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Andrew E. Christie
University of Washington

Kimberly K. Kutz-Naber
University of Wisconsin-Madison

Elizabeth A. Stemmler
Bowdoin College

Alexandra Klein
University of Washington

Daniel I. Messinger
University of Washington

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Midgut epithelial endocrine cells are a rich source of the neuropeptides APSGFLGMRamide (*Cancer borealis* tachykinin-related peptide Ia) and GYRKPPFNGSIFamide (Gly^1-SIFamide) in the crabs *Cancer borealis*, *Cancer magister* and *Cancer productus*

Andrew E. Christie[1,2,*], Kimberly K. Kutz-Naber[3], Elizabeth A. Stemmler[4], Alexandra Klein[1], Daniel I. Messinger[1], Christopher C. Goiney[1], Anna J. Conterato[4], Emily A. Bruns[4], Yun-Wei A. Hsu[1], Lingjun Li[3,5] and Patsy S. Dickinson[6]

[1]*Department of Biology, University of Washington, Box 351800, Seattle, WA 98195-1800, USA,* [2]*Mount Desert Island Biological Laboratory, PO Box 35, Old Bar Harbor Road, Salisbury Cove, ME 04672, USA,* [3]*Department of Chemistry, University of Wisconsin-Madison, 1101 University Avenue, Madison, WI 53706-1369, USA,* [4]*Department of Chemistry, Bowdoin College, 6600 College Station, Brunswick, ME 04011, USA,* [5]*School of Pharmacy, University of Wisconsin-Madison, 777 Highland Avenue, Madison, WI 53705-2222, USA and* [6]*Department of Biology, Bowdoin College, 6500 College Station, Brunswick, ME 04011, USA*

*Author for correspondence (e-mail: crabman@u.washington.edu)

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Summary

Over a quarter of a century ago, Mykles described the presence of putative endocrine cells in the midgut epithelium of the crab *Cancer magister* (Mykles, 1979). In the years that have followed, these cells have been largely ignored and nothing is known about their hormone content or the functions they play in this species. Here, we used a combination of immunohistochemistry and mass spectrometric techniques to investigate these questions. Using immunohistochemistry, we identified both SIFamide- and tachykinin-related peptide (TRP)-like immunopositive cells in the midgut epithelium of *C. magister*, as well as in that of *Cancer borealis* and *Cancer productus*. In each species, the SIFamide-like labeling was restricted to the anterior portion of the midgut, including the paired anterior midgut caeca, whereas the TRP-like immunoreactivity predominated in the posterior midgut and the posterior midgut caecum. Regardless of location, label or species, the morphology of the immunopositive cells matched that of the putative endocrine cells characterized ultrastructurally by Mykles (Mykles, 1979). Matrix-assisted laser desorption/ionization-Fourier transform mass spectrometry identified the peptides responsible for the immunoreactivities as GYRKPPFNGSIFamide (Gly^1-SIFamide) and APSGFLGMRamide [*Cancer borealis* tachykinin-related peptide Ia (CabTRP Ia)], respectively, both of which are known neuropeptides of *Cancer* species. Although the function of these midgut-derived peptides remains unknown, we found that both Gly^1-SIFamide and CabTRP Ia were released when the midgut was exposed to high-potassium saline. In addition, CabTRP Ia was detectable in the hemolymph of crabs that had been held without food for several days, but not in that of fed animals, paralleling results that were attributed to TRP release from midgut endocrine cells in insects. Thus, one function that midgut-derived CabTRP Ia may play in *Cancer* species is paracrine/hormonal control of feeding-related behavior, as has been postulated for TRPs released from homologous cells in insects.

Key words: brain-gut peptide, immunohistochemistry, laser-scanning confocal microscopy, matrix-assisted laser desorption/ionizaton-Fourier transform mass spectrometry, MALDI-FTMS.

Introduction

In both invertebrates and vertebrates, peptides originally isolated from neural tissues have been found subsequently in endocrine cells of the gut epithelium, and *vice versa*. This 'brain-gut' distribution has been extensively documented in insects, where many neuropeptides, including tachykinin-related peptides (TRPs), A-type allatostatins and FLRFamide-related peptides have been found in endocrine cells of the midgut epithelium (e.g. Reichwald et al., 1994; Sehnal and Zitnan, 1996; Davey et al., 1999; Pabla and Lange, 1999; Winther et al., 1999; Winther and Nässel, 2001). The peptides released from these insect midgut endocrine cells have been implicated in the paracrine/hormonal regulation of many physiological processes, including, but not limited to, the control of heart function,

hemolymph circulation, digestion and water and ion transport (e.g. Duve et al., 1994; Lee et al., 1998; Zudaire et al., 1998; Winther and Nässel, 2001).

Although crustaceans are closely related to insects, and neuroendocrine regulation is an important component of control systems in both taxa, the endocrine role of the gut epithelium has not been widely studied in crustaceans (Mykles, 1979; Chung et al., 1999; Webster et al., 2000). As in insects, the digestive tract of decapod crustaceans, including the crabs *Cancer borealis*, *Cancer magister* and *Cancer productus* (the subjects of our investigation), can be subdivided into three distinct regions: the foregut, the midgut and the hindgut (Fig. 1). The midguts of brachyurans, including those of *Cancer* species, consist of the midgut proper (sometimes referred to as the intestine), the highly branched hepatopancreas (also referred to as the midgut gland) and three associated caeca: the paired anterior midgut caeca (AMCs), which arise laterally, with one on either side of the midgut just posterior to the pyloric region of the foregut, and the single posterior midgut caecum (PMC), which arises dorsally, at or just anterior to the midgut-hindgut transition (Fig. 1).

The ultrastructural organization of the *C. magister* midgut epithelium is similar to that of other transporting epithelial tissues (Mykles, 1979). Specifically, the epithelial cells possess microvillar borders and contain a baso-centrally located nucleus, numerous basally located mitochondria and an extensive system of smooth endoplasmic reticulum (Mykles, 1979). In addition to the epithelial cells, Mykles also noted that hemocytes, terminals of putative neurosecretory neurons, and putative endocrine cells are also contained within the midgut epithelium (Mykles, 1979). In *C. magister*, as well as in the lobsters *Homarus americanus* and *Homarus gammarus*, the putative endocrine cells were identified throughout the midgut proper, as well as in its associated caeca (Mykles, 1979). In terms of their ultrastructure, these cells were found to be essentially identical to their insect counterparts (Reinhardt, 1976; Hecker, 1977; Endo and Nishiitsutsuji-Uwo, 1982; Brown et al., 1985; Leite and Evangelista, 2001; Neves et al., 2003). Specifically, in *C. magister* they exhibited a slightly enlarged basal region, which contains the nucleus, an extensive rough endoplasmic reticulum and Golgi complex, as well as a slender apical extension that projects toward the midgut lumen (Mykles, 1979).

Although not explicitly stated in his study, the transmission electron micrographs of Mykles show that many types of dense-core vesicles are contained within the putative endocrine cells of the *C. magister* midgut [i.e. figs 13 and 14 in Mykles (Mykles, 1979)]. Moreover, some of the vesicles shown in the micrographs appear to be docked to, or are in the process of fusing with, the plasma membrane. Collectively, these observations suggest that the midgut epithelial endocrine cells of crustaceans contain and secrete diverse hormones. However, in contrast to the wealth of information on the hormonal contents of insect midgut endocrine cells, nothing is known about the identity of the paracrines/hormones present in the putative midgut epithelial endocrine cells of any decapod species. Here, we have begun an immunohistochemical and mass spectrometric investigation to determine the extent to which crustacean neuropeptide paracrines and hormones are located in and released by midgut epithelial endocrine cells of *Cancer* species, focusing on the tachykinin-related peptides (TRPs), which are well-documented brain-gut peptides in insects (reviewed by Nässel, 1999), and the SIFamides, a newly described family of neuropeptides present in both insects and crustaceans (Janssen et al., 1996; Vanden Broeck, 2001; Huybrechts et al., 2003; Sithigorngul et al., 2002; Verleyen et al., 2004; Yasuda et al., 2004; Messinger et al., 2005; Christie et al., 2006). Some of these data have appeared previously in abstract form (Christie et al., 2005).

Materials and methods

Animals

Dungeness crabs (*Cancer magister* Dana) and red rock crabs (*Cancer productus* Randall) were collected by hand or trap at multiple locations throughout the San Juan Archipelago and greater Puget Sound areas of Washington State, USA. Jonah crabs (*Cancer borealis* Stimpson) were purchased from J&A Seafood (Brunswick, ME, USA), Allen's Seafood (Harpswell, ME, USA), Downeast Lobster Pound (Trenton, ME, USA) or the Marine Biological Laboratory (Woods Hole, MA, USA).

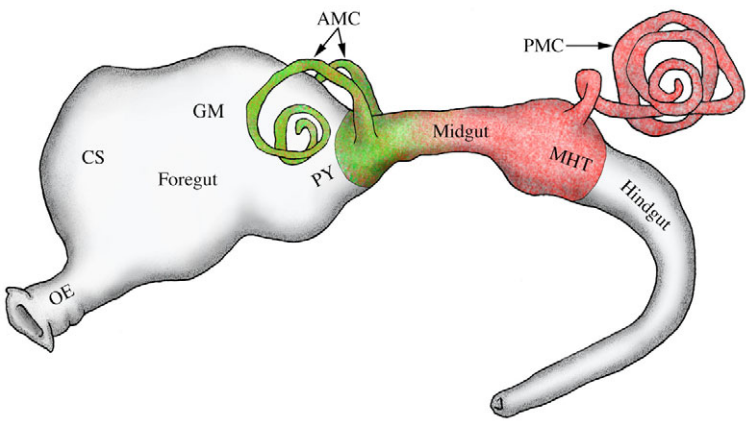

Fig. 1. Schematic representation of the digestive tract, including the midgut, of *Cancer* crabs. The digestive tract of the investigated *Cancer* species can be divided into three distinct regions: the foregut [comprised of the oesophagous (OE), the cardiac sac (CS), the gastric mill (GM) and the pylorus (PY)], the midgut (colored) and the hindgut. The midgut region is comprised of midgut proper, the highly branched hepatopancreas (not included in this schematic) and three associated caeca: the paired anterior midgut caeca (AMC), which arise laterally, one on either side of the midgut just posterior to the pylorus, and the single posterior midgut caecum (PMC), which arises dorsally, at or just anterior to the midgut/hindgut transition (MHT). Regions of the midgut where SIFamide-like immunoreactivity has been localized are shown in green; those in which tachykinin-like labeling was seen are colored red.

