analyzed two neuroendocrine organs, the SG (which has not generated TRP-immunopositive responses in previous studies; Christie *et al.*, 1995; Fu *et al.*, 2005; Messinger *et al.* 2005) and the PO (which has been shown to contain TRPs in some but not all *Cancer* species; Christie *et al.*, 1995; Li *et al.*, 2003; Fu *et al.*, 2005; Messinger *et al.* 2005).

Using direct tissue MALDI-FTMS we assayed neural tissues (CoGs, STG, SGs, and POs) from *C. irroratus* for the presence of TPSGFLGMRamide using the following criteria: (i) detection of the peptide's $[M + H]^+$ ion at *m/z* 964.50 and (ii) detection of the $[MH–C_2H_4O]^+$ and $[MH–C_2H_4O–NH_3]^+$ fragment ions at *m/z* 920.48 and *m/z* 903.46, respectively, with mass measurement errors that were lower than 3 ppm. All spectra were internally calibrated using PPG polymer calibrant and, for all CoG spectra, tissues from a minimum of three animals were examined.

A representative direct tissue MALDI-FT mass spectrum of a CoG from *C. irroratus* is shown in Fig. 3a. Here, we detected both APSGFLGMRamide and TPSGFLGMR amide, the latter with its characteristic fingerprint ions at both *m/z* 920.48 and *m/z* 903.46. For the CoG samples, we found that the peptide profiles varied upon desorption from different locations on the MALDI sample, but the variability suggested co-localization of the two TRP isoforms. Specifically, upon desorption from some regions of the sample we detected high relative abundances of both peptides, while in other regions, signals from both peptides were lower relative to other peptides in the sample. Both APSGFLGMRamide and TPSGFLGMRamide were also detected in the STG of *C. irroratus*, although the abundance of the peptides, relative to other peptides in the STG, was lower than that observed in the CoGs. As the calibrated

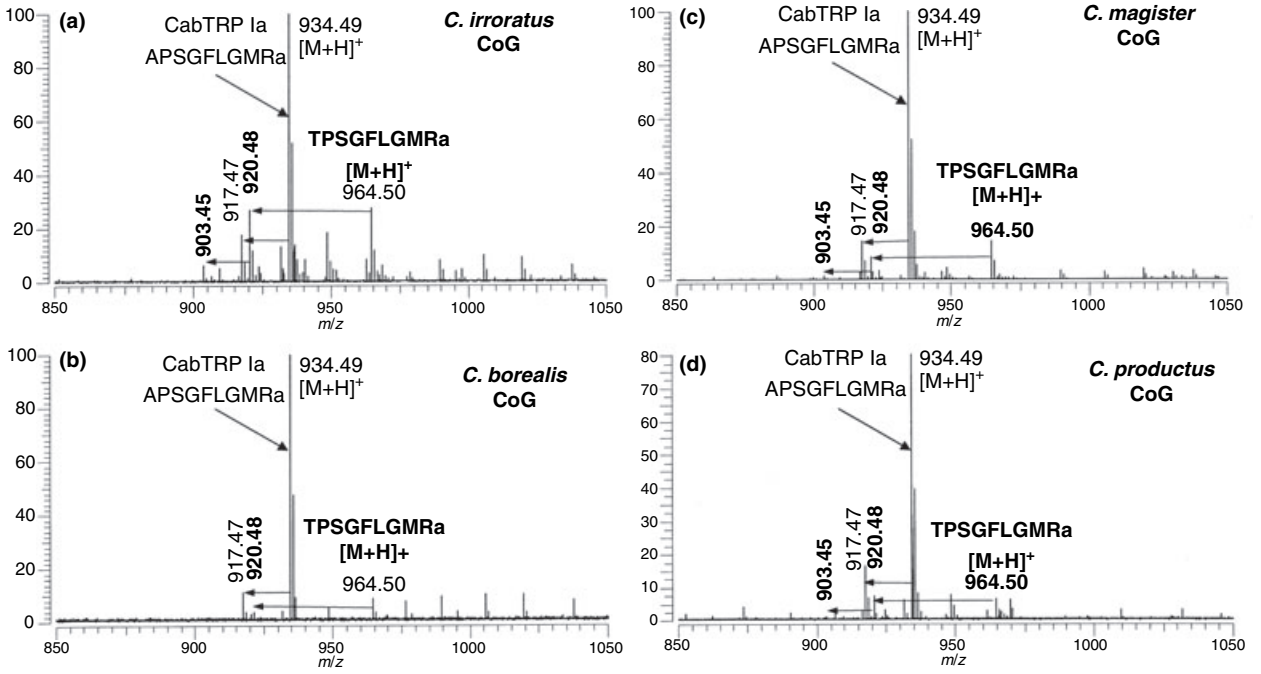

Fig. 3 Direct MALDI-FTMS spectra of small pieces of freshly dissected and desheathed single commissural ganglion (CoG) from four *Cancer* crabs. (a) *Cancer irroratus,* (b) *Cancer borealis*, (c) *Cancer magister*, and (d) *Cancer productus*. All spectra were measured using DHB as the matrix and conditions optimized for accumulation of *m/z* 1500. Labeled peaks are those that correspond to the $[M + H]^+$ ions for APSGFLGMRamide and the TPSGFLGMRamide isoform, as well as MALDI-induced fragments at *m/z* 917.47 ($[MH–NH_3]^+$ for APSGFLGMRamide) and *m/z* 920.48 and 903.45 ($[MH–C_2H_4O]^+$ and $[MH–C_2H_4O–NH_3]^+$ for TPSGFLGMRamide).

masses for both peptides in the CoG and STG tissues were less than 2 ppm (see Table 1), we feel confident that both peptides are present in the STNS of *C. irroratus*. In contrast with data for the CoGs and STG, ions characteristic of the two TRP isoforms were not detected when SG or PO tissues were analyzed.

