The Journal of Experimental Biology 209, 3241-3256
Published by The Company of Biologists 2006
doi:10.1242/jeb.02372

Members of the crustacean hyperglycemic hormone (CHH) peptide family are differentially distributed both between and within the neuroendocrine organs of *Cancer* crabs: implications for differential release and pleiotropic function

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Accepted 6 June 2006

Summary

The crustacean hyperglycemic hormone (CHH) family of peptides includes CHH, moult-inhibiting hormone (MIH) and mandibular organ-inhibiting hormone (MOIH). In the crab *Cancer pagurus*, isoforms of these peptides, as well as CHH precursor-related peptide (CPRP), have been identified in the X-organ–sinus gland (XO–SG) system. Using peptides isolated from the *C. pagurus* SG, antibodies to each family member and CPRP were generated. These sera were then used to map the distributions and co-localization patterns of these peptides in the neuroendocrine organs of seven *Cancer* species: *Cancer antennarius*, *Cancer anthonyi*, *Cancer borealis*, *Cancer gracilis*, *Cancer irroratus*, *Cancer magister* and *Cancer productus*. In addition to the XO–SG, the pericardial organ (PO) and two other neuroendocrine sites contained within the stomatogastric nervous system, the anterior cardiac plexus (ACP) and the anterior commissural organ (ACO), were studied. In all species, the peptides were found to be differentially distributed between the neuroendocrine sites in conserved patterns: i.e. CHH, CPRP, MIH and MOIH in the XO–SG, CHH, CPRP and MOIH in the PO, and MOIH in the ACP (no immunolabeling was found in the ACO). Moreover, in *C. productus* (and probably in all species), the peptides present in the XO–SG and PO were differentially distributed between the neurons within each of these neuroendocrine organs (e.g. CHH and CPRP in one set of XO somata with MIH and MOIH co-localized in a different set of cell bodies). Taken collectively, the differential distributions of CHH family members and CPRP both between and within the neuroendocrine organs of crabs of the genus *Cancer* suggests that each of these peptides may be released into the circulatory system in response to varied, tissue-specific cues and that the PO- and/or ACP-derived isoforms may possess functions distinct from those classically ascribed to their release from the SG.

Key words: *Cancer* crabs, sinus gland, (SG), pericardial organ (PO), anterior cardiac plexus (ACP), anterior commissural organ (ACO), stomatogastric nervous system (STNS), stomatogastric ganglion (STG), neurohormone, crustacean hyperglycemic hormone (CHH), crustacean hyperglycemic hormone precursor-related peptide (CPRP), mandibular organ-inhibiting hormone (MOIH), moult-inhibiting hormone (MIH), immunohistochemistry.

Introduction

Research on crustaceans has played a major role in advancing our understanding of neuroendocrine structure and control. Nearly 80 years ago, Koller (Koller, 1925; Koller, 1927) demonstrated that hemolymph-borne agents were responsible for color change in shrimp. In the decade that followed, Hanström (Hanström, 1931; Hanström, 1933; Hanström, 1935; Hanström, 1937) identified a structure located in the eyestalk, the sinus gland (SG) as the source of these substances. Later, Bliss (Bliss, 1951) and Passano (Passano, 1951) independently demonstrated the neurohemal nature of the SG, a first for any neural structure in the animal kingdom. In recent years, work from many laboratories has shown that the SG is one of the major neuroendocrine organs in crustaceans and a number of hormones contained within it have been identified, including a group of structurally related peptides that have collectively been termed the crustacean hyperglycemic hormone (CHH) family.

In crustaceans, the CHH family includes CHHs, moult-inhibiting hormones (MIHs), gonad-inhibiting hormones (GIHs), vitellogenesis-inhibiting hormones (VIHs) and mandibular organ-inhibiting hormones (MOIHs), all of which are large peptides (72–78 amino acids) that share considerable amino acid sequence and structural homology, including six conserved cysteine residues and three disulfide bridges (Keller, 1992; de Kleijn and van Herp, 1995; Soyez, 1997; van Herp, 1998; Webster, 1998; Chan et al., 2003; Chen et al., 2005; Fanjul-Moles, 2006). As their diverse names imply, members of the CHH family have been shown to influence a broad array of physiological processes including, but not limited to, the regulation of metabolism, somatic growth and reproductive maturation (Sedlmeier and Keller, 1981; Sedlmeier, 1982; Sedlmeier, 1988; Keller, 1992; Charmantier-Daures et al., 1994; Wainwright et al., 1996; Santos et al., 1997; Khayat et al., 1998; van Herp, 1998; Webster, 1998; Chung et al., 1999; Spanings-Pierrot et al., 2000; Sonobe et al., 2001; Chan et al., 2003; Serrano et al., 2003).

Based on preprohormone organization, the CHH family can be divided into two sub-groups: (1) CHH and (2) MIH/GIH/VIH/MOIH (de Kleijn and van Herp, 1995; Lacombe et al., 1999; Chan et al., 2003; Chen et al., 2005; Fanjul-Moles, 2006). The preprohormones of the CHH sub-group always contain a second peptide, commonly referred to as CHH precursor-related peptide or CPRP, which is C-terminally flanked by the CHH (Weidemann et al., 1989; Tensen et al., 1991; de Kleijn et al., 1994a; de Kleijn et al., 1995; Ohira et al., 1997a; Davey et al., 2000; Toullec et al., 2002; Marco et al., 2003; Chen et al., 2004; Mettulio et al., 2004; de la Iglesia et al., 2005; Hsu et al., 2005a; Toullec et al., 2006). The preprohormones of the MIH/GIH/VIH/MOIH sub-group lack precursor-related peptides (Klein et al., 1993; de Kleijn et al., 1994b; Sun, 1994; Lee et al., 1995; Ohira et al., 1997b; Chan et al., 1998; Gu and Chan, 1998; Umphrey et al., 1998; Tang et al., 1999; Lu et al., 2001; Yang and Rao, 2001; Edomi et al., 2002; Krungkasem et al., 2002; Ohira et al., 2005). Although CPRP has been shown to circulate in the hemolymph, the physiological role(s) it plays in crustaceans remains unknown (Wilcockson et al., 2002).