Regardless of species, animals were maintained in aerated natural seawater aquaria chilled to approximately 10°C.

Tissue and hemolymph collection

For tissue collection, crabs were anesthetized by packing in ice for 30–60 min, after which the dorsal carapace was removed and the midgut with its associated caeca (Fig. 1) was dissected from each animal in chilled (approximately 10°C) physiological saline [composition: 440 mmol l^{-1} NaCl, 11 mmol l^{-1} KCl, 13 mmol l^{-1} $CaCl_2$, 26 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} Hepes (pH adjusted to 7.4 with NaOH)]. The hepatopancreas was not investigated in our study. Isolated midguts were pinned in Sylgard 184 (World Precision Instruments, Inc., Sarasota, FL, USA; catalog #SYLG184)-lined Petri dishes and processed for either immunohistochemistry or mass spectrometry as described below.

For the collection of hemolymph, size/weight-matched adult male *C. productus* were housed in individual seawater tanks and held without food for one week. At the end of the starvation period, half of the animals were allowed to feed *ad libitum* on chopped fresh fish, whereas the remaining animals were kept unfed. Two hours after the initiation of feeding, hemolymph was collected from both the fed and unfed animals by inserting a 22-gauge needle attached to a 3-ml plastic syringe through the junction of the thorax and abdomen into the pericardial chamber. Approximately 2 ml of hemolymph was drawn from each animal. A fresh needle and syringe was used for each hemolymph draw. Immediately after its collection, hemolymph was processed for mass spectrometry as described below.

Whole-mount immunohistochemistry

Whole-mount immunoprocessing

All preparations were processed for immunohistochemistry as whole-mounts. In brief, tissues were fixed overnight (12–24 h) at 4°C in a freshly made solution of 4% paraformaldehyde (EM grade; catalog #15710; Electron Microscopy Sciences, Hatfield, PA, USA) in 0.1 mol l^{-1} sodium phosphate (P) buffer (pH 7.4) followed by five rinses (at 1-h intervals) in a solution of P containing 0.3% Triton X-100 (P-Triton). After rinsing, tissues were incubated in a primary antibody (see below) for approximately 24–72 h. Dilution of primary antiserum was done in P-Triton, with 10% normal donkey serum (NDS; Jackson ImmunoResearch; catalog #017-000-121) added to diminish nonspecific binding. Following incubation in primary antibody, tissues were again rinsed five times at 1-h intervals in P-Triton and then incubated overnight in secondary antibody (see below). As with the primary antibody, secondary antibody incubation was done in P-Triton containing 10% NDS. For all double-labeled preparations, the primary antibodies were applied simultaneously, as were the secondary antibodies used for double-labeling. After secondary antibody incubation, tissues were rinsed five times over approximately 5 h in P and then mounted between a glass microscope slide and coverslip using Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA; catalog #H-1000). To determine the location of nuclei within the midgut epithelium, some preparations were mounted in Vectashield mounting medium commercially premixed with DAPI (Vector Laboratories; catalog #H-1200). Incubation in both primary and secondary antibody was done at 4°C using gentle agitation. All rinses were done at room temperature (18–22°C) without agitation. Secondary antibody incubation, and all subsequent processing, was performed in the dark. Likewise, slides were stored in the dark at 4°C until examined.

Antibodies

A rabbit polyclonal antibody generated against VYRKPPFNGSIFamide [Val^1-SIFamide; antibody code 3423-30 (Christie et al., 2006)] was used for the detection of SIFamides. A rat monoclonal antibody generated against substance P [clone NC1/34 HL; Abcam Inc., Cambridge, MA, USA; catalog #ab6338 (Cuello et al., 1979)] was used for the detection of TRPs. Unless noted otherwise, the SIFamide antibody was used at a final dilution of 1:1000, whereas the substance P antibody was used at a final dilution of 1:500. Donkey anti-rabbit immunoglobulin G (IgG) conjugated with Alexa-Fluor 488 (Molecular Probes, Eugene, OR, USA; catalog #A-21206) was used to visualize the SIFamide antibody, whereas donkey anti-rat IgG conjugated with Alexa-Fluor 594 (Molecular Probes; catalog #A-21209) or Rhodamine Red-X (Jackson ImmunoResearch Laboratories, West Grove PA, USA; catalog #712-295-153) was used for visualization of the substance P antibody. Unless otherwise noted, all secondary antibodies were used at final dilutions of 1:300.

Preadsorption controls

For use in preadsorption controls, GYRKPPFNGSIFamide (Gly^1-SIFamide) and APSGFLGMRamide [*Cancer borealis* tachykinin related-peptide Ia (CabTRP Ia)] were synthesized by AC Scientific (Duluth, GA, USA) and the Protein Chemistry Laboratory of the University of Pennsylvania School of Medicine (Philadelphia, PA, USA), respectively. These peptides are known to be present in the neural tissues of *Cancer* species (Christie et al., 1997; Huybrechts et al., 2003; Messinger et al., 2005), and, as we show here, in the midguts of these animals as well (see Results). In our controls, we preadsorbed working dilutions of each antibody with either Gly^1-SIFamide (10^{-5} mol l^{-1}) or CabTRP Ia (10^{-5} mol l^{-1}) for 2 h at room temperature prior to applying the solution to the tissue. Immunostaining was then performed as described above, except that the incubation time in the preadsorbed antibody solution was limited to approximately 24 h so as to minimize degradation of the blocking peptide. Owing to a limited supply of synthetic peptides, preadsorption controls were performed only on *C. productus* midguts.

Confocal and epifluorescence microscopy

After immunolabeling, preparations were viewed with a Nikon (Tokyo, Japan) Eclipse E600 epifluorescence microscope, and digital images were collected using a Bio-Rad

Radiance 2000 laser scanning confocal microscope (Bio-Rad Microscience Ltd, Hemel Hempstead, UK). The Nikon Eclipse E600 epifluorescence microscope was equipped with Nikon PlanFluor 10× 0.30NA, PlanFluor 20× 0.50NA and PlanFluor 40× 0.75NA dry objective lenses and ENDOW GFP HYQ (EX, 450–490 nm; DM, 495 nm; BA, 500–550 nm) and G-2E/C TRITC (EX, 528–553 nm; DM, 565 nm; BA, 600–660 nm) filter sets. The Bio-Rad Radiance 2000 system was equipped with a modified Nikon Eclipse E600FN microscope and a krypton/argon mixed gas laser (488 and 568 nm excitation lines used). Imaging on this system was done using Nikon PlanApo 10× 0.45NA DIC dry, PlanApo 20× 0.75NA DIC dry and PlanApo 60× 1.4NA DIC oil immersion objective lenses, Bio-Rad-supplied HQ515/30 and/or E600LP emission filters, a 560DCLPXR dichroic mirror (for imaging double-labeled preparations) and Bio-Rad LaserSharp 2000 software. For imaging preparations labeled with DAPI, a Mai Tai laser (Spectra Physics, Fremont, CA, USA) tuned to 800 nm was used with the Radiance confocal system.

Direct tissue MALDI-FTMS

For direct tissue matrix-assisted laser desorption/ionization-Fourier transform mass spectrometry (MALDI-FTMS), midguts were dissected as described above, then small pieces of AMC, PMC or midgut proper were isolated. Tissue fragments were rinsed sequentially in two 12-μl droplets of 0.75 mol l^{-1} fructose (Sigma-Aldrich, St Louis, MO, USA; 99%), placed on one face of a 10-faceted probe tip, and then sliced 10–20 times with a 0.1 mm needle. The macerated tissue was then gathered together and covered with a 0.5 μl droplet of 1.0 mol l^{-1} 2,5-dihydroxybenzoic acid (DHB; Sigma-Aldrich; 98%, sublimed prior to use), prepared in 1:1 acetonitrile [Fisher Scientific, Pittsburg, PA, USA; high-performance liquid chromatography (HPLC) grade] and water containing 2% (v/v) phosphoric acid. All midgut samples were analyzed using a HiResMALDI Fourier transform mass spectrometer (IonSpec, Lake Forest, CA, USA) equipped with a Cryomagnetics (Oak Ridge, TN, USA) 4.7 Tesla actively shielded superconducting magnet (Department of Chemistry, Bowdoin College, Brunswick, ME, USA), as described for neural tissues (Christie et al., 2006). Internal mass calibration was performed using selective in-cell accumulation of calibrant as previously described (Stemmler et al., 2005). Poly(propylene glycol) 725 and 2000 (PPG; Sigma-Aldrich) was used as the calibrant for most measurements; angiotensin II (Sigma-Aldrich) was used to calibrate *C. productus* AMC samples, with known phospholipid peaks used to calibrate samples from the midgut proper.

Release experiments

To assess whether the SIFamide and/or TRP present in midgut epithelial endocrine cells were releasable, chemical depolarization experiments similar to those employed by Winther and Nässel were undertaken (Winther and Nässel, 2001). Assessment of release was determined by both quantitative immunohistochemistry and mass spectrometry.