Conservation of midgut-neural distributions for both APSGFLGMRamide and TPSGFLGMRamide in other Cancer species

To determine if TPSGFLGMRamide is a species-specific isoform of TRP, or if it is present in the gut and/or neural tissues of other *Cancer* species, direct tissue MALDI-FTMS analysis on PMCs and CoGs from *C. borealis, C. productus,* and *C. magister* was conducted. Identification of TPSGFLGMRamide was based, as above, upon the detection of the $[M + H]^+$ ion at *m/z* 964.50 and the $[MH–C_2H_4O]^+$ and $[MH–C_2H_4O–NH_3]^+$ fragment ions at *m/z* 920.48 and *m/z* 903.46, respectively. All spectra were also examined for the presence of APSGFLGMRamide via detection of its $[M + H]^+$ ion at *m/z* 934.49. For each species, tissues from a minimum of three animals were examined. Representative spectra for PMC and CoG tissues are shown in Figs 1 and 3, and exact mass measurements for these tissues are summarized in Table 1. These data provide definitive evidence that both isoforms of TRP are present in the CoG and PMC tissues of all *Cancer* species examined in this study.

The abundance of TPSGFLGMRamide relative to APSGFLGMRamide varies between species but is similar for gut and CoG tissues within each species

As shown in Figs 1 and 3, the direct MALDI-FT mass spectra of PMC and CoG samples showed TPSGFLGMRamide peak intensities that varied among the Brachyuran species analyzed. For example, we observed high relative intensities for the TPSGFLGMRamide peptides in both PMC and CoG tissues from *C. irroratus* (Figs 1a and 3a), while the relative TPSGFLGMRamide peak intensities were consistently lower for PMC and CoG tissues from *C. magister* (Figs 1c and 3c). Within each species, the abundance of TPSGFLGMRamide relative to that of APSGFLGMRamide in PMC tissues was similar to that observed in CoG tissues.

Although MALDI is not generally considered to be a quantitative technique, we assumed that the structural similarity between the two TRPs would yield similar ionization efficiencies, and that the relative ion intensities observed in the MALDI-FT mass spectra should reflect the relative amounts of the two TRPS in direct tissue samples. To quantify the percentage of TPSGFLGMRamide, we analyzed MALDI-FT mass spectra of PMC and CoG tissues and extracted the ion

intensity for TPSGFLGMRamide ($[M + H]^+$ ion and fragments) and compared that to the ion intensity ($[M + H]^+$ ion and fragments) for (TPSGFLGMRamide + APSGFLGMRamide). To assure that we accounted for variability derived from the MALDI ionization process, as well as animal to animal variations, we included in our data set ion intensities extracted from multiple spectra with reasonable signal to noise ratios from a single animal (two to six spectra) and compared the averaged data from at least three different animals ($n = 4$ for the PMC of *C. borealis*). The results presented below were derived from a statistical analysis of those mean values; a full analysis that included all individual measurements from all animals did not change these conclusions.

As shown in Fig. 4, the relative peptide intensities were strongly correlated for the two types of tissues, CoGs and PMCs, and no significant differences were observed between the two types of tissues within a given species of *Cancer* ($p > 0.05$). However, the percentage of TPSGFLGMRamide in CoG and PMC tissues from *C. irroratus* were significantly different ($p < 0.001$) from the percentages found in either PMC or CoG tissues from the remaining three species of *Cancer, C. productus, C. borealis*, and *C. magister* (Fig. 4), while no significant differences ($p > 0.05$) were found between tissues within *C. productus, C. borealis*, and *C. magister*.

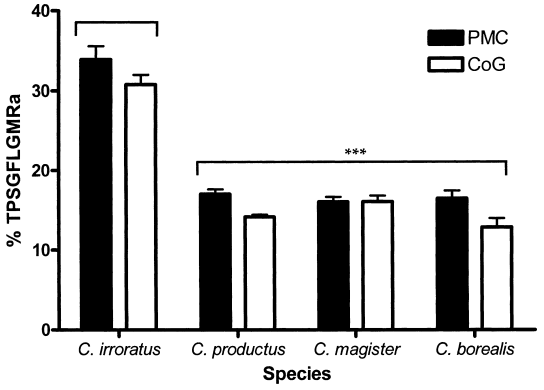

Fig. 4 Percentage of TPSGFLGMRamide relative to the sum of TPSGFLGMRamide and APSGFLGMRamide in PMC and CoG tissues. Each peptide was quantified as the sum of the $[M + H]^+$ and fragment ion intensities (*m/z* 934.49 and *m/z* 917.47 for APSGFLGMRamide; *m/z* 964.50, *m/z* 920.48, and *m/z* 903.45 for TPSGFLGMRamide). The percentages shown are the mean values from three animals (four for the PMC from *Cancer borealis*). The mean for each animal was calculated by averaging results from two to nine spectra. Statistical analysis (two-way ANOVA) was performed using the mean values for each animal. The percentage of TPSGFLGMRamide for PMC and COG tissues from *Cancer irroratus* were significantly different ($p < 0.001$) than the percentages observed for tissues from the three other *Cancer* crabs. No significant difference ($p > 0.05$) was observed between PMC and COG tissues for any species. ***Significance level of $p < 0.001$. Error bars indicate SE.