In brachyuran crabs, particularly those of the genus *Cancer*, much work has been done in an effort to identify and characterize the actions of the native CHH-family members and CPRPs in the X-organ (XO)–SG system. In this genus, four distinct CHH isoforms have been identified [two from *Cancer pagurus* and two from *Cancer productus* (Chung et al., 1998; de la Iglesia et al., 2005; Hsu et al., 2005a); Table 1] as have two isoforms of MIH [one from *Cancer magister* and the other from *C. pagurus* (Chung et al., 1996; Umphrey et al., 1998); Table 1] and two isoforms of MOIH [both from *C. pagurus* (Wainwright et al., 1996); Table 1]. Similarly, nine isoforms of CPRP have been characterized from the SG of *Cancer* species [one from *C. pagurus* and four each from *C. productus* and *Cancer borealis* (Chung et al., 1998; de la Iglesia et al., 2005; Fu et al., 2005a; Fu et al., 2005b; Hsu et al., 2005a); Table 1]. As Table 1 shows, the isoforms of any given group are nearly identical in amino acid sequence, even when comparing isoforms from different species.

Although most studies concerning CHH-family members and CPRPs have focused on the SG, there is evidence that some of these peptides are also present in other regions of the crustacean nervous system (de Kleijn et al., 1995; Sun, 1995; Chang et al., 1999; Dircksen et al., 2001; Lu et al., 2001; Gu et al., 2002; Wilcockson et al., 2002; Basu and Kravitz, 2003; Chen et al., 2004; Fu et al., 2005b; Ohira et al., 2005; Toullec et al., 2006). CHH- and CPRP-like immunoreactivities have been found in the neuroendocrine pericardial organ (PO) of *C. pagurus* (Wilcockson et al., 2002) and putative CPRP fragments have been sequenced from this structure in *C. productus* (Fu et al., 2005b). The functional relevance of this dual XO–SG/PO distribution of CHH in these crabs is currently unknown. However, it has been postulated that the signals governing its release from each site are distinct. Moreover, the isoforms present in one *versus* the other structure may also be distinct (Dircksen et al., 2001; Toullec et al., 2006) and subserving different physiological roles, as has been shown for the CHHs isolated from the SG and PO of the crab *Carcinus maenas* (Dircksen et al., 2001).

Recently, we began a study to identify and compare the hormone complements present in the neuroendocrine organs of several *Cancer* species (Christie et al., 2004; Christie and Messinger, 2005; de la Iglesia et al., 2005; Fu et al., 2005a; Fu et al., 2005c; Hsu et al., 2005a; Hsu et al., 2005b; Messinger et al., 2005a; Messinger et al., 2005b; Cruz-Bermúdez et al., 2006). As a first step toward determining the identity, tissue distributions and physiological roles played by each CHH-family member and CPRP in the neuroendocrine systems of these species, we undertook the immunohistochemical mapping studies presented in this report. Specifically, antisera previously generated against native *C. pagurus* peptides (Webster, 1996; Wilcockson et al., 2002) were used to map the distributions and co-localization patterns of the CHH family and CPRP in the neuroendocrine organs of *Cancer antennarius*, *Cancer anthonyi*, *C. borealis*, *Cancer gracilis*, *Cancer irroratus*, *C. magister* and *C. productus*. As the data that follow show, most members of the CHH family and CPRP were immunologically detected in multiple neuroendocrine organs, with the complement of peptides present in each site conserved across species. In at least *C. productus*, the peptides detected in the XO–SG and PO were also differentially distributed among the neurons comprising each site. Collectively, the data presented here represent the most extensive and complete immunohistochemical survey of CHH family members and CPRP in crustacean neuroendocrine organs to date. Moreover, our findings support the hypotheses that: (1) members of CHH family and CPRP are released in response to varied, tissue-specific cues and (2) the isoforms released from non-XO–SG sites probably possess functions distinct from those ascribed to them when secreted from the SG. Some of these data have appeared previously in abstract form (Hsu et al., 2004).

Table 1. *Isoforms of fully characterized crustacean hyperglycemic hormone-family members and crustacean hyperglycemic hormone precursor-related peptide from Cancer species*

Peptide	Amino acid sequence	
CHH		
Capa-CHH I	QIYDTSCKGVYDRGLFSDLEHVCDDCYNLYRNSYVASACRSNCYSNVVFRQCMEELLLMD	60
Capa-CHH II*	pEIYDTSCKGVYDRGLFSDLEHVCDDCYNLYRNSYVASACRSNCYSNVVFRQCMEELLLMD	60
Capr-CHH I	QIYDTSCKGVYDRGLFSDLEHVCDDCYNLYRNSYVASACRSNCYSNVVFRQCMEELLLME	60
Capr-CHH II	QIYDSSCKGVYDRGLFSDLEHVCDDCYNLYRNSYVASACRSNCYSNVVFRQCMEELLLME	60
	EFDKYARAVQIV	72
	EFDKYARAVQIV	72
	EFDKYARAVQIV	72
	EFDKYARAVQIV	72
MIH		
Capa-MIH*	RVINDDCPNLIGNRDLYKKVEWICEDCSNIFRNTGMATLCRKNCFFNEDFLWCVYATERT	60
Cama-MIH	RVINDDCPNLIGNRDLYKRVEWICEDCSNIFRNTGMATLCRKNCFFNEDFLWCVYATERT	60
	EEMSQLRQWVGILGAGRE	78
	EEMSQLRQWVGILGAGRE	78
MOIH		
Capa-MOIH I*	RRINNDCQNFIGNRAMYEKVDWICKDCANIFRKDGLLNNCRSNCFYNTEFLWCIDATENT	60
Capa-MOIH II	RRINNDCQNFIGNRAMYEKVDWICKDCANIFRQDGLLNNCRSNCFYNTEFLWCIDATENT	60
	RNKEQLEQWAAILGAGWN	78
	RNKEQLEQWAAILGAGWN	78
CPRP		
Capa-CPRP*	RSAQGMGKMERLLASYRGALEPSTPLGDLSGSLGHPVE	38
Capr-CPRP I	RSAQGMGKMEHLLASYRGALESNTPTGDLPGGLVHPVE	38
Capr-CPRP II	RSAQGMGKMERLLASYRGAVEPNTPLGDLPGGLVHPVE	38
Capr-CPRP III	RSAQGMGKMEHLLASYRGALESNTPLGDLPGGLVHPVE	38
Capr-CPRP IV	RSAQGMGKMERLLASYRAAVEPNTPLGDLPGGLVHPVE	38
Cabo-CPRP I	RSAQGLGKMERLLASYRGALEPNTPLGDLSGSVGHPVE	38
Cabo-CPRP II	RSAQGLGKMERLLASYRGALEPNTPLGDLSGSLGHPVE	38
Cabo-CPRP III	RSAQGLGKMEHLLASYRGALEPNTPLGDLSGSLGHPVE	38
Cabo-CPRP IV	RSAQGLGKMERLLVSYRGAVEPNTPLGDLSGSLGHPVE	38

CHH, crustacean hyperglycemic hormone; CPRP, CHH precursor-related peptide; MIH, moult-inhibiting hormone; MOIH, mandibular-inhibiting hormone.