Anatomical studies

For anatomical release experiments, the paired AMCs and the single PMC from individual crabs were isolated as described earlier. Each PMC was subsequently divided into two approximately equal pieces. Following their isolation, one AMC and one section of the PMC from a crab were loosely pinned in a Sylgard 184-lined Petri dish containing chilled (4°C) physiological saline (see above for composition), with the other AMC and the other portion of the PMC from the same individual loosely pinned in a separate Sylgard-lined Petri dish containing chilled physiological saline. Equal volumes of saline (approximately 3 ml) were placed in each dish and the tissues incubated in this saline for 1 h at 4°C. The saline bathing the tissues was continuously mixed using gentle agitation. After 1 h, the saline in one dish was replaced with a fresh sample of chilled physiological saline, whereas that in the other dish was replaced with an equal volume of chilled high-potassium (K^+) saline (composition identical to that of the physiological saline, except for the KCl being raised to 110 mmol l^{-1}, with the additional KCl replacing NaCl). Tissues were allowed to incubate in these solutions for 1 h at 4°C under gentle agitation and were then fixed for immunohistochemistry as described earlier. All tissues from a given individual were simultaneously immunoprocessed using a common set of reagents. The immunolabeling methods were identical to those presented earlier in this study, with the exceptions that final dilutions of the primary and secondary antibodies were lowered 5-fold (i.e. 1:5000 anti-SIFamide, 1:2500 anti-substance P and 1:1500 for either secondary antibody) and the incubation time in primary antibody was limited to 24 h. These modifications were made because they produced weak, but consistent, labeling in the tissues, therefore maximizing our ability to detect subtle changes in label intensity.

ImageJ 1.37 software (available free of charge at http://rsb.info.nih.gov/ij/download.html; National Institutes of Health, Bethesda, MD, USA) was used to determine the intensity of labeling in midgut endocrine cells from both the chemically depolarized tissues and their physiological saline counterparts. Specifically, confocal *z*-series from simultaneously immunoprocessed tissues were collected using the Bio-Rad Radiance confocal system described earlier, ensuring that the pixel values of the images were not saturated (i.e. no pixels with intensities of 0 or 255). For each tissue group from each species, a physiological saline-incubated caecum was imaged first with the mean pixel intensity of labeling in the endocrine cells set to a value of approximately 170. The same settings were then used to image all preparations from a given experimental grouping, and all image collection for each experiment was done during a single imaging session. After *z*-series were collected, the Bio-Rad .pic files were converted to .tif images using ImageJ. For each *z*-series, an optical section that contained immunopositive cells whose nuclei were clearly identifiable was selected. Within each selected section, the cytoplasmic region surrounding the nucleus of a given cell was delineated using the Freehand Draw tool of ImageJ; the mean pixel value of the delineated

cytoplasmic region was then calculated using the Analyze command of the software. For each tissue sample, the mean pixel value for each of 20 immunopositive cells from each of three different regions of each AMC (junction with the midgut proper, middle of the caecum and the distal tip) or PMC section (both ends and the middle of each segment) was determined using ImageJ. The mean pixel value of these 60 cells was then calculated to give a single value for each tissue sample. This value for each high-K^+ saline-treated tissue was then compared with its physiological saline-treated counterpart using a paired two-tailed Student's *t*-test.

As an additional control, six pairs of AMCs and six pairs of PMC sections were subjected to incubation in the same saline solution (i.e. physiological/physiological or high-K^+/high-K^+). These tissues were immunoprocessed, imaged and analyzed identically to the physiological/high-K^+ saline pairings.

Mass spectrometric studies

To further assess peptide release from the midgut epithelial endocrine cells, and to attempt to determine the directionality of release, the releasates from several experiments were assayed *via* MALDI-FTMS for the presence of SIFamides and TRPs. Here, a single AMC or PMC was removed and the two ends of the tissue were tied closed with suture silk. Two salines were used for releasate studies: 10 ml of physiological saline containing one tablet of Mini Complete EDTA-free protease inhibitor cocktail [Roche Applied Science, Indianapolis, IN, USA; catalog #1 836 170] or the same volume of high-K^+ saline containing one tablet of the same protease inhibitor cocktail. For each releasate experiment, a single caecum was placed in 300 μl of physiological saline for 1 h, transferred to a second 300 μl sample of physiological saline for 1 h, then transferred to 300 μl of high-K^+ saline for 1 h, at which time the tissue was removed. All experiments were performed at 4°C and the tissue-saline mixtures were continuously mixed using gentle agitation. Samples for MALDI-FTMS analysis were prepared by mixing 0.5 μl of either the high-K^+ or the second physiological saline solutions with 0.5 μl of 1.0 mol l^{-1} DHB (prepared as described earlier). Sample analysis was performed on the HiResMALDI Fourier transform mass spectrometer located at Bowdoin College using an accumulation of 30 laser shots and conditions optimized for the detection of *m/z* 1000.

MALDI-FTMS of hemolymph extracts

To assess the complement of peptides in circulation, hemolymph, collected as described earlier, was immediately placed in twice its volume of acidified methanol [90% methanol (Sigma-Aldrich; HPLC grade): 9% glacial acetic acid (Fisher Scientific; sequencing grade): 1% water (Sigma-Aldrich; HPLC grade)] and vortexed for 3 min at 10°C using a Thermolyne Maxi Mix II tabletop vortexer (Barnstead/Thermolyne, Dubuque, IA, USA). After vortexing, the hemolymph/acidified methanol mixture was centrifuged at 15 800 ***g*** for 5 min at 10°C using an Eppendorf 5415C tabletop centrifuge (Eppendorf AG, Hamburg, Germany). After centrifugation, the supernatant was removed, flash-frozen in liquid nitrogen, and stored at –80°C until used for mass spectrometry. Immediately prior to MALDI-FTMS, extracts were thawed and large proteins removed by placing 500 μl of a crude extract into a 10 000 Da molecular mass cutoff tube (Argos Technologies, Elgin, IL, USA) and centrifuging at 16 100 ***g*** for 10 min at room temperature. The resulting low-molecular-mass filtrates were concentrated using a Savant SC 110 SpeedVac concentrator (Thermo Electron Corporation, West Palm Beach, FL, USA) and then resuspended in 10 μl of 0.1% formic acid (Sigma-Aldrich; puriss grade). The acidified samples were desalted by aspirating them through a ZipTipC18 pipette tip (Millipore, Billerica, MA, USA) and then the bound peptides eluted with 4 μl of 50% acetonitrile. Desalted extracts were mixed 1:1 with DHB matrix (150 mg ml^{-1} in 50% methanol/deionized water) on a MALDI probe tip and allowed to crystallize at room temperature. MALDI-FTMS analysis was then performed as described in several recent publications (Kutz et al., 2004; Messinger et al., 2005) using an IonSpec HiResMALDI Fourier transform mass spectrometer equipped with a 7.0-T actively shielded superconducting magnet (School of Pharmacy, University of Wisconsin-Madison).

Figure production

Anatomical figures were produced using Photoshop software (version 7.0; Adobe Systems Inc., San Jose, CA, USA). Contrast and brightness were adjusted as required to optimize the clarity of the printed confocal micrographs. For the production of direct tissue and releasate MALDI-FTMS figures, mass spectral traces were scanned into and labeled with Microsoft Word (Microsoft Corporation, Redmond, WA, USA). For the production of MALDI-FTMS figures depicting the peptides present in hemolymph samples, mass spectra were exported as bitmaps into Macromedia Fireworks MX 2004 Version 7.0 (Macromedia Inc., San Francisco, CA, USA) using the Boston University Data Analysis (BUDA) program and then labeled in Fireworks.

Results

Immunohistochemical evidence for SIFamide and TRP in the midgut endocrine cells of Cancer crabs

Within the midguts of each of the investigated species ($N \geqslant 10$ preparations for each antibody in each species), both SIFamide- and TRP-like immunopositive cells were seen (Figs 2 and 3). Regardless of species, immunolabel or location within the midgut, the gross morphology of the stained cells was similar, and was essentially identical to that of the putative endocrine cells of *C. magister* described by Mykles (Mykles, 1979). Specifically, the immunopositive cells exhibited an enlarged basal region and extended a thin, beaded projection apically (Figs 2 and 3). Likewise, the relative location and shape of the nuclei in the immunopositive cells was identical to that of the putative endocrine cells described by Mykles (Mykles, 1979), i.e. basally located and relatively spherical *versus* distinctly elongated and more centrally located for the epithelial cells proper (Fig. 4). Although similar in overall

organization, the immunopositive cells present in the midgut caeca tended to possess longer apical processes than those present in the midgut proper (Figs 2 and 3), although this is probably a reflection of the relative thicknesses of the epithelium of the respective midgut regions. Likewise, the immunopositive cells in the midgut caeca tended to be more uniformly distributed and densely packed than were those in the midgut proper, where labeling was often patchy (Figs 2 and 3). Regardless of label or location within the midgut, most of the immunopositive cells appeared to span the entire thickness of the epithelial layer, abutting/contacting both the basal surface of the midgut (which is adjacent to the hemocoel) and the midgut lumen (Figs 2 and 3). Some of the labeled cells exhibited short, thin basal processes that projected along the outer surface of the midgut epithelium, abutting the hemocoel (arrows in Fig. 5B).

No SIFamide- or TRP-like immunopositive nerve terminals were seen on any portion of the midgut surface in any of the examined *Cancer* species, suggesting that their midguts are not innervated by either SIFamide- or TRP-containing neuronal processes.

SIFamide- and TRP-like immunopositive endocrine cells are regionally segregated within the midgut epithelium of Cancer species

In immunoprocessing the midguts of *C. magister*, *C. borealis* and *C. productus* with the SIFamide and substance P antibodies, a notable feature consistently seen in all species ($N \geqslant 10$ preparations for each antibody in each species) was a differential distribution of cells labeled by each antibody. Specifically, the SIFamide-immunostained cells were restricted to the epithelium of the anterior portion of the midgut proper and the AMCs (Fig. 1, Fig. 5A), whereas those labeled by the substance P antibody were concentrated in the posterior portion of the midgut proper and the PMC (Fig. 1, Fig. 5B). It should be noted that a small number of TRP-immunopositive cells were seen in the anterior portion of the midgut and the AMC (Fig. 1, Fig. 5A). From double-labeled preparations ($N \geqslant 5$ for each species), it was clear that the few TRP-like immunopositive cells seen in the anterior midgut and AMCs were not among those labeled by the SIFamide antibody, and *vice versa* (e.g. the presence of red and green, but not yellow, cells in Fig. 5A). Thus, the two immunoreactivities do not appear to colocalize in the midgut cells.