Identification of unprocessed APSGFLGMRG from midgut tissues of *Cancer borealis* and other *Cancer* species

When MALDI-FTMS was applied to a small piece of PMC freshly dissected from the Jonah crab, *C. borealis*, we observed signals derived from APSGFLGMRamide at *m/z* 934.49 and TPSGFLGMRamide at *m/z* 964.50; however, an additional, abundant peptide was present at *m/z* 992.50 in the MALDI-FT mass spectrum of the fresh tissue and a tissue extract. The same peak was present, but was much less abundant, in the PMCs of *C. irroratus* and the other two *Cancer* species. Because of its high relative abundance in *C. borealis*, we chose to analyze this peak in that species. A mass shift of 15.995 Da relative to *m/z* 992.50 to give an ion at *m/z* 1008.50, with a 63.998 Da loss to give *m/z* 944.50, was observed following exposure to air, indicating that this peptide also contained methionine. Using internal calibration with PPG, the exact mass of the new peptide was determined (see Table 1). The mass difference relative to APSGFLGMRamide, 58.0057 Da, was consistent with a glutamate/alanine or aspartate/glycine substitution; alternatively, it could result from a longer peptide sequence containing an additional glycine residue, coupled with a change from an amide to an acid at the C-terminus.

When the monoisotopic ion at *m/z* 992.50 was isolated and subjected to SORI-CID, the mass spectrum showed peaks with the same *m/z* values as those observed in the spectrum of APSGFLGMRamide. Specifically, ions identified as b-type ions and internal fragments in the SORI-CID spectrum of APSGFLGMRamide (see Supplementary materials) were also detected in the spectrum of *m/z* 992.50. In contrast, the y-series ions detected for APSGFLGMRamide were detected at masses shifted by 58.01 and associated losses of H_2O, not NH_3, were more prominent. Collectively, these observations supported the identification of *m/z* 992.50 as APSGFLGMRG, an extended form of APSGFLGMRamide with an addition of glycine and a C-terminus acid, rather than an amide.

The identification of the *m/z* 992.50 peak as APSGFLGMRG was further supported by the analysis of the synthetic peptide by SORI-CID. Monoisotopic isolation of *m/z* 992.50 followed by SORI-CID produced a mass spectrum of the standard (Fig. 5c) with identical masses and similar ion intensities to that of the neuropeptide found in the *C. borealis* PMC (Fig. 5b). In summary, the accurate mass measurements, SORI-CID experiments, and the close agreement between the synthetic standard and tissue-derived peptide spectra support the identification of APSGFLGMRG as an immature form of APSGFLGMRamide that has not been processed to the C-terminal amide in *C. borealis*.

We also used MALDI-FTMS to determine if APSGFLGMRG was present in the STNS (STG or CoG) or

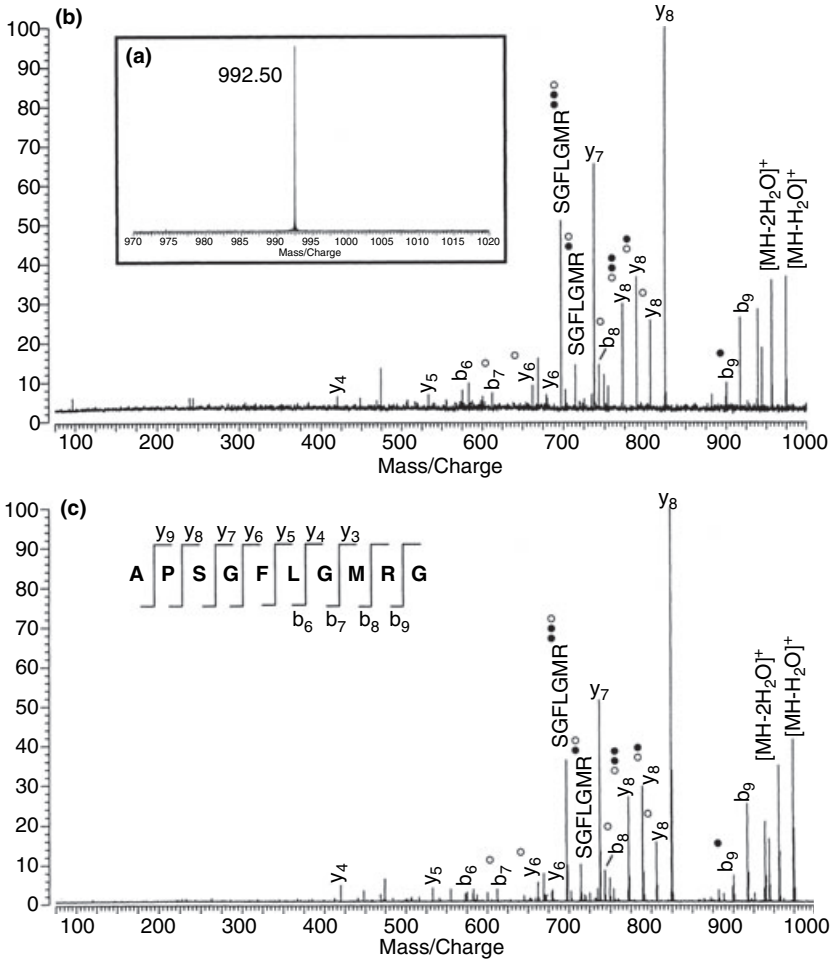

Fig. 5 SORI-CID MALDI-FTMS spectra of the *m/z* 992.50 peak, identified as APSGFLGMRG, detected in a single tissue extract of the PMC from *Cancer borealis* and the APSGFLGMRG standard. (a) Monoisotopic isolation of the *m/z* 992.50, $[M + H]^+$ ion from the PMC extract and (b) SORI-CID of the *m/z* 992.50, $[M + H]^+$ ion from the PMC extract. Ar was used as the collision gas with an excitation amplitude of 7.5 V_{bp}, $n = 10$; (c) SORI-CID of the *m/z* 992.50, $[M + H]^+$ ion from the APSGFLGMRG standard. Ar was used as the collision gas with an excitation amplitude of 6.5 V_{bp}, $n = 1$. Ions that have lost NH_3 are shown with a filled circle and ions that have lost H_2O are shown with an open circle.