Cancer pagurus CHH I and II [Capa-CHH I and II (Chung et al., 1998)], *Cancer productus* CHH I and II [Capr-CHH I and II (Hsu et al., 2005)], *C. pagurus* MIH [Capa-MIH (Chung et al., 1996)], *Cancer magister* MIH [Cama-MIH (Umphrey et al., 1998)], *C. pagurus* MOIH I and II [Capa-MOIH I and II (Wainwright et al., 1996)], *C. pagurus* CPRP [Capa-CPRP (Chung et al., 1998)], *C. productus* I to IV [Capr-CPRP I to IV (Fu et al., 2005a)] and *Cancer borealis* [Cabo-CPRP I to IV (Fu et al., 2005b)].

In each group of aligned sequences, amino acid residues identical to that used to generate the antibodies, indicated with an asterisk, are shaded.

Materials and methods

Animals

Brown rock crabs *Cancer antennarius* Stimpson and yellow rock crabs *Cancer anthonyi* Rathbun were purchased from Coastal Catch (Santa Barbara, California, USA). Jonah crabs *Cancer borealis* Stimpson were purchased from the Marine Biological Laboratory (Woods Hole, Massachusetts, USA). Graceful crabs *Cancer gracilis* Dana were collected by hand at False Bay, San Juan Island, Washington, USA. Atlantic rock crabs *Cancer irroratus* Say were purchased from J&A Seafood (Brunswick, Maine, USA). Dungeness crabs *Cancer magister* Dana and red rock crabs *Cancer productus* Randall were collected by hand, trap or trawl at multiple locations throughout the San Juan Archipelago and greater Puget Sound areas of Washington State, USA. Regardless of species, animals were maintained in either aerated natural seawater aquaria chilled to 10°C (Department of Biology, University of Washington, Seattle, Washington,

USA) or in flow-through natural seawater tables [Friday Harbor Laboratories, Friday Harbor, Washington, USA (locally caught animals only); ambient water temperature was approximately 8–12°C, depending on season].

Tissue collection

For the collection of tissue, crabs were anesthetized by packing in ice for 30–60 min. After anesthesia, the eyestalks were isolated, then the dorsal carapace was removed and the foregut and the lateral walls of the pericardial chamber were dissected free 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 7.4 (adjusted with NaOH)]. To obtain the XO–SG system, the carapace encasing an eyestalk was split both dorsally and ventrally and one half of the split shell was gently teased away from the other half. The remaining half of the eyestalk was then pinned in a wax-lined Pyrex dish filled with chilled physiological saline and the optic ganglia containing the XO–SG system were subsequently isolated. POs were obtained by pinning the isolated walls of the pericardial chamber in a wax-lined Pyrex dish containing chilled physiological saline. The nerve roots forming each PO were then dissected free from the muscles and connective tissues of the pericardial wall. For isolating the stomatogastric nervous system (STNS), which contains two recently identified neuroendocrine organs (Christie et al., 2004; Messinger et al., 2005a), the anterior cardiac plexus (ACP) and the anterior commissural organ (ACO), the foregut was flattened by making a longitudinal cut from the esophagus to the pylorus on its ventral side followed by a pair of medial cuts directed along the ossicles of the cardiac sac/gastric mill. After its opening, the teeth of the gastric mill were removed and the flattened foregut was pinned, inside down, in a wax-lined Pyrex dish containing chilled physiological saline. The STNS was then dissected free from the muscles and connective tissues of the foregut.

Antibodies and antibody production

Primary antibodies

All of the primary antibodies used in our study were rabbit polyclonal antibodies generated against native *C. pagurus* peptides (Table 1). These peptides were purified from a common pool of approximately 2000 SGs as described in several previous publications (Chung et al., 1996; Wainwright et al., 1996; Webster, 1996; Chung et al., 1998). For production of the CHH antibody, native *C. pagurus* (Capa)-CHH II was used. The development of this antibody is described elsewhere (Webster, 1996). For the production of the CPRP antibody, the antigen was *C. pagurus* (Capa)-CPRP conjugated to bovine thyroglobulin using either glutaraldehyde or 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. The production of this antibody is also described elsewhere (Wilcockson et al., 2002). For the production of the MIH and MOIH antibodies, methods similar to those employed for the production of the CHH antibody were used. Specifically, New Zealand white rabbits (one for each peptide) were injected subcutaneously at multiple sites with 5 nmol of native *C. pagurus* (Capa)-MIH or native *C. pagurus* (Capa)-MOIH I, each dissolved in 0.3 ml of phosphate-buffered saline and emulsified with an equal volume of complete Freund's adjuvant. Four weeks after the initial immunization, a booster of 5 nmol of peptide (Capa-MIH or Capa-MOIH I) in Freund's incomplete adjuvant was injected into each rabbit. Similarly, 8 weeks after the initial immunization, rabbits were boosted again, this time with 3 nmol of peptide (Capa-MIH or Capa-MOIH I) in Freund's incomplete adjuvant. Twelve weeks after the initial immunization, rabbits were terminally exsanguinated under anesthesia and serum was collected from the retracted clots. Production of all antisera used in this study was done at the University of Wales Bangor (Bangor, UK).

Secondary and tertiary antibodies

The secondary and tertiary antibodies used in our study were kind gifts from either Jackson ImmunoResearch Laboratories, Inc. (West Grove, Pennsylvania, USA) or Molecular Probes (Eugene, Oregon, USA). These included donkey anti-rabbit IgG labeled with Alexa Fluor 488 (Molecular Probes; catalog #A-21206), Alexa Fluor 594 (Molecular Probes; catalog #A-21207), FITC (Jackson ImmunoResearch; catalog # 711-095-152) or Rhodamine Red X (Jackson ImmunoResearch; catalog # 711-295-152); goat anti-rabbit IgG labeled with Alexa Fluor 488 (Molecular Probes; catalog # A11008) or Texas Red (Jackson ImmunoResearch; catalog #111-075-144); unlabeled monovalent goat anti-rabbit IgG antigen-binding (Fab) fragments (Jackson ImmunoResearch; catalog # 111-007-003) or donkey anti-goat IgG labeled with FITC (Jackson ImmunoResearch, catalog # 705-095-147).