Preadsorption controls

To assess the specificities of the immunolabeling just described, preadsorption controls for each antibody were performed using the only known peptide hormones present in the midgut (i.e. Gly^1-SIFamide and CabTRP Ia; see Results). For the Val^1-SIFamide antibody, a complete block of immunolabeling was achieved only when the antibody was adsorbed with Gly^1-SIFamide (N=3 preparations; data not shown). When this antibody was pretreated with CabTRP Ia (N=3 preparations; data not shown) no effect was seen in immunolabeling for SIFamide. Similarly, a complete block of TRP-like

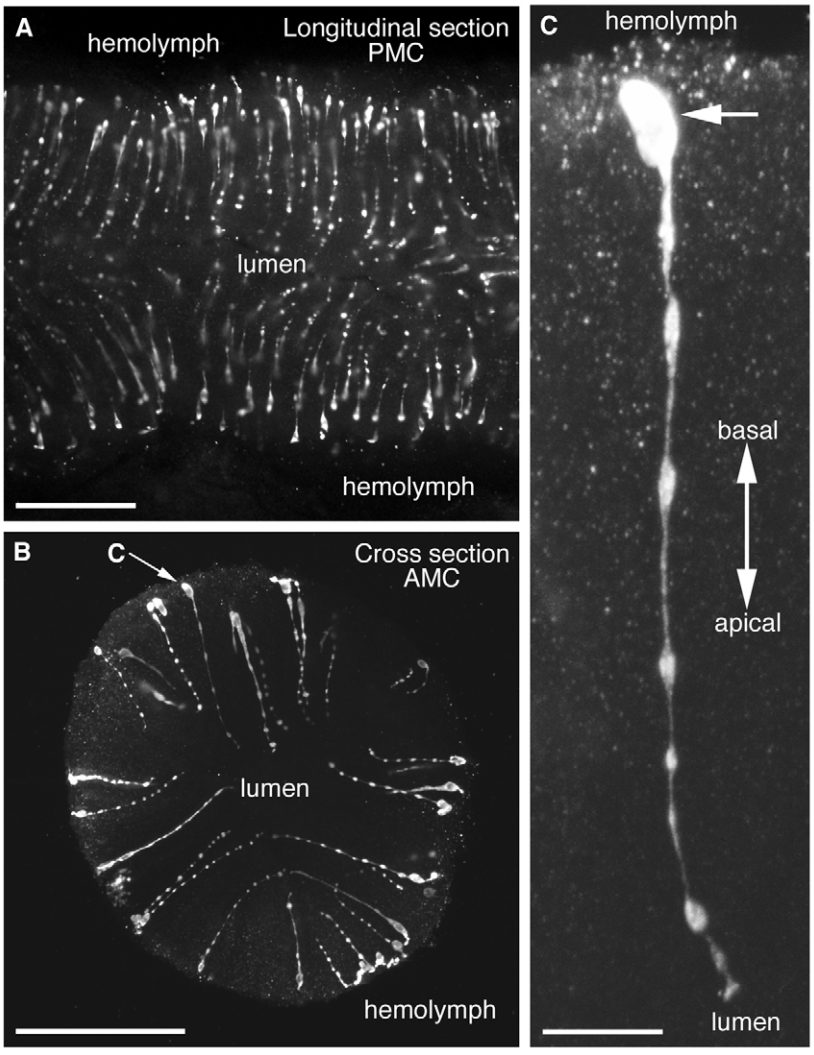

Fig. 2. General organization and morphology of peptidergic endocrine cells in the *Cancer* anterior and posterior midgut caeca epithelia. Regardless of species (*Cancer productus* shown), immunolabel (anti-substance P in A and anti-SIFamide in B and C) or location within the midgut caeca (posterior midgut caecum in A and anterior midgut caecum in B and C), the gross organization and morphology of the intrinsic peptidergic endocrine cells was similar. Specifically, all cells possessed an enlarged basal region and extended a thin, beaded projection apically toward the midgut lumen. This organization is shown in longitudinal-section in A and in cross-section in B. The morphology of one peptidergic cell from B (arrow) is shown at higher magnification in C. (A) A single optical section. (B) A brightest pixel projection of 42 optical sections collected at 1.05-μm intervals. (C) A brightest pixel projection of 22 optical sections collected at 0.75-μm intervals. Scale bars, 200 μm (A,B) and 25 μm (C).

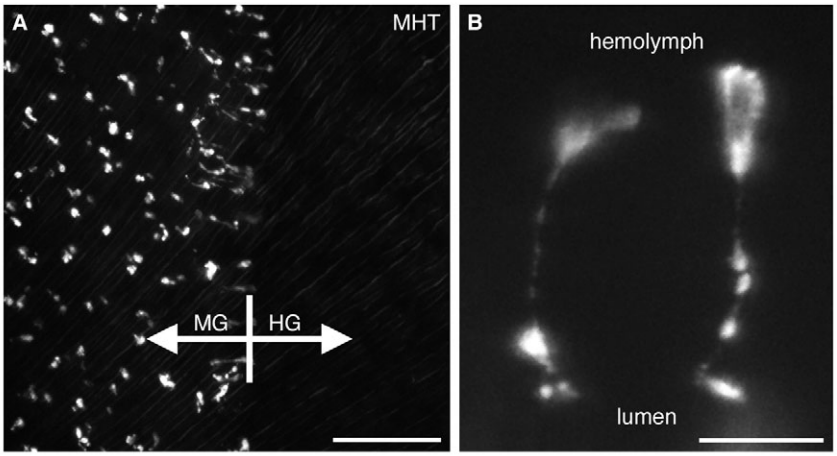

Fig. 3. General organization and morphology of peptidergic epithelial endocrine cells in the midgut proper (MG) of *Cancer* crabs. Regardless of species (*Cancer productus* shown), immunolabel (anti-substance P shown) or location within the midgut proper (posteriormost portion of the midgut shown), the gross organization and morphology of the intrinsic peptidergic endocrine cells was similar. All cells possessed an enlarged basal region and extended a short beaded projection apically toward the midgut lumen. This organization is shown in a low-magnification view of the flattened midgut in A and in a high-magnification view of two immunolabeled cells in B. (A) A brightest pixel projection of 61 optical sections collected at 2.1-μm intervals taken at the midgut/hindgut transition (MHT); the boundary of each region is delineated. Note that no immunolabeling is present in the hindgut (HG). In this image, faint autofluorescence can be seen in the muscle fibers overlying both the MG and HG. (B) A brightest pixel projection of nine optical sections collected at 1.95-μm intervals showing two labeled endocrine cells at high magnification. Note that both immunopositive cells appear to span the entire epithelium, abutting both the midgut lumen and the hemocoel. Scale bars, 200 μm (A) and 25 μm (B).

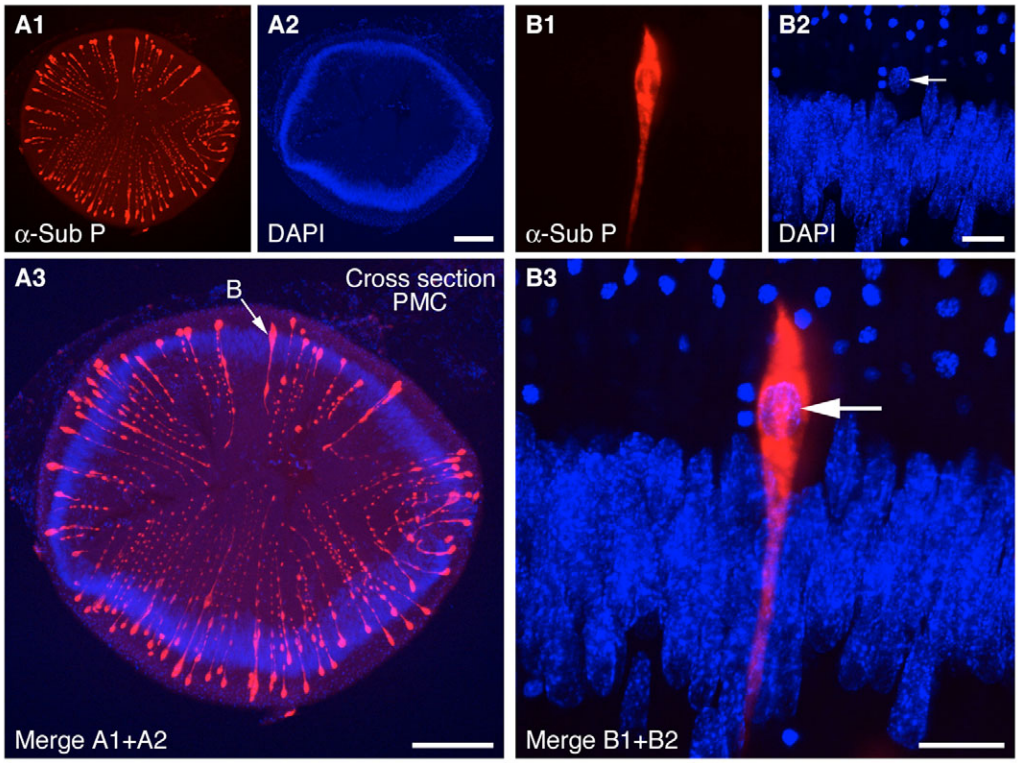

Fig. 4. The nuclei of *Cancer* midgut epithelial endocrine cells are located within their enlarged basal region [*Cancer magister* posterior midgut caecum (PMC) is shown as an example]. (A) Cross-section of the posterior midgut caecum showing the overall distribution of (A1) substance P-like immunoreactivity (pseudocolored red) and (A2) DAPI labeling (pseudocolored blue) in this structure. A3 is a pseudocolored merger of images A1 and B2. All images in this set are brightest pixel projections of 40 optical sections collected at 1.95-μm intervals, with the substance P and DAPI images collected sequentially. A1 and A2 are shown at the same scale, with the scale bar in A2 equal to 200 μm. The scale bar in A3 is also equal to 200 μm. (B) One immunopositive endocrine cell from A shown at higher magnification. When the images of the substance P immunoreactivity (B1) and DAPI label (B2) are merged (B3), the nucleus of the epithelial endocrine cell (arrow in B2 and B3) can clearly be seen to reside in the enlarged basal region. It should be noted that many other nuclei are present in this micrograph. The large elongate nuclei in the lower portion of B2 and B3 are probably those of epithelial cells, whereas the small, round nuclei in the upper portion of the image may be those of hemocytes. As in A, the substance P and DAPI images shown in B were collected sequentially. All images in this set are brightest pixel projections of 28 optical sections collected at 1.05-μm intervals. B1 and B2 are shown at the same scale, with the scale bar in B2 equal to 25 μm. The scale bar in B3 is also equal to 25 μm.