in either of the two neuroendocrine organs (SG or PO) of *C. irroratus*. No evidence for this peptide was found in any *C. irroratus* tissues that we examined other than the PMC (Table 1). Similarly, we consistently detected APSGFLGMRG in PMC, but not CoG tissues of the other *Cancer* species included in this study (Table 1). We found no evidence for the unprocessed form of the TPSGFLGMRamide in PMC tissues from any of the *Cancer* species examined in this study; however, this form, if present, may have been below the detection limit of our measurements given the lower abundance of the processed peptide.

MALDI-FTMS detection of APSGFLGMRamide but not TPSGFLGMRamide in the commissural ganglion of the spiny lobster *Panulirus interruptus* and the crayfish *Procambarus clarkii*

Our identification of both APSGFLGMRamide and TPSGFLGMRamide in the nervous systems of crabs *C. irroratus*, *C. borealis, C. magister* and *C. productus* was surprising, as only the former isoform had been identified previously in crustacean neural tissues (Christie *et al.* 1997; Nieto *et al.* 1998; Huybrechts *et al.* 2003; Yasuda-Kamatani and Yasuda 2004; Messinger *et al.* 2005; Stemmler *et al.* 2005). Moreover, cloning of preproTRP cDNAs from two species, the spiny lobster *P. interruptus* and the crayfish *P. clarkii*, identified only APSGFLGMRamide, which is present as multiple copies in the neural TRP precursor protein (Yasuda-Kamatani and Yasuda 2004). Given the predicted lack of TPSGFLGMRamide in the neural tissues of *P. interruptus* and *P. clarkii*, we conducted direct tissue MALDI-FTMS analysis on CoGs from these species as a control for our identification of the novel isoform identified in the *Cancer* crabs. As Fig. 6 shows, a peak at *m/z* 934.49 was seen in spectra collected from *P. interruptus* CoG tissues, and the calibrated mass (Table 1) confirms the presence of APSGFLGMRamide in this tissue. In no spectra, however, were peaks seen at *m/z* 964.50, *m/z* 920.48 or *m/z* 903.46, corresponding to the $[M + H]^+$ ion and the $[MH–C_2H_4O]^+$ and $[MH–C_2H_4O–NH_3]^+$ fragment ions, respectively, of TPSGFLGMRamide (predicted peak positions are shown with arrows in Fig. 6a). These results reflect measurements made for over 20 animals. We also measured direct tissue spectra for a pair of CoGs from a single *P. clarkii*. As was the case for *P. interruptus*, we detected only the peak at

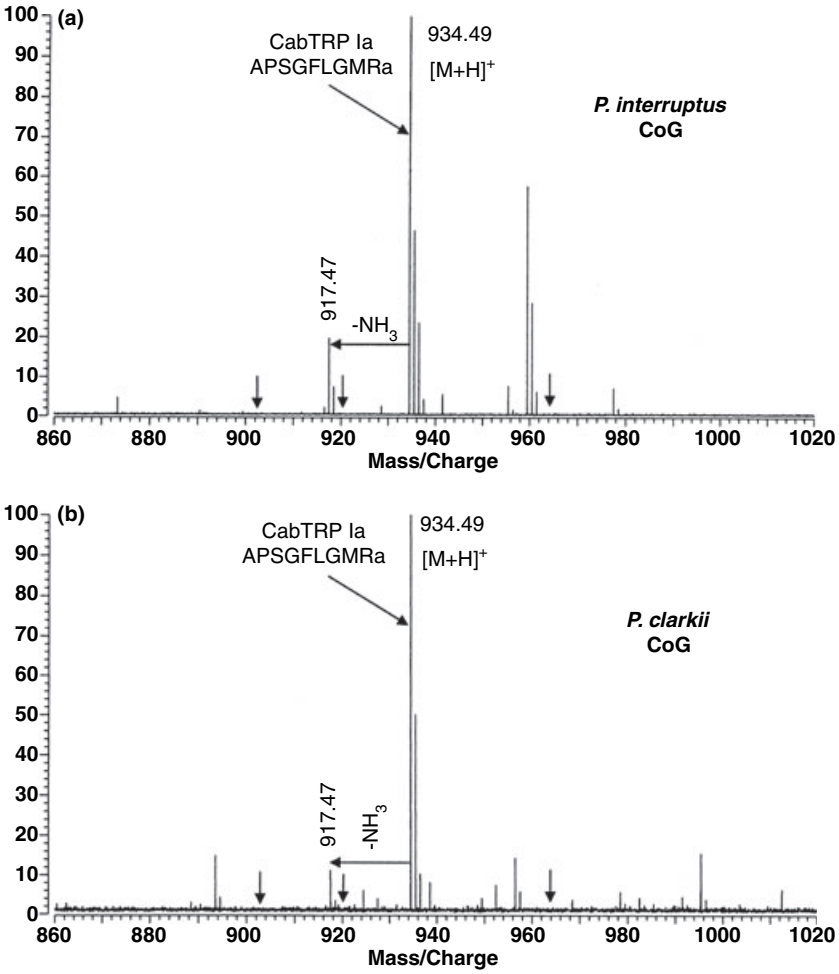

Fig. 6 Direct MALDI-FTMS spectra of small pieces of freshly dissected desheathed single commissural ganglion (CoG) from (a) the spiny lobster, *Panulirus interruptus* and (b) the red swamp crayfish, *Procambarus clarkii.* All spectra were measured using DHB as the matrix, with conditions optimized for accumulation of *m/z* 1500. Peaks for the $[M + H]^+$ and $[MH–N-H_3]^+$ ions for APSGFLGMRamide are labeled. Arrows indicate where the $[M + H]^+$ and MALDI-induced fragment peaks from the TPSGFLGMRamide isoform would appear, if they were present. The spectrum for the *P. interruptus* CoG is representative of spectra measured from more than 20 animals. The spectrum for *P. clarkii* is representative of the signals observed from a single animal.