Whole-mount immunohistochemistry

Single labeling

For single labeling, tissue was fixed overnight in a freshly made solution of 4% paraformaldehyde (EM grade; Electron Microscopy Sciences, Hatfield, Pennsylvania, USA; catalog #15710) in 0.1 mol l^{-1} sodium phosphate buffer (pH 7.4) followed by five rinses (at 1 h intervals) in a solution of sodium phosphate buffer containing 0.3% Triton X-100 (P-Triton). After rinsing, tissue was incubated with gentle agitation in a 1:5000 dilution of primary antibody for approximately 72 h. Primary antiserum was diluted in P-Triton, with 10% normal donkey serum (NDS; Jackson ImmunoResearch; catalog #017-000-121) or normal goat serum (NGS; Jackson ImmunoResearch; catalog #005-000-121) added to diminish nonspecific binding. Following incubation in primary antibody, tissue was again rinsed five times at 1 h intervals in P-Triton and then incubated overnight in a 1:300 dilution of secondary antibody. As with the primary antibody, secondary antibody incubation was done in P-Triton with 10% NDS or NGS, using gentle agitation. After secondary antibody incubation, each preparation was rinsed five times over approximately 5 h in sodium phosphate buffer and then mounted between a glass microscope slide and coverslip using Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, California,

USA; catalog # H-1000). Fixation and incubation in both primary and secondary antibody was done at 4°C. All rinses were done at room temperature (approximately 20°C) without agitation. Secondary antibody incubation, and all subsequent processing, was conducted in the dark. Likewise, slides were stored in the dark at 4°C until examined.

To increase our confidence that the immunoreactivities reported here were specific for peptides related to the antigen used for the production of each antiserum, we conducted a series of adsorption controls. In these experiments, native Capa-CHH II, Capa-CPRP, Capa-MIH and Capa-MOIH I isolated from the SG of *C. pagurus* (Table 1) were used as blocking agents. It should be noted that due to a limited supply of these peptides, adsorption controls were only conducted on *C. productus* tissues and that the adsorptions used for labeling one preparation were sometimes recycled for use in blocking studies on other samples. In each adsorption experiment, the antibody was incubated with a blocking peptide (approximately 10^{-5} mol l^{-1}) for 2 h at room temperature prior to applying the solution to the tissue. For each tissue in which an antibody produced labeling, a complete block of staining was achieved only when the antiserum was adsorbed with the peptide used for its production (three preparations for each peptide and antibody in each tissue; data not shown). Adsorption of an antibody with the other peptides had no appreciable effect on immunolabeling (three preparations for each peptide and antibody in each tissue; data not shown).

Double labeling

For preparations that were double-labeled (*C. productus* tissues only), the staining protocol for single labeling was modified in order to permit the use of primary antibody pairs that were both developed in the same host species (rabbit in this case). In brief, POs and SGs were dissected, fixed and incubated in the first primary antibody as described above for 1 or 2 days, respectively. Following incubation in the first primary antibody, tissues were rinsed with P-Triton as described for single labeling, then the SGs were incubated overnight in a 1:25 dilution of monovalent goat anti-rabbit IgG Fab fragments that also contained 10% NDS whereas the POs were incubated for 2 days in a 1:10 dilution of goat anti-rabbit IgG Fab fragments containing 10% NDS. For both tissues, this incubation with Fab fragments was done to sterically cover and immunologically convert the first primary antibody from rabbit to goat. After incubation with the Fab fragments, tissues were rinsed every hour for 5 h and then incubated overnight in a 1:300 dilution of FITC-conjugated donkey anti-goat IgG containing 10% NDS. Following this overnight incubation, tissues were rinsed as before then incubated in the second primary antibody for 1 (PO) or 2 (SG) days. After incubation in the second primary antibody, tissues were rinsed and then incubated overnight in a 1:300 dilution of Rhodamine Red X-conjugated donkey anti-rabbit IgG containing 10% NDS. All subsequent processing was the same as for the single labeling described above.

To assess the veracity of our double-labeling protocol, we conducted controls to determine the extent to which the Fab fragments were capable of sterically covering, and hence masking, the first primary antibody from detection *via* the second secondary antibody. Specifically, a single primary was applied to a tissue, sterically converted to goat *via* incubation in Fab fragments and then incubated in the Rhodamine Red X-conjugated donkey anti-rabbit IgG. For all antibodies and all tissues in which double labeling was undertaken (3 preparations for each antibody in each tissue), a complete masking of the primary antibody was achieved, and hence little or no cross-talk between the first and second primary antibody sets should have occurred in our double-labeled preparations (data not shown).

Confocal and epifluorescence microscopy

After immunoprocessing, preparations were viewed and data were collected using one of two Bio-Rad MRC 600 laser scanning confocal microscopes (Bio-Rad Microscience Ltd., Hemel Hempstead, UK), a Bio-Rad Radiance 2000 laser scanning confocal microscope or a Nikon 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., 1997; Christie et al., 2003; Christie et al., 2004; Messinger et al., 2005a).

Figure production

For the production of figures, Bio-Rad.*pic* files collected using the MRC 600 or Radiance 2000 systems were converted to *tif* files using ImageJ (available free of charge from the National Institutes of Health at http://rsb.info.nih.gov/ij/). Individual micrographs and composite figures were produced from the *tif* files using a combination of ImageJ and Photoshop (version 7.0; Adobe Systems Inc., San Jose, California, USA). Schematic diagrams were produced using Canvas (version 8.0; Deneba Systems Inc., Miami, Florida, USA). It should be noted that the contrast and brightness of final figures were adjusted as needed to optimize the clarity of printed images.

Results

The X-organ-sinus gland system

As in other brachyurans, the XO–SG system of *Cancer* species is located within the eyestalk (Figs 1, 2), with the XO (a loosely associated cluster of somata) situated in the proximoventral corner of the medulla terminalis and the SG (the neurosecretory terminals derived from the XO somata) present at the junction of the medulla interna and medulla externa (Cooke and Sullivan, 1982; Beltz, 1988; Fingerman, 1992). As stated earlier, isoforms of all members of the CHH family (i.e. CHH, MIH and MOIH) have been isolated from the XO–SG system of *C. pagurus*, as has an isoform of CPRP (Chung et al., 1996; Wainwright et al., 1996; Chung et al., 1998). Using antibodies generated against *C. pagurus* peptides (Webster et al., 1996; Wilcockson et al., 2002; this study), we immunolabeled eyestalks from *C. antennarius*, *C. anthonyi*, *C. borealis*, *C. gracilis*, *C. irroratus*, *C. magister* and *C. productus*

to determine if the XO–SG system of these species also contains members of these peptide groups.