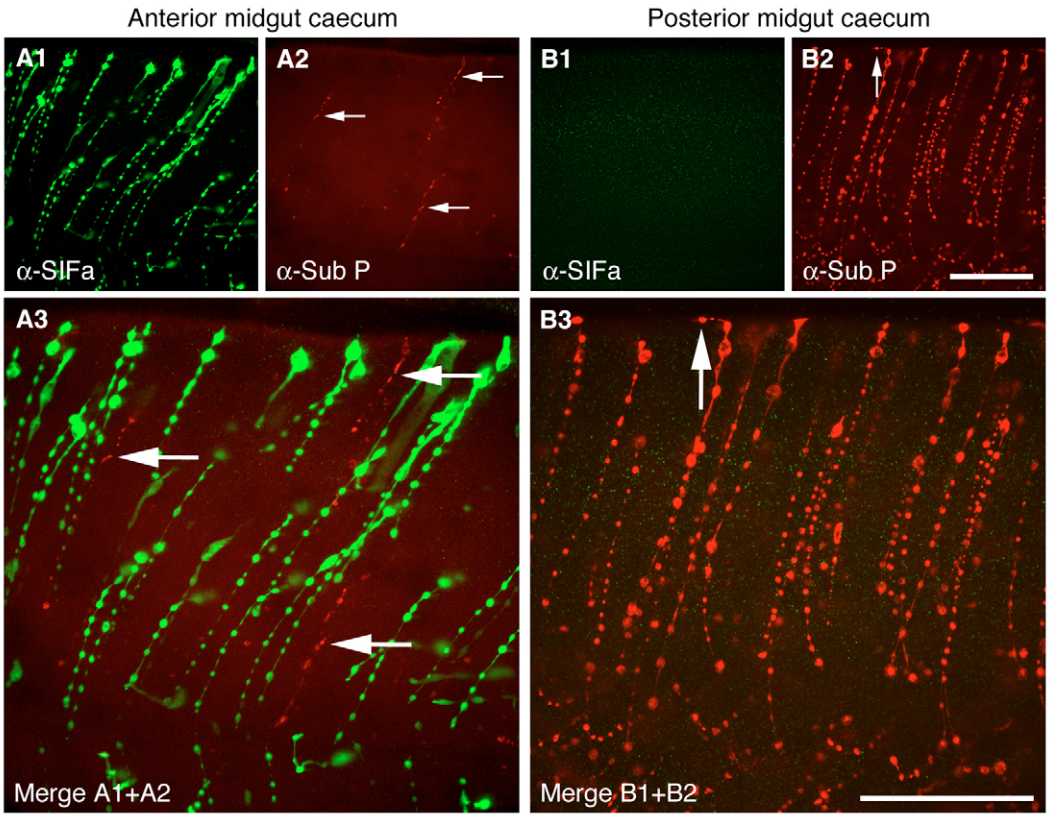

Fig. 5. SIFamide- and tachykinin-related peptide-like immunopositive endocrine cells are differentially distributed within the *Cancer* midgut epithelium. In each of the species investigated (*Cancer borealis* shown), the cells labeled by the SIFamide and substance P antibodies were differentially distributed. Specifically, the SIFamide (SIFa)-stained cells (pseudocolored green) were restricted to the epithelium of the anterior portion of the midgut proper and the anterior midgut caeca (shown in A), whereas substance P (Sub P) immunopositive cells (pseudocolored red) were concentrated in the posterior portion of the midgut proper and the posterior midgut caecum (shown in B). As the few substance P-like immunopositive cells seen in the anterior midgut (arrows) were not among those labeled by the SIFamide antibody, and *vice versa* (e.g. the red and green, but not yellow, cells present in A), no colocalization of the two peptides is apparent in the midgut. It should be noted that some epithelial endocrine cells possess a short, thin, basal process that projects along the outer surface of the midgut (arrows in B). This type of projection was seen in a subset of both the SIFamide- and TRP-like immunopositive cells (TRP cell shown). A1 (anti-SIFamide) and A2 (anti-substance P) are brightest pixel projections of 28 optical sections collected at 1.05-μm intervals. Both labels were imaged simultaneously. A3 is a merge of A1 and A2. B1 (anti-SIFamide) and B2 (anti-substance P) are brightest pixel projections of 26 optical sections collected at 1.05-μm intervals. Both labels were imaged simultaneously. B3 is a merge of B1 and B2. A1, A2, B1 and B2 are all shown at the same scale, with the scale bar in B2 equal to 200 μm. A3 and B3 are shown at the same scale, with the scale bar in B3 also representing 200 μm.

immunoreactivity was achieved when the substance P antibody was preadsorbed with CabTRP Ia (N=3 preparations; data not shown), but not when this antibody was pretreated with Gly^1-SIFamide (N=3 preparations; data not shown).

Direct tissue MALDI-FTMS identification of Gly^1-SIFamide and CabTRP Ia in Cancer midgut tissues

Although the immunohistochemistry described above strongly supported the presence of both SIFamide- and TRP-like peptides in midgut epithelial endocrine cells, the identity of the specific isoforms present remained unknown. To identify these substances, we performed direct tissue MALDI-FTMS on epithelial samples isolated from either the AMC or PMC of each of the *Cancer* species used in this study, as well as from the midgut proper of *C. borealis*. In the spectra collected from small pieces of the PMC ($N\geqslant3$ samples per species), an intense peak appearing at *m/z* 934.49 was consistently detected at a high relative abundance in all species. A representative spectrum from *C. borealis* is shown in Fig. 6A. The *m/z* 934.49 peak was identified as CabTRP Ia based upon the *m/z* value measured using internal calibration with PPG (see Table 1). This assignment was further substantiated by isolation and measurement of MS/MS spectra that showed excellent agreement with that of a CabTRP Ia standard (data not shown). Spectra of the PMC samples showed no indication of a peak corresponding to Gly^1-SIFamide (i.e. *m/z* 1381.74) or any other known SIFamide isoform (i.e. Ala^1-SIFamide and Val^1-SIFamide).

In spectra collected from the AMC of each species ($N\geqslant3$ samples per species), all peptide peaks were consistently less intense than peaks derived from the PMC; a representative spectrum from *C. borealis* is shown in Fig. 6B. Peaks at both

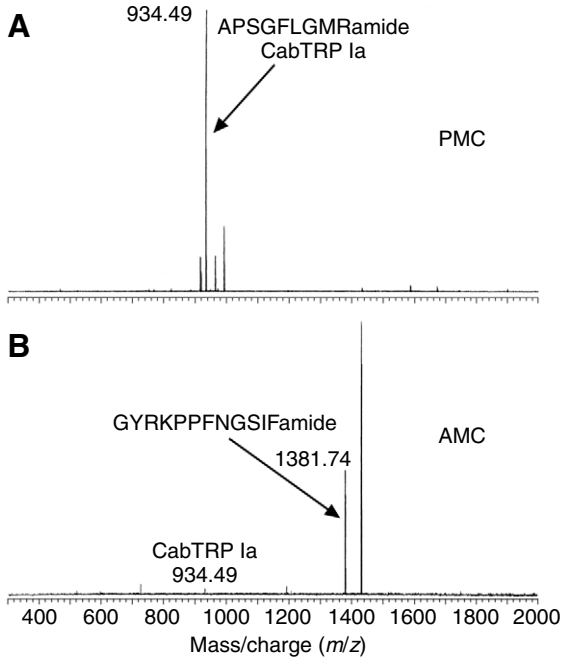

Fig. 6. Direct MALDI-FTMS analysis of midgut tissues. Data presented in this figure are from *Cancer borealis*, although identical peptide identifications were achieved from both *Cancer magister* and *Cancer productus*. Regardless of species or tissue, spectra were measured using DHB as the matrix, with conditions optimized for accumulation of *m/z* 1500. (A) A representative spectrum from a small piece of posterior midgut caecum (PMC). In PMC samples, an intense peak appearing at *m/z* 934.49 was consistently detected at a high relative abundance. This peak was identified as APSGFLGMRamide (CabTRP Ia), based upon the *m/z* value measured using internal calibration with poly(propylene glycol). Spectra of the PMC samples showed no indication of a peak corresponding to GYRKPPFNGSIFamide (Gly^1-SIFamide), i.e. *m/z* 1381.74, or any other known SIFamide isoform. (B) A representative spectrum from a small piece of anterior midgut caecum (AMC). In AMC samples, a peak at *m/z* 1381.74 (corresponding to the $[M+H]^+$ ion for Gly^1-SIFamide) was detected in approximately 95% of the spectra measured; a peak at *m/z* 934.49 (corresponding to the $[M+H]^+$ ion for CabTRP Ia) was detected in approximately 40% of the spectra. Because of the low intensities of these peptide peaks, only accurate mass measurements were used for peptide identification in this tissue.