m/z 934.49 (Fig. 6b); the calibrated mass of this peak (Table 1) confirms the presence of APSGFLGMRamide in this tissue. No evidence for peaks corresponding to TPSGFLGMRamide were observed (predicted peak positions are shown with arrows in Fig. 6b). Thus, as predicted from the molecular analysis done on the *P. interruptus* and *P. clarkii* nervous systems, the only isoform of TRP we could identify in CoG tissues was APSGFLGMRamide.

Adsorption of tachykinin-related peptide-like immunolabeling by TPSGFLGMRamide

Previous studies have demonstrated the presence of TRP-like immunoreactivity in the PMC (Christie *et al.* 2007) as well as in the CoGs and STG (Goldberg *et al.* 1988; Christie *et al.* 1997; Messinger *et al.* 2005) of a number of *Cancer* species. Likewise, previous work has shown that this immunoreactivity is abolished by pre-adsorption with APSGFLGMRamide (Christie *et al.* 1997; Messinger *et al.* 2005; Christie *et al*. 2007). As we have shown here that TPSGFLGMRamide is present along with APSGFLGMRamide in both of these tissue, we hypothesized that some of the TRP-like immunoreactivity seen is due to the presence of this peptide. To test this theory, we conducted antibody adsorptions using synthetic TPSGFLGMRamide as a blocking agent. In PMC, CoG and STG tissues from *C. productus* ($n = 3$ preparations for each tissue), adsorption of the substance P antibody with 10^{-5} mol/L TPSGFLGMRamide prior to its use for labeling abolished all staining in all profiles (data not shown). Likewise, in the STG of *C. borealis* ($n = 3$ preparations), preadsorption of the antibody with 10^{-5} mol/L TPSGFLGMRamide abolished all immunoreactivity (data not shown). In contrast, labeling with antibody held at room temperature for two hours was robust and identical to that previously reported for the PMC (Christie *et al.* 2007), CoGs and STG (Christie *et al.* 1997; Messinger *et al.* 2005) ($n = 3$ preparations for each tissue; data not shown). Collectively these results show that TPSGFLGMRamide, like APSGFLGMRamide, is detected by the substance P antibody and since all TRP labeling in the STG originates from a single pair of axons (Goldberg *et al.* 1988; Christie *et al.* 1997), that both peptides must be co-localized in the same neurons, at least in this ganglion.

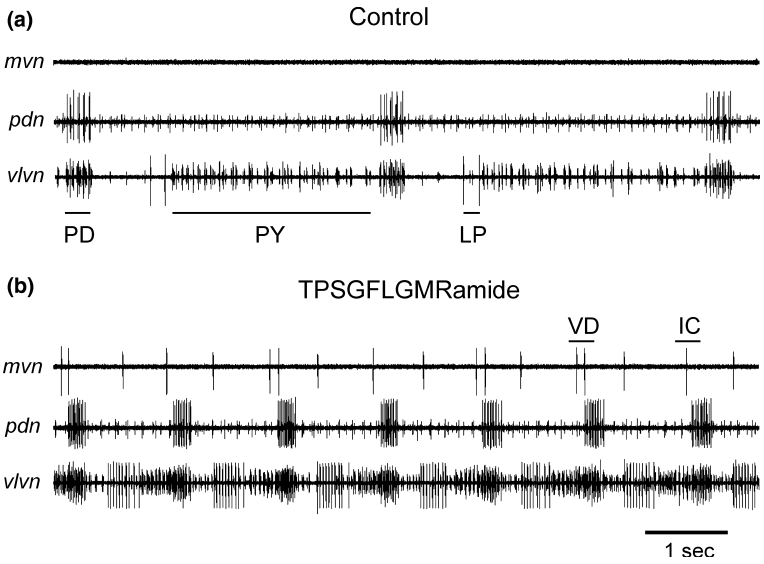

Fig. 7 TPSGFLGMRamide (10^{-6} mol/L) activated the pyloric motor pattern. Modulatory input to the STG was eliminated by blocking impulse conduction in the stomatogastric nerve (*stn*), which is the primary input nerve to the ganglion. (a) In control conditions, with modulatory input blocked, the pyloric pattern was slow and weak. The core triphasic pattern (i.e., pyloric dilator neuron- lateral pyloric neuron-pyloric neuron [PD–LP–PY]) was present, but neither the ventricular dilator neuron (VD) nor the inferior cardiac neuron (IC) fired. (b) In the presence of TPSGFLGMRamide, pyloric cycle frequency consistently increased, the IC and VD neurons, recorded on the medial ventricular nerve (*mvn*), were activated, and bursting was enhanced in the PD, PY, and LP neurons. Other abbreviations: pyloric dilator nerve (*pdn),* ventral lateral ventricular nerve (*vlvn*).