Immunohistochemical detection of CHH, MIH, MOIH and CPRP in the XO–SG system

For each antibody, intense labeling was evident in nerve terminals in the SG (Figs 3, 4) as well as in approximately three-dozen somata in the XO (Fig. 3; MIH labeling shown) in each species ($N \geq 4$ preparations for each antibody in each species). Within the SG, the nerve terminals labeled by the CHH and CPRP antisera (Fig. 4A,B) had a tendril-like appearance, whereas the terminals labeled by the MIH and MOIH antisera (Fig. 4C,D) appeared more oval and blob-like. Regardless of antibody or species, all labeling in the SG could be traced back to the immunopositive XO somata *via* labeled axons in the sinus gland tract (sgt), suggesting that these somata are the sole source of the SG staining.

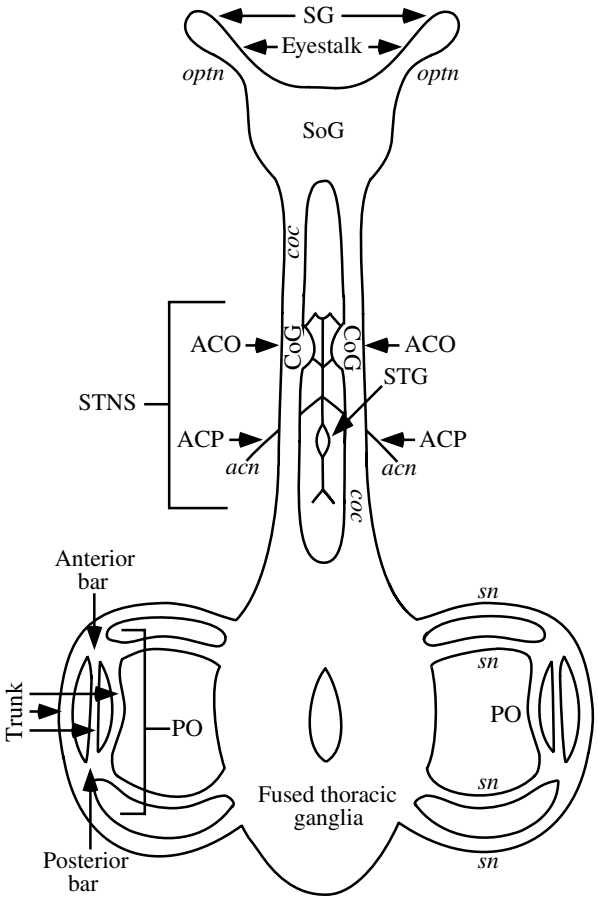


Fig. 1. Schematic representation of the nervous system of a brachyuran crab illustrating the relative locations of the known neuroendocrine organs of *Cancer* species. The central nervous system (CNS) of brachyurans is generally considered to consist of the supraesophageal (SoG) and fused thoracic ganglia, which are connected *via* the circumesophageal connectives (*coc*s). The optic nerves (*optn*s) link the SoG with the ganglia of the eyestalks, the location of the neuroendocrine sinus gland (SG). Another well-known neuroendocrine site is the pericardial organ (PO), which is located in the pericardial chamber surrounding the heart. The POs consist of elaborations of the segmental nerves (*sn*s), which project from the fused thoracic ganglia. Two additional neuroendocrine sites, the anterior cardiac plexus (ACP) and the anterior commissural organ (ACO), are contained within the stomatogastric nervous system (STNS), an offshoot from the CNS that overlies the foregut. The ACPs are located on the anterior cardiac nerves (*acn*s) and the ACOs are located within the commissural ganglia (CoGs). For the sake of future discussion, the stomatogastric ganglion (STG) is also shown in this schematic. It should be noted that this illustration is not drawn to scale and that other portions of the nervous system have been excluded for the sake of simplicity.

Patterns of co-localization in the XO–SG system of C. productus

As just described, the SG terminals labeled by the CHH and CPRP antibodies were tendril-like whereas those labeled by the MIH and MOIH antibodies were distinctly blob-like in appearance in all species (Fig. 4). For *C. productus*, we quantified and compared the number of somata labeled by each antibody. In this species, the numbers of XO somata labeled

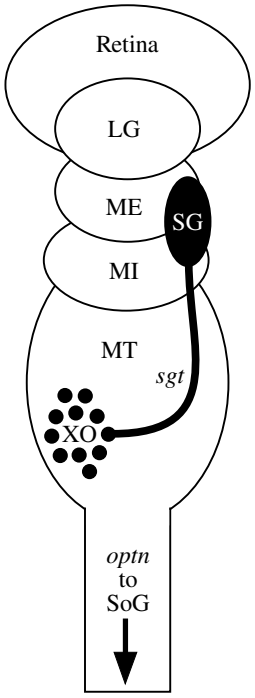


Fig. 2. Schematic diagram of the optic ganglia of *Cancer* species highlighting the location and organization of the X-organ–sinus gland (XO–SG) system. The nervous system contained within the eyestalk consists of several distinct regions, including the medulla terminalis (MT), the medulla interna (MI), the medulla externa (ME), the lamina ganglionaris (LG) and the retina. This system of ganglia is connected to the supraesophageal ganglion (SoG) *via* the optic nerve (*optn*). Located in the MT is a loosely associated collection of neurosecretory somata that are collectively termed the X-organ (XO). The release site of hormones produced by these somata is the sinus gland (SG), which is located at the junction of the MI and ME. The sinus gland tract (*sgt*) links the XO and SG.

by the CHH and CPRP antibodies were essentially identical [anti-CHH: mean=40±1.90 somata/XO, range=36–49, 6 preparations; anti-CPRP: mean=41±2.12 somata/XO, range=37–47, 4 preparations (Student's *t*-test, $P>0.05$)] as were the number of somata labeled by the MIH and MOIH sera [anti-MIH: mean=32±1.38 somata/XO, range=25–36, 8 preparations; anti-MOIH: mean=29±1.92 somata/XO, range=22–35, 6 preparations (Student's *t*-test, $P>0.05$)]. By contrast, the number of somata stained by either the CHH or CPRP antibody was statistically different from those labeled by either the MIH or MOIH antibody (ANOVA, $P<0.0005$; Tukey's test, $P<0.05$). Collectively, these findings suggested the potential for CHH/CPRP and MIH/MOIH co-localization in the XO–SG system, and simultaneously implied that the former two peptides were contained in subpopulations of neurons distinct from those producing the latter two peptides. To directly assess the patterns of co-localization present among the CHH family members and CPRP in *C. productus*, we developed a double-immunohistochemistry method utilizing primary antisera generated in a common host species and conducted double-immunolabeling for all possible combinations of the four antisera using this method.