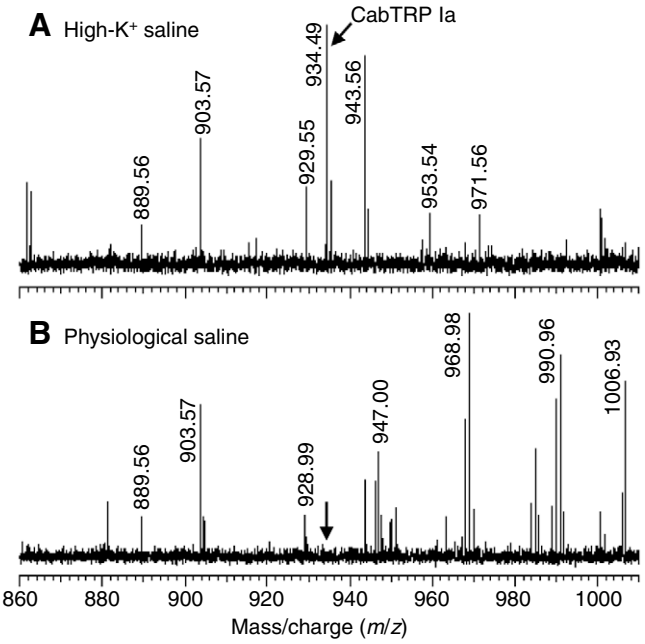

Fig. 7. Detection of APSGFLGMRamide (CabTRP Ia) in the releasate from posterior midgut caecum (PMC) samples. (A) MALDI-FTMS spectrum of 0.5 μl of sample taken after a single PMC sample was immersed in high-K^+ saline containing an inhibitor cocktail for 1 h at 4°C. (B) MALDI-FTMS spectrum of 0.5 μl of sample taken after a single PMC sample was immersed in physiological saline containing an inhibitor cocktail for 1 h at 4°C prior to tissue transfer to high-K^+ saline. The arrow indicates the *m/z* position where CabTRP Ia would be found, if present in the sample. Spectra were measured using DHB as the matrix, and conditions were optimized for ions of *m/z* 1000, using the accumulation of 30 laser shots. Both A and B are shown at the same (*m/z*) scale.

m/z 1381.74 and *m/z* 934.49 were detected in at least one spectrum from each animal, but the peak at *m/z* 1381.74 was seen more consistently than was the *m/z* 934.49 peak. Because of the low intensities of the peptide peaks present in AMC spectra, only accurate mass measurements were used for peptide identification. The measured masses were consistent with the assignment of these peaks as Gly^1-SIFamide and CabTRP Ia (Fig. 6B, Table 1). In all species, the relative intensity of the peak corresponding to Gly^1-SIFamide was greater than that of CabTRP Ia (Fig. 6B).

For *C. borealis*, we also examined small pieces of tissue taken from the anterior midgut near the AMC junction, from the central portion of the midgut and from the posterior midgut near the PMC junction. We consistently detected a peak corresponding to that of CabTRP Ia (i.e. *m/z* 934.49) in tissue samples collected from the midgut near the base of the posterior midgut caeca, as well as in most, but not all, samples taken from the posterior and middle region of the midgut proper (Table 1). Peaks corresponding to both CabTRP Ia and Gly^1-SIFamide (*m/z* 1381.74) were consistently detected from midgut tissue collected from near the base of the AMC (Table 1). No peak corresponding to Gly^1-SIFamide was detected in other midgut tissue samples (Table 1), and that corresponding to CabTRP Ia was detected in fewer samples at the anterior relative to the posterior end of the midgut proper.

Immunohistochemical evidence for peptide release from Cancer midgut endocrine cells

To determine whether Gly^1-SIFamide and/or CabTRP Ia can be released from midgut endocrine cells, we compared the relative intensity of SIFamide- and TRP-like immunoreactivity in tissue samples that had been incubated in either physiological or high-K^+ saline. Specifically, one of the paired AMCs from an individual was incubated in physiological

Table 1. *Exact mass measurements for APSGFLGMRamide (CabTRP Ia) and GYRKPPFNGSIFamide (Gly^1-SIF) detected in the direct analysis of tissues using MALDI-FTMS*

		Peptide identity:	APSGFLGMRa	GYRKPPFNGSIFa
		Ion identity:	$[M+H]^+$	$[M+H]^+$
		Calculated *m/z*:	934.49273	1381.73753
Species	Tissue[a]		Measured *m/z*[b,*]	Measured *m/z*[b,*]
Cancer borealis	PMC		934.49398 (1.3)	ND
	MG^P		934.49075 (–2.1)	ND
	MG^C		934.49265 (–0.1)	ND
	MG^A		934.49194 (–0.8)	1381.73142 (–4.4)
	AMC		934.49232 (–0.4)	1381.73669 (–0.6)
Cancer productus	PMC		934.49284 (0.1)	ND
	AMC		934.49283 (0.1)	1381.73754 (0.01)
Cancer magister	PMC		934.49328 (0.6)	ND
	AMC		934.49392 (1.3)	1381.73837 (0.6)

*Figure given in parentheses is the mass measurement error (p.p.m.).
[a]PMC, posterior midgut caecum; MG^P, posterior midgut; MG^C, central midgut; MG^A, anterior midgut; AMC, anterior midgut caecum.
[b]Internal calibration with poly(propylene glycol), angiotensin II or with known phospholipid peaks.
ND, not detected.

Table 2. *Depolarization-induced changes in immunolabeling intensity*

Species	Tissue/label	Control	High K^+	Change (%)	*P* value
Cancer borealis	AMC/SIFa	169.8±5.5	164.5±6.6	–3.1±1.9	0.0088
	PMC/TRP	158.8±8.5	150.2±7.1	–5.4±1.7	0.0009
Cancer productus	AMC/SIFa	171.8±6.3	165.0±6.0	–4.0±0.6	<0.0001
	PMC/TRP	168.7±4.6	160.2±5.0	–5.0±2.3	0.0032
Cancer magister	AMC/SIFa	164.7±9.3	158.3±7.6	–3.8±2.2	0.0083
	PMC/TRP	161.7±8.6	155.0±9.1	–4.1±0.9	<0.0001

Paired anterior midgut caeca (AMCs) and the single posterior midgut caecum (PMC) from six individual crabs were isolated, and each PMC was divided into two pieces. After 1 h of incubation in control saline, the saline surrounding one member of each tissue pair was replaced with control saline, whereas the other was replaced with high-K^+ saline. After 1 h, all tissues were simultaneously immunoprocessed for either SIFamide (SIFa)- or substance P (TRP)-like labeling. Values shown are the mean pixel values for each of the six preparations. Paired two-tailed Student's *t*-tests were used to compare labeling intensity in control and high-K^+ saline (*P* value). No statistically significant differences were seen in *C. borealis* tissue pairs when both AMCs or PMC sections were incubated in the same saline (*N*=6 pairs per tissue and saline; data not shown).

saline, whereas the other was incubated in high-K^+ saline (*N*=6 pairings for each species). Likewise, one half of the PMC from an individual was incubated in physiological saline, whereas the other half of the caecum was incubated in high-K^+ saline (*N*=6 pairings for each species). Tissue pairs were then simultaneously immunoprocessed for either SIFamide-like (AMCs) or TRP-like (PMC) labeling. For the SIFamide- and TRP-like immunoreactivity in each species, a small, but consistent, decrease in label intensity was seen in the tissue samples chemically depolarized by exposure to the high-K^+ saline (Table 2). Pair-wise two-tailed paired Student's *t*-tests showed that this drop in label intensity was statistically significant (Table 2). By contrast, no statistically significant differences were seen in the *C. borealis* tissue pairs where both AMCs or both PMC sections were incubated in the same saline [AMC physiological/physiological, *P*=0.415; AMC high-K^+/high-K^+, *P*=0.7089; PMC physiological/physiological, *P*=0.8797; PMC high-K^+/high-K^+, *P*=0.3774 (*N*=6 pairs per tissue and saline)].

Mass spectrometric evidence for peptide release from midgut endocrine cells

To further assess peptide release from the midgut, and to determine whether the released peptides are secreted into the solution surrounding the exterior of the tissue, we exposed single *C. borealis* AMC and PMC samples to physiological and high-K^+ saline in the presence of a protease inhibitor cocktail at 4°C. Each sample was exposed to physiological saline for 1 h, transferred to a fresh sample of physiological saline for 1 h and then transferred to and incubated for 1 h in high-K^+ saline.

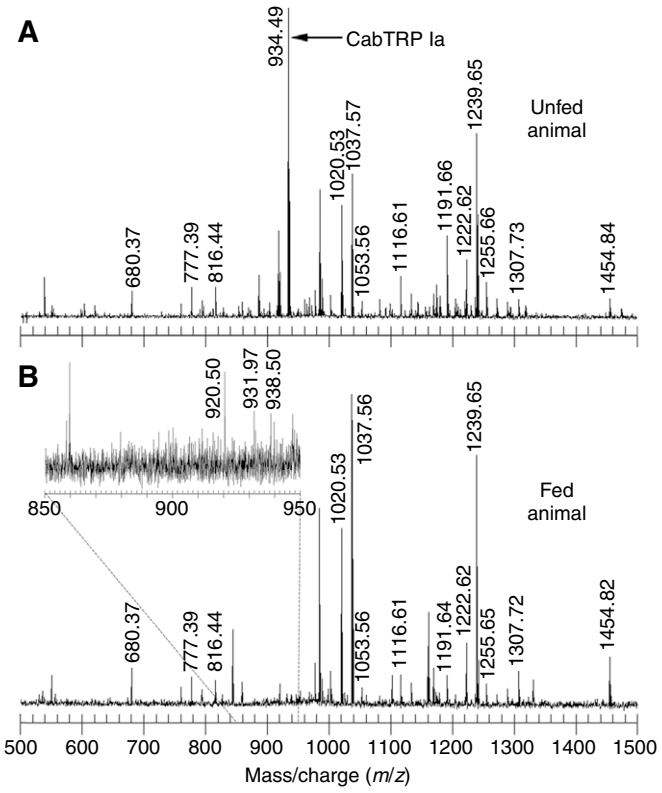

Fig. 8. Detection of APSGFLGMRamide (CabTRP Ia) in the hemolymph of unfed and fed *Cancer productus* using MALDI-FTMS. To assess whether or not circulating levels of Gly^1-SIFamide and/or CabTRP Ia are influenced by the feeding status of an individual animal, hemolymph samples were collected and analyzed from animals held without food for approximately seven days (A), as well as from those held without food for approximately one week but allowed to feed at will for 2 h prior to sampling (B). As can be seen in the representative spectra, a peak corresponding to the $[M+H]^+$ ion for CabTRP Ia, i.e. *m/z* 934.49, was detectable only in the hemolymph of unfed animals. Gly^1-SIFamide was not detected in spectra from either unfed or fed individuals. With the exception of the inset, both A and B are shown at the same (*m/z*) scale.