APSGFLGMRamide and TPSGFLGMRamide activate the pyloric motor pattern in *C. borealis*; APSGFLGMRG has no effect

To determine whether the newly identified TRP isoform is biologically active, we chose to examine its physiological effects on the pyloric central pattern generator of the crab, *C. borealis*. The core of the pyloric pattern generator, located in the STG, is a network composed of four types of neurons (one interneuron, the anterior burster [AB]; and three types of motor neuron: the pyloric dilator [PD], lateral pyloric [LP], and pyloric [PY] motor neurons) that produce the rhythmic motor output that drives movements of the pyloric filter in crustaceans. Previous studies have shown that APSGFLGMRamide increases activity in this neural network (Christie *et al.* 1997). Moreover, in the STG, all local TRP input is provided by a single pair of input axons (Christie *et al.* 1997), which we have shown via mass spectrometric and blocking studies must contain both TRP isoforms. To examine the effects of TPSGFLGMRamide, we first blocked axonal conduction in the stomatogastric nerve (*stn*), which is the sole input nerve to the STG. This removed all modulatory inputs, and resulted in a relatively weak pyloric pattern. When TPSGFLGMRamide was then bath applied, it caused a clear activation of the pyloric pattern. In the representative traces shown in Fig. 7, it can be seen that cycle period increased, as did the intensity of firing in all three neuronal types shown (PD, LP, PY). We quantified the activity in the pyloric dilator (PD) neurons, which serve as the pacemakers for the pyloric pattern, and found that not only overall pyloric cycle period, but also the PD neuron duty cycle and the number of spikes per PD burst increased significantly in TPSGFLGMRamide relative to control saline (ANOVA, $p < 0.05$, $n = 5$). However, neither the duration of the PD action potential burst nor the frequency of firing within the burst showed a significant change. Thus, it is clear that this TRP isoform is biologically active.

The effects of TPSGFLGMRamide appeared to be very similar to those previously reported for APSGFLGMRamide (Christie *et al.* 1997). However, because our experimental protocol was somewhat different from that used previously, our control values for cycle frequency were lower than those reported by Christie *et al.* (1997). We therefore applied APSGFLGMRamide to the same preparations, so that we could compare the effects of APSGFLGMRamide and TPSGFLGMRamide directly (Fig. 8). The effects of the two isoforms were virtually identical for all parameters measured. In contrast, APSGFLGMRG had no effect on any preparation ($n = 7$; data not shown).

Discussion

Multiple tachykinin relared-peptide isoforms are present in the nervous systems of Cancer species

In the majority of the invertebrates thus far examined, multiple TRP isoforms have been identified. For example, fifteen and fourteen TRPs have been described in the cockroaches *Periplaneta americana* and *Leucophaea maderae,* respectively, with seven isoforms identified in the honeybee *Apis mellifera* and the echiuroid worm *Urechis unitinctus*, six isoforms in the fruitfly *Drosophila melanogaster* and five isoforms in the locust *Locusta migratoria* (Schoofs *et al.* 1990a,b, 1993; Muren and Nässel 1996, 1997; Kawada *et al.* 1999, 2000; Siviter *et al.* 2000; Takeuchi *et al.* 2003; Predel *et al.* 2005). In contrast, a very different picture has emerged in decapod crustaceans. Here, previous studies have identified only a single TRP isoform: APSGFLGMRamide or CabTRP Ia. This peptide has been identified in authentic form in six species spanning four infraorders: i.e., the crabs *C. borealis*