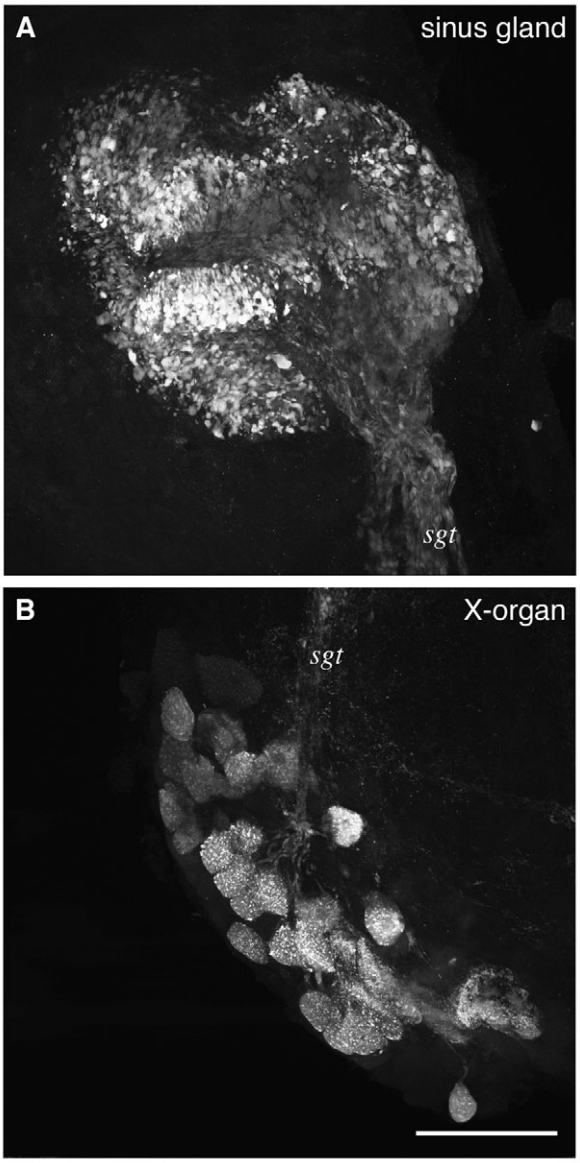


Fig. 3. Moult-inhibiting hormone (MIH)-like immunoreactivity in the X-organ-sinus gland (XO–SG) system of *Cancer productus*. (A) MIH-like labeling in the SG. Whole-mount immunolabeling of the nervous system contained within the eyestalk with antibody to MIH consistently stained nerve terminals in the SG. This labeling could be unambiguously traced *via* immunopositive axons in the sinus gland tract (*sgt*) to somata in the XO. This micrograph is a brightest pixel projection of 29 optical sections taken at 1.95 μm intervals. (B) MIH-like labeling in the XO. Immunoprocessing using anti-MIH consistently labeled 30 or so somata in the XO. This micrograph is a brightest pixel projection of 19 optical sections taken at 1.95 μm intervals. A and B are taken from the same preparation and are shown at the same magnification. Scale bar in B, 150 μm.

As Fig. 5 shows, co-localization of CHH and CPRP as well as of MIH and MOIH (shown in Fig. 5A) was seen in XO somata in each of the *C. productus* eyestalks labeled using these antibody combinations (6 preparations for each combination). In none of the eyestalks labeled with any other combination of primary antisera was any co-localization evident in XO somata (Fig. 5B; 6 preparations for each antibody combination). All somata that expressed CHH-like immunoreactivity were co-labeled by the CPRP antibody, a finding that is not surprising given their production from the same preprohormone in *C. productus* (de la Iglesia et al., 2005; Hsu et al., 2005a). Interestingly, complete co-localization was also seen in the double-labels pairing of the MIH and MOIH antibodies (Fig. 5A), suggesting that all XO somata that produce MIH also produce MOIH.

The pericardial organ

Another crustacean neuroendocrine system known to contain CHH-related peptides is the PO (Dircksen et al., 2001; Wilcockson et al., 2002; Fu et al., 2005b). In brachyuran species, including *C. productus*, the PO consists of two or more longitudinal nerve trunks that are connected by vertical nerve bars (Fig. 1). The trunks and bars that form each PO are elaborations of the segmental nerves (*sn*s) that originate from the fused thoracic ganglia (Fig. 1). Both intrinsically and extrinsically located cell bodies contribute to the release terminals that lie under the epineurium that covers the PO (Cooke and Sullivan, 1982; Beltz, 1988; Fingerman, 1992). As stated earlier, both CHH and CPRP have been detected in the PO of *C. pagurus* using immunohistochemistry (Wilcockson et al., 2002). In this species, all labeling for both peptides originates from three intrinsic somata located in or near the anterior bar of the PO (Wilcockson et al., 2002). To assess if CHH-, CPRP-, MIH- or MOIH-like peptides were present in the POs of other species of *Cancer* and to determine their distributions and patterns of co-localization in *C. productus*, we immunoprocessed this tissue with the same set of antisera used in our mapping of these substances in the SG.