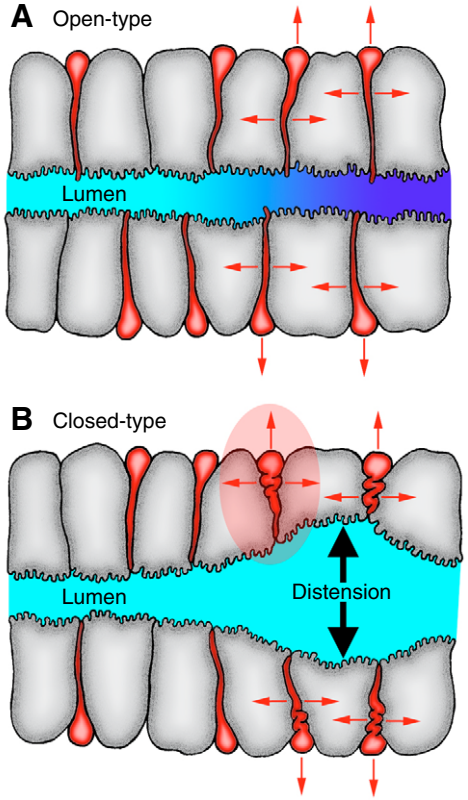

Fig. 9. Schematic representations of possible triggers for peptide release, including that of CabTRP Ia, from *Cancer* midgut epithelial endocrine cells. In these schematics, intrinsic endocrine cells are colored red and epithelial cells are colored grey. At present, the cues triggering secretion of paracrines/hormones from the intrinsic endocrine cells of the crab midgut epithelium are unknown. However, two classes of epithelial endocrine cells ('open-type' and 'closed-type') have been proposed, based on ultrastructural morphology (Endo and Nishiitsutsiji-Uwo, 1981; Fujita et al., 1988). (A) In open-type endocrine cells, the apical projections span the entirety of the epithelium, projecting into the gut lumen. It is proposed that these cells monitor the extracellular environment of the lumen, initiating (red arrows) or stopping secretion of hormones/paracrines when a threshold level of some chemical/ionic cue is achieved (shown here as a color gradient in the lumen). (B) In closed-type cells, the apical projections do not extend into the lumen. These cells are believed to be mechanosensory, monitoring changes in distension, which trigger (red arrows) or stop the release of hormonal/paracrine signaling agents. It should be noted that regardless of cell type, it is unclear how large the sphere of influence (pink oval in B) might be for a peptide released from gut epithelial endocrine cells. Likewise, it is not clear whether there is a directionality to release from these cells. Given that our study shows that circulating levels of CabTRP Ia are elevated in starved animals, and previous work has demonstrated a myotropic action for it on the musculature of the foregut (Messinger et al., 2005), we hypothesize that the TRP released from the midgut endocrine cells may play a crucial role in ensuring foregut muscle contraction in times of limited food availability. It should be noted that the endocrine cells in both panels of this schematic are highly stylized and should not be interpreted as representative of the morphology of epithelial endocrine cells in a general sense.

We then assayed the second physiological saline and the high-K^+ saline releasates, as well as unexposed saline standards, for evidence of Gly^1-SIFamide and CabTRP Ia using MALDI-FTMS. In three of four high-K^+ PMC releasates, we detected a peak at *m/z* 934.49, which corresponds to that of CabTRP Ia (Fig. 7A). This peak was not evident in any of the physiological saline releasate (Fig. 7B), nor was it present in either of the saline standards. In no releasate sample (physiological or high-K^+ saline) was a peak corresponding to Gly^1-SIFamide (i.e. *m/z* 1381.74) detectable.

The presence of CabTRP Ia in the circulatory system is correlated to feeding status

To determine whether the levels of Gly^1-SIFamide and/or CabTRP Ia in the hemolymph of *C. productus* are related to the feeding status of an animal, we sampled and assayed for their

presence in the hemolymph of both starved and recently fed individuals. *C. productus* was chosen for hemolymph analysis as neither of the classically ascribed *Cancer* neuroendocrine organs, i.e. the X-organ-sinus gland (XO-SG) system and the pericardial organ (PO), possess TRPs in this species (Fu et al., 2005), whereas in *C. borealis*, TRPs are present in the PO (Christie et al., 1995; Li et al., 2003) and in *C. magister* the distribution of TRPs is unknown. In hemolymph samples from two of the three crabs that were held without food, an abundant peak corresponding to that of CabTRP Ia (i.e. *m/z* 934.49) was detected using MALDI-FTMS (Fig. 8A). In the third unfed crab we were unable to make a positive identification of CabTRP Ia because of a low intensity of all signals in the collected spectra. In none of the three fed individuals (Fig. 8B) were we able to detect CabTRP Ia in the hemolymph, although intense signals from other peptides were consistently seen. Although the lack of a peak does not necessarily indicate the complete absence of the peptide, it does indicate that the concentration is below our detection threshold, showing that the relative hemolymph concentrations of CabTRP Ia differ in fed *versus* unfed animals. In no sample (unfed or fed) was Gly^1-SIFamide detected.

Discussion

The crustacean midgut: a major endocrine center

In his ultrastructural analysis of the *C. magister* midgut, Mykles (Mykles, 1979) demonstrated the presence of cells within the epithelium that were similar in organization to endocrine cells described earlier in the midgut epithelia of insects (Reinhardt, 1976; Hecker, 1977). Because the *C. magister* cells contained numerous dense-core vesicles and exhibited morphological correlates of dense-core vesicle release, they too were hypothesized to serve an endocrine role (Mykles, 1979). Although no investigation prior to our study has focused on these crustacean midgut cells, much work has been done on their insect counterparts, and the endocrine function of those cells is now well recognized (Winther and Nässel, 2001). Here, we have used a combination of immunohistochemical and mass spectrometric methods to investigate further the endocrine nature of the putative midgut epithelial endocrine cells of *C. magister*, as well as those of two related crabs, *C. borealis* and *C. productus*. In each of the species examined, we found populations of SIFamide- and TRP-like immunopositive cells that exhibited morphologies identical to those of the cells described as putatively endocrine by Mykles (Mykles, 1979). Accurate mass measurements of midgut tissues made using MALDI-FTMS identified the midgut SIFamide as GYRKPPFNGSIFamide and the TRP as APSGFLGMRamide in all of the investigated species. Release experiments showed that both peptides can be secreted from the endocrine cells of the midgut, with at least CabTRP Ia secreted *in vivo* into the media bathing the outer surface of the midgut, which *in vitro* would be the hemolymph. As both of Gly^1-SIFamide and CabTRP Ia are known to be biologically active in crustaceans (Christie et al., 1997; Swensen and Marder, 2000; Wood et al., 2000; Swensen and Marder, 2001; Thirumalai and Marder, 2002; Messinger et al., 2005; Christie et al., 2006), we feel that our work supports Mykles' hypothesis that the crustacean midgut possesses intrinsic endocrine cells (Mykles, 1979). Moreover, given the size of the midgut in *Cancer* species and the density of endocrine cells present there, it appears that this portion of the digestive tract may represent a major endocrine site in this decapod genus, as it has been shown to be in many insect species (Sehnal and Zitnan, 1996).

What physiological cues trigger hormone release from the midgut epithelium?

Broadly speaking, gut epithelial endocrine cells are typically classified as being either 'open-' or 'closed-type' based on their ultrastructural morphology (Endo and Nishiitsutsiji-Uwo, 1981; Fujita et al., 1988). 'Open-type' endocrine cells extend their apical processes to the gut lumen, which typically exhibits a microvillar border. Cells of this type are hypothesized to monitor the ionic environment within the lumen and to release their paracrines/hormones in response to changes in the chemical composition of this environment (Fig. 9A). By contrast, 'closed-type' epithelial endocrine cells do not have their apical extensions in direct contact with the gut lumen. Here, rather than responding to chemical cues, it is hypothesized that the cells monitor and release their paracrine/hormone complements in response to changes in gut tension (Fig. 9B). It is not yet clear as to which class the epithelial endocrine cells of the *Cancer* midgut belong. In his description of the ultrastructure of the *C. magister* midgut epithelium, Mykles did not state whether or not a direct contact with midgut lumen is made by these cells, and from the electron micrographs shown in his study, it is not possible for us to determine this (Mykles, 1979). Our immunohistochemical images indicate that a direct contact is likely, suggesting the cells are 'open-type', but this cannot be shown unequivocally with the methodology used here. Thus, further analysis will be required to determine whether the epithelial endocrine cells of the midgut of *Cancer* species are chemosensory, mechanosensory or perhaps both. Moreover, the direction of release of peptides from these midgut endocrine cells has not been determined. From our study it is clear that Gly^1-SIFamide or CabTRP Ia are distributed throughout the cells. This distribution of peptide could allow for paracrine release throughout the midgut epithelium as well as secretion into the hemolymph. Webster et al. found morphological evidence for hormone secretion in the basal region of fore- and hindgut endocrine cells in the crab *Carcinus maenas*, substantiating the proposed release into the hemolymph for these cells (Webster et al., 2000). The micrographs of Mykles also show vesicles docked to, and/or in the process of fusing with, the basal plasma membrane of the endocrine cells of the *C. magister* midgut (Mykles, 1979) (i.e. fig. 13), suggesting that here too, release into the hemolymph is likely. In our chemical depolarization studies, we showed *via* immunohistochemistry that both Gly^1-SIFamide and CabTRP Ia are releasable from the midgut epithelium, although only CabTRP Ia was detectable *via* MALDI-FTMS in the releasate bathing the outer surface of the

midgut. Although the latter finding may be a result of the threshold for mass spectrometric detection of the two peptides, it raises the possibility that some midgut-derived peptides may function solely as a paracrine or as a hormone, whereas others may serve dual roles.