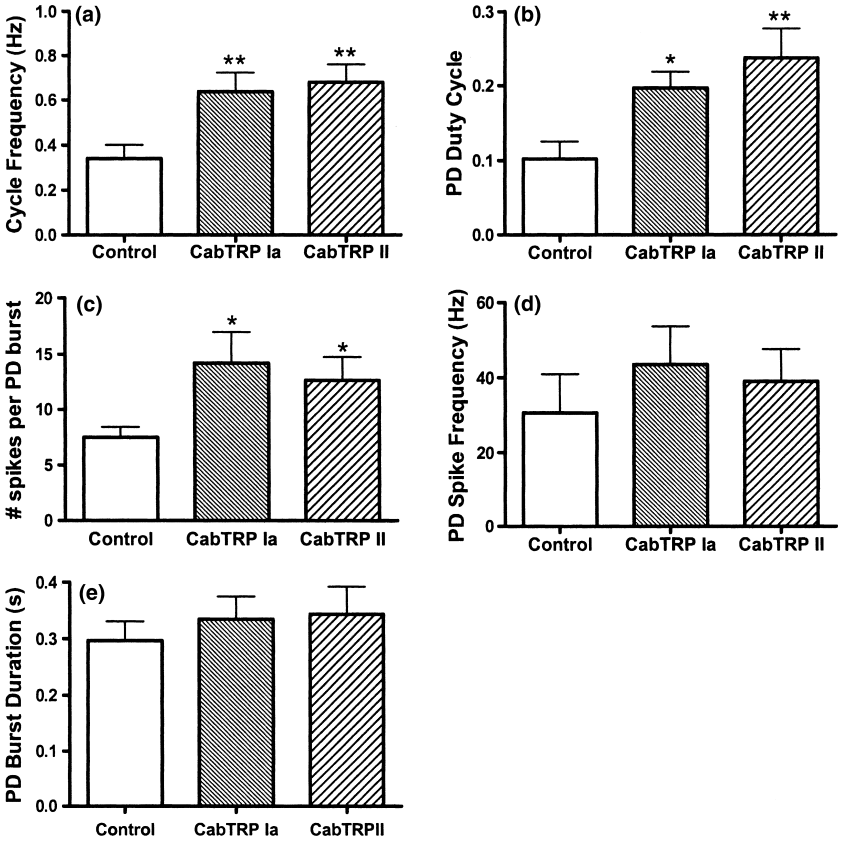

Fig. 8 (a) Pyloric cycle frequency, (b) pyloric dilator neuron (PD) duty cycle and (c) number of spikes per PD burst all increased significantly in both TPSGFLGMRamide (CaiTRP/CabTRP II) and APSGFLGMRamide (Cab TRP I), but there were no significant differences between the two peptides. Although there were clear trends, neither PD spike frequency within a burst (d) nor PD burst duration (e) increased significantly. All data were analyzed using one-way repeated measures Analysis of Variance, with *post hoc* Tukey tests to compare pyloric parameters in the absence of all modulatory input (blocked), and when superfused with either CabTRP Ia, or CaiTRP/CabTRP II., $N = 8$, (*) = $p < 0.05$, (**) = $p < 0.01$.

and *C. productus* (infraorder Brachyura; Christie *et al.* 1997; Messinger *et al.* 2005), the shrimp *Penaeus vannamei* (infraorder Penaeoidea; Nieto *et al.* 1998), the freshwater crayfish *P. clarkii* and the chelate marine lobster *Homarus americanus* (infraorder Astacidae; Yasuda-Kamatani and Yasuda 2004; Stemmler *et al.* 2005) and the spiny lobster *P. interruptus* (infraorder Palinura; Yasuda-Kamatani and Yasuda 2004). Moreover, cDNAs encoding preproTRPs were recently cloned from both the crayfish *P. clarkii* and the spiny lobster *P. interruptus* (Yasuda-Kamatani and Yasuda 2004). In both species, the preproTRP was found to encode seven copies of APSGFLGMRamide, with no other TRP isoforms predicted. Interestingly, not only was the number of TRP sequences conserved between the preprohormones, but the structural organization of the preproTRPs was also identical. Taken collectively, the data thus far available from decapod species has led to the hypothesis that members of this arthropod order, unlike other invertebrate groups, possess but a single, common TRP, namely APSGFLGMRamide (Yasuda-Kamatani and Yasuda 2004).

In our study, we present data that indicates that the common and unique decapod TRP hypothesis is, in fact, incorrect, at least for a subset of species. Using direct tissue MALDI-FTMS in combination with SORI-CID, we identified both APSGFLGMRamide and TPSGFLGMRamide, a TRP isoform reported here for the first time, from midgut and neural tissues of the crabs *C. irroratus, C. borealis*, *C. magister* and *C. productus*. Thus, in at least *Cancer* species, the existence of multiple TRP isoforms appears to be a common phenomenon.

Processing of tachykinin-related peptides from the non-amidated precursor

The non-amidated form of APSGFLGMRamide identified in this study, i.e., APSGFLGMRG, suggests that this peptide, like other amides, is initially synthesized as a slightly longer peptide sequence, which contains an additional glycine residue and is not amidated, then is converted to APSGFLGMRamide by the enzyme peptidylglycine α-amidating monooxygenase. While we did not detect a comparable G-acid form of TPSGFLGMRamide, detection of this form may have been prevented by the lower abundance of the new isoform relative to APSGFLGMRamide. Interestingly, we only found the unprocessed peptide, APSGFLGMRG, in the PMC, but not in CoG tissues. It seems likely that the unprocessed peptides are formed from the same precursor in all tissues, but that these precursors are simply present at concentrations too low to detect in the neural tissues, which could be the result of differences in enzymatic concentrations or rates of conversion in different tissues, or differences in the rate of synthesis of the precursor relative to the rate of conversion. We cannot at present distinguish between these alternatives, though as additional studies are conducted, it

will be interesting to see which of these alternative hypotheses is borne out experimentally.

Unlike APSGFLGMRamide, TPSGFLGMRamide does not appear to be conserved in all decapods

In addition to the three *Cancer* species, MALDI-FTMS spectra collected from *P. interruptus* and *P. clarkii* CoGs were analyzed for their TRP complement. As predicted from cloning studies (Yasuda-Kamatani and Yasuda 2004), the only TRP-like mass detected in either the *P. interruptus* or the *P. clarkii* spectra was that corresponding to APSGFLGMRamide. Thus, it appears that the distribution of TPSGFLGMRamide is more restricted than that of APSGFLGMRamide, bringing into question whether the presence of multiple TRP isoforms, such as we have shown for the *Cancer* crabs, is the exception rather than the rule. While the phylogeny of decapods is somewhat in dispute, there is a general consensus that the Brachyurans (true crabs) are more derived than are the Palinurans (spiny lobsters) or Astacideans (freshwater crayfish and clawed lobsters). Perhaps APSGFLGMRamide represents the common ancestral decapod TRP and the presence of additional isoforms represents an evolutionary development in derived animals. Additional studies, both mass spectrometric and molecular, encompassing many more species and infraorders, will be necessary to test this hypothesis, but if one considers crustaceans to be ancestral to insects (with their myriad of TRPs), this hypothesis is an intriguing one.