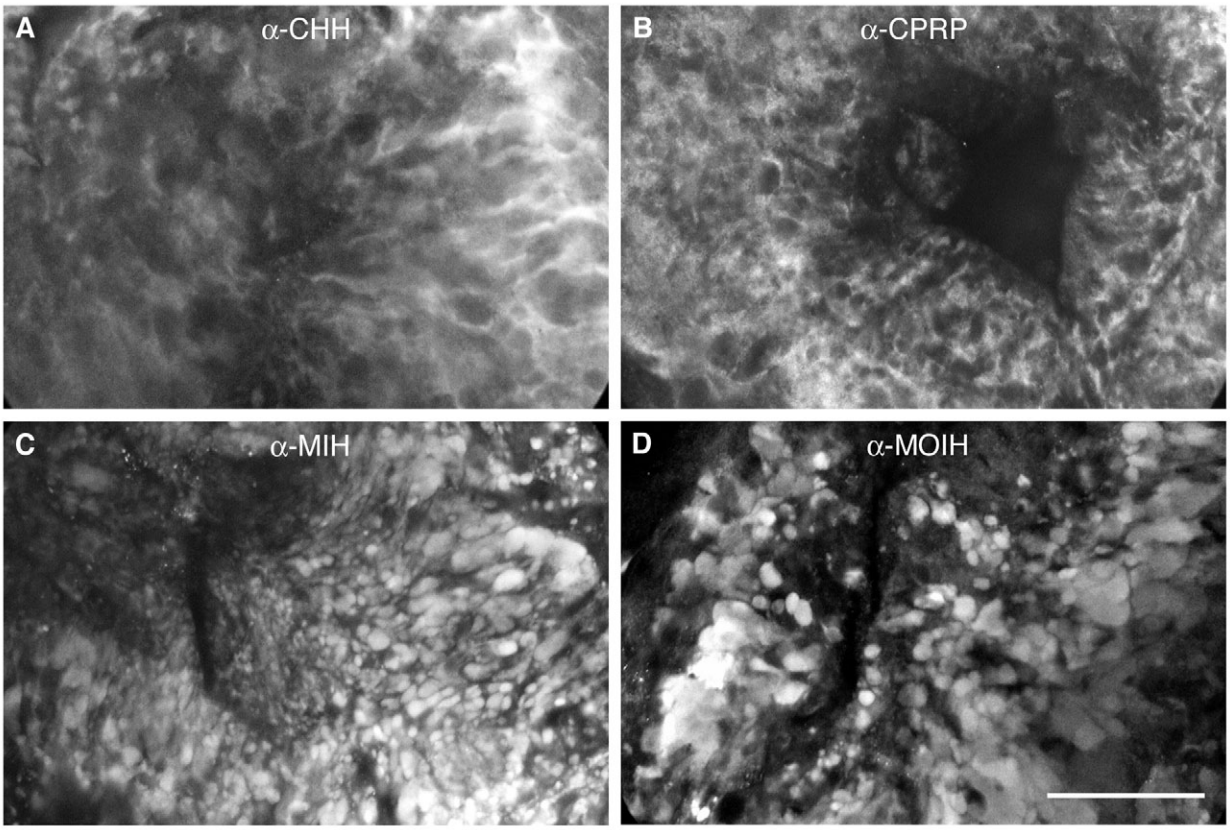


Fig. 4. The distribution of crustacean hyperglycemic hormone (CHH)-like and CHH precursor-related peptide (CPRP)-like labels in the sinus gland (SG) differ from those of moult-inhibiting hormone (MIH) and mandibular organ-inhibiting hormone (MOIH) in *Cancer productus*. Within the SG, terminals labeled by the CHH and CPRP antibodies had the appearance of flocculent tendrils, whereas those revealed by the MIH and MOIH antibodies appeared more oval and blob-like. (A) CHH-like immunopositive terminals in the SG. (B) CPRP-like immunopositive terminals in the SG. (C) MIH-like immunopositive terminals in the SG. (D) MOIH-like immunopositive terminals in the SG. All micrographs are single optical sections shown at the same magnification. Scale bar in D, 50 μm.

Immunohistochemical detection of CHH, CPRP and MOIH, but not MIH in the PO

Immunoprocessing of POs with the CHH, CPRP and MOIH antisera consistently produced labeling within this tissue in each of the *Cancer* species investigated (Fig. 6; $N \geqslant 3$ preparations for each antibody in each species). By contrast, no staining was seen when POs of any crab were processed with the MIH antiserum ($N \geqslant 3$ preparations for each species). For both the CHH and CPRP antibodies, labeling in the POs of each species consisted of up to three somata (usually two bipolar and one multipolar) in the anterior bar or in the *sn*s just anterior to it, as well as fine processes and peripherally located nerve terminals covering extensive regions of the anterior and posterior bars and portions of all three nerve trunks (Fig. 6A,B). For both antibodies, the vast majority of the immunopositive fine fibers and nerve terminals appeared to originate from the arborizations of the intrinsic somata. In each of the CHH-immunopositive somata, labeling was cytoplasmic, uniformly diffuse and often extended for a considerable distance into the axons emanating from these cell bodies. By contrast, labeling in the CPRP-immunopositive somata in all species was cytoplasmic and granular, with little immunoreactivity evident in their projecting axons.

In all animals, MOIH-like staining was weak in the axons and in patches of superficially located nerve terminals in both the anterior and posterior bars, as well as in the nerve trunks (Fig. 6D). Whereas no MOIH-like staining was seen in any intrinsic PO somata in any of the species, approximately four MOIH-immunopositive cell bodies were present in the fused thoracic ganglia of *C. productus* (3 preparations; data not shown). As the thoracic ganglia are known to be the source of many of the inputs to the PO (Cooke and Sullivan, 1982; Beltz, 1988; Fingerman, 1992), these somata are one potential source of the MOIH staining seen in the PO of this species, and possibly the others as well.

Patterns of co-localization in the PO of C. productus

In all of the species investigated, the distribution of CHH- and CPRP-like immunoreactivity in the POs was essentially identical with the exception of the appearance of labeling in the three intrinsic somata. To determine the extent of co-localization of the two peptides in *C. productus*, double-immunolabeling, similar to that in the SG, was undertaken.