Paracrine roles for midgut-derived peptides in the crab midgut

Work from many laboratories has shown the epithelium of the crustacean midgut to be multifunctional (for reviews, see Vonk, 1960; Dall and Moriarty, 1983; Icely and Nott, 1992). Lipid absorption and storage are well-documented roles played by this tissue. The midgut epithelium is also known to be the site of synthesis of the peritrophic membrane, a permeable barrier that separates the food bolus from the epithelial cells of the midgut, protecting them from mechanical damage and attack by toxic/pathenogenic agents. The transport of sugars, amino acids, ions and water from the gut to the hemolymph are also controlled by the cells that comprise the midgut epithelium. Here, we provide evidence in support of another function for the midgut epithelium in *Cancer* crabs, namely paracrine/endocrine signaling.

Prior ultrastructural analyses and physiological studies provide several possible paracrine targets for midgut-derived CabTRP Ia and Gly^1-SIFamide. In the locust, TRPs have been shown to stimulate contractions of the circular muscles in the midgut (Pabla and Lange, 1999) and, although untested, the same may be true in *Cancer* crabs. It is also possible that the nerve terminals abutting the basal surface of the midgut epithelial cells (Mykles, 1979) may be a paracrine target of the CabTRP Ia and/or Gly^1-SIFamide released from the intrinsic endocrine cells as both peptides are known to serve neuromodulatory roles in crustaceans (Christie et al., 1997; Swensen and Marder, 2000; Wood et al., 2000; Swensen and Marder, 2001; Thirumalai and Marder, 2002; Christie et al., 2006). Moreover, midgut epithelial cells themselves may be paracrine targets of their endocrine neighbors, thereby modulating the ability of the gut to absorb and store lipids, synthesize membrane and transport ions, water and other materials. Clearly, our study opens the door for future investigations on the paracrine actions of substances secreted from midgut endocrine cells.

In his description of the *C. magister* midgut epithelium, Mykles noted few structural differences between the cells present in different regions of the midgut (Mykles, 1979). In fact, the only major difference noted was that the cells in the midgut caeca tended to possess longer apical processes than those present in the midgut proper. In contrast to their apparent conserved morphology, we have found that at least a subset of the endocrine cells present in the anterior midgut are neurochemically distinct from their more posteriorly located counterparts (i.e. SIFamide predominating *versus* exclusively TRP-positive). Although the significance of this neurochemical compartmentalization remains to be determined, it may manifest functionally in the site-specific paracrine control of the midgut, as has been postulated for a similar chemical segregation seen in the midgut of the mosquito *Aedes aegypti* (Veenstra et al., 1995). In that report, TRP-like immunoreactivity was found in endocrine cells of the anterior midgut and the most frontal portion of the posterior midgut. It was hypothesized that this distribution of midgut TRP cells could result in a localized cinching of the muscles of both the anterior- and posterior-most portions of the posterior midgut, thereby holding a blood meal in the posterior midgut long enough to ensure complete digestion. Moreover, RFamide-like immunoreactivity, possibly reflecting the presence of peptides related to vertebrate cholecystokinin (CCK)/gastrins, i.e. a sulfakinin, is restricted to the posterior midgut in *A. aegypti*. As this same region is known to be the site of trypsin synthesis and release, it was postulated that the RFamides were involved in local paracrine regulation of the biosynthesis and/or release of this protease, as has been shown to be the case for CCK/gastrins in the vertebrate gut. Thus, as future studies are directed at the paracrine regulation of the crab midgut by endocrine cell-derived factors, it will be interesting to determine whether the neurochemical regionalization we report does in fact manifest itself functionally.

Hormonal roles for midgut-derived peptides

The ultrastructure of *Cancer* midgut endocrine cells suggests that they release peptides directly into the hemocoel (Mykles, 1979). Our release studies support this hypothesis, at least for CabTRP Ia. If so, the peptides released from midgut endocrine cells should be able to act not only as paracrines, but also as hormones. Previous studies on the physiological effects of CabTRP Ia in *Cancer* species suggest that one hormonal role that is almost certainly played by this peptide is neuro/myomodulation of the stomatogastric neuromuscular system of the foregut (Christie et al., 1997; Swensen and Marder, 2000; Wood et al., 2000; Swensen and Marder, 2001; Thirumalai and Marder, 2002; Messinger et al., 2005). In *C. borealis*, CabTRP Ia has been shown to activate or enhance the activity of several neuronal elements participating in the gastric mill circuit, which drives chewing by a set of internally located teeth, and the pyloric circuit, which controls the movement of the pyloric filter (Christie et al., 1997; Swensen and Marder, 2000; Wood et al., 2000; Swensen and Marder, 2001). The thresholds for these actions are approximately 10^{-9}–10^{-8} mol l^{-1}, which is within the range typically viewed as hormonal in this species. Moreover, in *C. productus*, a hormonally relevant concentration of CabTRP Ia has also been shown to enhance the excitatory junctional potentials in several gastric mill and pyloric muscles, as well as to increase the size of contraction in at least a subset of them (Messinger et al., 2005). In at least *C. productus*, no CabTRP Ia is present in either of the two crustacean neuroendocrine organs typically viewed as the major sources of circulating peptide hormones, namely the XO-SG and the PO (Fu et al., 2005). Thus, the TRP-containing midgut endocrine cells described here are prime candidates for the source of the CabTRP Ia that hormonally modulates the stomatogastric system.

In addition to its likely actions on the foregut, hormonally

delivered CabTRP Ia may well influence a variety of other targets in *Cancer* species, as has been shown for midgut-derived TRPs in insects. For example, TRPs have been shown to be myostimulatory on the hindgut (reviewed by Nässel, 1999). In all species thus far examined, all isoforms of this peptide family have been shown to induce contractions of the midgut, including increases in both the rate and amplitude of muscle contractions. In fact, the effects of TRPs on the cockroach midgut are so pronounced that it has commonly been used as a bioassay for tracking TRPs during the process of their purification from both insect and non-insect species [e.g. CabTRP Ia from *C. borealis* (Christie et al., 1997)]. In at least a subset of insects, no local TRP innervation of the hindgut has been found and no hormonal source other than midgut endocrine cells has been identified (Winther and Nässel, 2001). Similarly, in the beetles *Tenebrio molitor* and *Zophobas atratus*, TRPs have been shown to be cardiostimulatory (Sliwowska et al., 2001). In both species, exogenous application of several TRP isoforms (at hormonally relevant concentrations) increased heart-beat frequency. Moreover, in *Z. atratus* these peptides also increased the amplitude of heart contractions. As no TRP innervation of the heart was found in either beetle species, the cardiotropic actions of TRP were attributed to circulating peptides, probably originating from endocrine cells in the midgut (Sliwowska et al., 2001). Clearly both the hindgut and heart of *Cancer* species too are also potential targets of circulating CabTRP Ia and as investigations are conducted on them, it will be interesting to see how extensive the influence of this midgut-derived peptide may be in crabs.

In contrast to the wealth of knowledge on the physiological actions of TRPs in arthropods, only a single study exists on the physiological effects of the SIFamides in this phylum. Here, the action of Val^1-SIFamide on the stomatogastric system of the American lobster *H. americanus* was investigated, and like CabTRP Ia, this peptide too was found to be a potent neuromodulator (Christie et al., 2006). In *Cancer* species, neither the XO-SG nor the PO contains Gly^1- or any other SIFamide isoform (Fu et al., 2005). Thus, if the stomatogastric neural circuits and/or the foregut musculature are modulated by low concentrations of this peptide, the immunopositive midgut endocrine cells described here are a possible source of the hormone.

A putative function for feeding-regulated release of CabTRP Ia in C. productus

The gastric mill and pyloric rhythms produced by the stomatogastric nervous system (STNS) of decapod species, including members of the genus *Cancer*, are highly variable in their expression. Work from many laboratories has shown that much of this variation in motor pattern expression is because of the modulatory actions of peptides released both locally within the ganglia that comprise the STNS and delivered to it *via* the hemolymph. Although the stomatogastric neural circuits are modulated by peptides delivered both locally and hormonally, most of the foregut musculature is likely to be influenced only by hormonally delivered substances, as there appears to be little direct innervation of it by peptidergic axons.

The work of Jorge-Rivera and Marder suggests that the actions of circulating peptides on the foregut musculature play a crucial role in ensuring foregut movement when ongoing motor patterns are weak, such as when there is little or no food present in the system and hence the activation of the stretch/chemosensory receptors is minimal or non-existent (Jorge-Rivera and Marder, 1996). It is under these conditions that peptidergic modulation of muscle contractions is at its strongest; without such modulation each burst of motor neuron activity produces a relatively small contraction, one that is unlikely to produce much, if any, muscle movement. Given that we have shown that the circulating levels of CabTRP Ia are elevated in starved animals, and that this peptide is myotropic on the musculature of the foregut (Messinger et al., 2005), we postulate that TRP release from the midgut endocrine cells may play a crucial role in ensuring foregut muscle contraction in times of limited food intake.

Brain-gut peptides in crustacea: rule or exception?

In addition to providing evidence in support of an endocrine role for the midgut epithelium, we have also shown that two known crustacean neuropeptides, Gly^1-SIFamide and CabTRP Ia, are among the complement of signaling molecules present in the epithelial endocrine cells of that tissue. Before this study, only two crustacean brain-gut peptides had been identified: crustacean hyperglycemic hormone and crustacean hyperglycemic hormone precursor-related peptide, both of which are found in the nervous system and in the foregut and midgut of the crab *C. maenas* (Kegel et al., 1989; Weidemann et al., 1989; Tensen et al., 1991; Chung et al., 1999; Webster et al., 2000; Dircksen et al., 2001). Our identification of Gly^1-SIFamide and CabTRP Ia in the *Cancer* midgut now brings to four the number of fully characterized brain-gut peptides in decapod species, and suggests the possibility that there may be a myriad of such peptides in the midgut epithelium of decapod crustaceans, as there are in insects.

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