TPSGFLGMRamide likely originates from a single nucleotide substitution in the coding sequence for APSGFLGMRamide

Given the extensive conservation of the peptide APSGFLGMRamide in decapods, as well as the identical structures of the preproTRPs, each containing seven copies of TRP, from crayfish *P. clarkii* and spiny lobster *P. interruptus,* it is interesting to postulate how TPSGFLGMRamide arose and why it has persisted in at least *Cancer* species. With respect to the former question, the answer undoubtedly lies in the structure of the gene/mRNA encoding the peptides, specifically in nucleotide sequences in the codons specifying the amino acid alanine versus those specifying threonine. Four codons encode each of these amino acids: GCT, GCC, GCA and GCG for alanine, and ACT, ACC, ACA and ACG for threonine. It is thus clear that a single nucleotide substitution of guanine for adenine at position one of each of the alanine codons would result in the substitution of the amino acid threonine for alanine in the resulting peptide. We therefore predict that such a nucleotide substitution in the coding sequence for at least one of the peptide copies in the *Cancer* preproTRP is responsible for the generation of TPSGFLGMRamide from the ubiquitous APSGFLGMRamide. While MALDI-FTMS is not quantitative, the relative abundance of TPSGFLGMRamide versus APSGFLGMRamide suggests that only a minority of TRPs present in the preprohormone are likely of the former isoform, assuming the organization of the *Cancer* preproTRP is homologous to those of *P. clarkii* and *P. interruptus*. Given the higher proportion of TPSGFLGMRamide in *C. irroratus* tissues than in those of the other *Cancer* species, we might expect a larger number of the seven copies of TRP within the gene to be TPSGFLGMRamide than in the other *Cancer* species.

The structural variation in tachykinin-related peptide isoforms does not result in functional variation at the level of receptor binding

The extensive conservation of APSGFLGMRamide, coupled with apparent lack of additional isoforms in some/most species, has lead to the postulation that the structural conservation of this isoform is significant for the physiological actions of the peptide, most likely because of its affinity to the crustacean TRP receptor or receptors (Yasuda-Kamatani and Yasuda 2004). Credence to this hypothesis is provided by the finding that a naturally occurring truncation of APSGFLGMRamide, SGFLGMRamide, is biologically inactive in the crustacean systems thus far tested (Christie *et al.* 1997). Lack of physiological action by this two amino acid truncation also suggests that the N-terminus plays an important role in receptor binding in decapods; however, the physiological activity of TPSGFLGMRamide demonstrates that the substitution of a hydrophilic polar amino acid (threonine) for a hydrophobic non-polar one (alanine) at the N-terminus can be tolerated. Clearly, it is impossible at this point to answer questions of receptor stringency satisfactorily without knowledge of the structure of the receptor(s) in question, and none have yet been cloned from any crustacean species. Perhaps the receptors are more promiscuous than initially proposed and/or the threonine for alanine substitution is not deleterious to receptor binding (both peptides are relatively weak with respect to hydrophilic/hydrophobic and polar/non-polar natures). Clearly our identification of more than one TRP isoform in decapod species raises many questions concerning the evolution of tachykinins in this diverse and important arthropod order.

A note on crustacean tachykinin-related peptide nomenclature

The first TRPs described from crustaceans, APSGFLGMRamide and SGFLGMRamide, were isolated from *C. borealis* (Christie *et al.* 1997). Because of their origin, and the fact that the latter peptide appeared to be a truncation of the former one, the peptides were named *Cancer borealis* tachykinin-related peptide Ia or CabTRP Ia and *Cancer borealis* tachykinin-related peptide Ib or CabTRP Ib, respectively. In the present study we have shown that APSGFLGMRamide is also present in *C. irroratus*, *C. magister* and *C. productus*. Given the presence of this peptide in these species, we propose the names *Cancer irroratus* tachykinin-related

peptide I (CaiTRP I), *Cancer magister* tachykinin-related peptide I (CamTRP I), and *Cancer productus* tachykinin-related peptide I (CapTRP I) as synonyms for APSGFLGMRamide, particularly when discussing the presence, distribution and/or physiological actions of this peptide in the relevant species. Similarly, in our study we have reported the identification of the novel TRP isoform TPSGFLGMRamide, originally from *C. irroratus* and subsequently from *C. borealis*, *C. magister* and *C. productus*. We propose the names *Cancer borealis* tachykinin-related peptide II (CabTRP II), *Cancer irroratus* tachykinin-related peptide II (CaiTRP II), *Cancer magister* tachykinin-related peptide II (CamTRP II), and *Cancer productus* tachykinin-related peptide II (CapTRP II) be used as synonyms for this TRP.

Given the identification of TPSGFLGMRamide, the name originally ascribed to SGFLGMRamide, i.e., CabTRP Ib, is now somewhat problematic as this truncated TRP may be derived from the enzymatic degradation of either APSGFLGMRamide or TPSGFLGMRamide. We therefore propose that this peptide be referred to as Cab-/Cai-/Cam-/CapTRP I/II 3-9 to better reflect its potential origin from both full-length TRP isoforms. To this end we also favor dropping the 'a' from CabTRP Ia and simply referring to this *C. borealis* TRP isoform as CabTRP I.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1 SORI-CID MALDI-FTMS spectra of the *m/z* 934.49 peak, identified as APSGFLGMRamide, detected in a single tissue extract of the PMC from *C. irroratus*. (a) Monoisotopic isolation of *m/z* 934.49, $[M + H]^+$ ion; (b) SORI-CID of the *m/z* 934.49, $[M + H]^+$ ion. Ar was used as the collision gas with an excitation amplitude of 7.5 V_{bp}, $n = 1$; (c) Expansion of the spectrum shown in (b). Ions that have lost NH_3 are shown with a filled circle, ions that have lost H_2O are shown with an open circle, and ions that have lost CH_2O are shown with an open square.

Table S1 Ions observed by SORI-CID of putative APSGFLGMRamide from the posterior midgut caecum (PMC) of *C. irroratus* and a standard of TPSGFLGMRamide.

Table S2 Ions observed by SORI-CID of putative APSGFLGMRG from a *C. borealis* posterior midgut caecum (PMC) extract and an APSGFLGMRG standard.

This material is available as part of the online article from http://www.blackwell-synergy.com

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