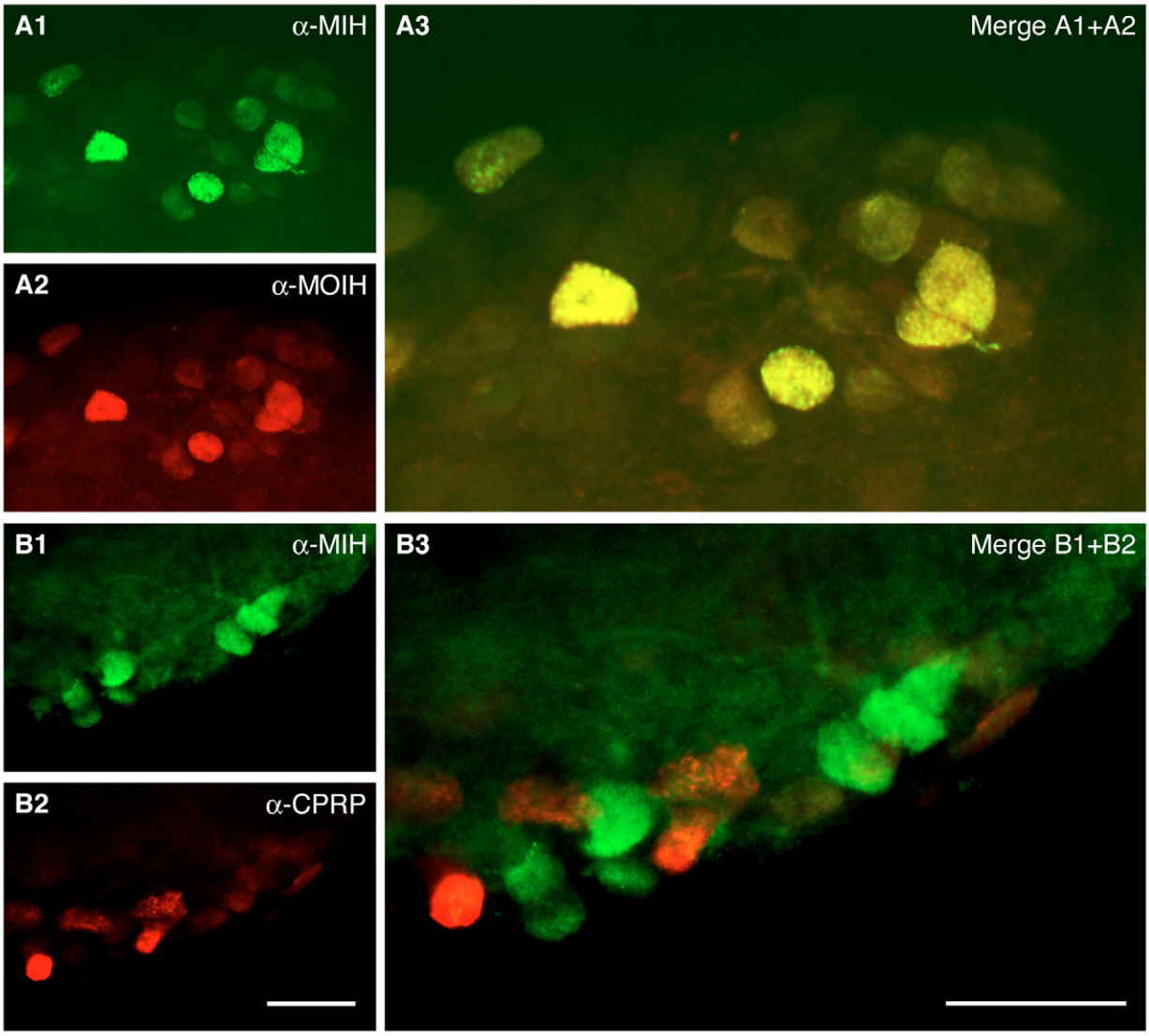


Fig. 5. Double-immunolabeling of the X-organ (XO) with antisera to crustacean hyperglycemic hormone (CHH)-family members and CHH precursor-related peptide (CPRP) in *Cancer productus*. (A1–3) Moult-inhibiting hormone (MIH)/mandibular organ-inhibiting hormone (MOIH) double labeling in the XO. This series of micrographs consists of pseudo-colored single optical sections collected from a single focal plane in an XO labeled with both anti-MIH (A1, pseudo-colored green) and anti-MOIH (A2, pseudo-colored red). When A1 and A2 were merged (A3), complete overlap in the labeled structures was revealed (i.e. yellow, but not green or red, coloration is seen in all immunopositive structures in the micrograph), suggesting that the MIH- and MOIH-like labels are contained in a common set of XO somata. The same result was seen in preparations pairing anti-CHH/anti-CPRP (data not shown). (B1–3) MIH/CPRP double labeling in the XO. This series of micrographs consists of pseudo-colored single optical sections collected from a single focal plane in an XO labeled with both anti-MIH (B1, pseudo-colored green) and anti-CPRP (B2, pseudo-colored red). When B1 and B2 were merged (B3), no overlap in the labeled structures was revealed (i.e. a lack of yellow colored structures in the micrograph), suggesting that the MIH- and CPRP-like labels are contained in distinct sets of XO somata. Though not shown, the same result was seen in preparations pairing anti-MIH/anti-CHH, anti-MOIH/anti-CHH and anti-MOIH/anti-CPRP. A1,2 and B1,2 are all shown at the same scale. Likewise A3 and B3 are shown at the same magnification. Scale bars in B2 and B3, 100 μm.

As Fig. 7 shows, a complete overlap of the two immunoreactivities was found in this tissue (6 preparations). CHH/MOIH (6 preparations) and CPRP/MOIH (6 preparations) double-labelings were also conducted. However, the relative weakness of the MOIH label with respect to either the CHH or CPRP labels made assessment of co-localization difficult. In most POs, the immunoreactivities appeared distinct, whereas in others, weak co-localization in a small population of terminals was evident (data not shown). Given the complete overlap of the CHH and CPRP labels and the fact that they appeared to be derived from a set of somata not labeled by the MOIH antiserum, we believe that the profiles labeled by the MOIH antibody are distinct from those containing CHH/CPRP.

The stomatogastric nervous system

The STNS (Fig. 1) of decapod species is situated over the foregut and is responsible for controlling the movement of food items through this portion of the digestive tract (Selverston and Moulins, 1987; Harris-Warrick et al., 1992). Recently, we identified two neuroendocrine organs within the STNS of *C. productus* (Christie et al., 2004; Christie and Messinger, 2005; Messinger et al., 2005a; Messinger et al., 2005b). One of these, the ACP, is located on the anterior cardiac nerves (*acn*s) overlying the cardiac sac region of the foregut (Christie et al., 2004) (Fig. 1). This site consists of a dense collection of nerve terminals located just below the epineurium covering the *acn*s (Christie et al., 2004), with all innervation to the site originating from four somata, one pair in each commissural ganglion (CoG) (Christie and Messinger, 2005; Messinger et al., 2005b). Based on their innervation of the ACP, these neuron pairs were named anterior commissural neurons 1 and 2 (ACN1/2) (Christie and Messinger, 2005; Messinger et al., 2005b). The other site, the ACO, is located within the anterior medial quadrant of the CoG (Fig. 1) and consists of a dense collection of nerve terminals that envelop an extensive network of hemolymph lacunae that invaginate deep into the ganglion (Messinger et al., 2005a). The origin of the fibers that produce the ACO remain unknown, though somata in the thoracic ganglia are likely candidates (Messinger et al., 2005a). Both the ACP and ACO are also present in the STNSs of *C. antennarius*, *C. anthonyi*, *C. borealis*, *C. gracilis*, *C. irroratus* and *C. magister* (A.E.C., D.I.M. and E. Savage, unpublished observations). To determine if the ACP and/or ACO contain

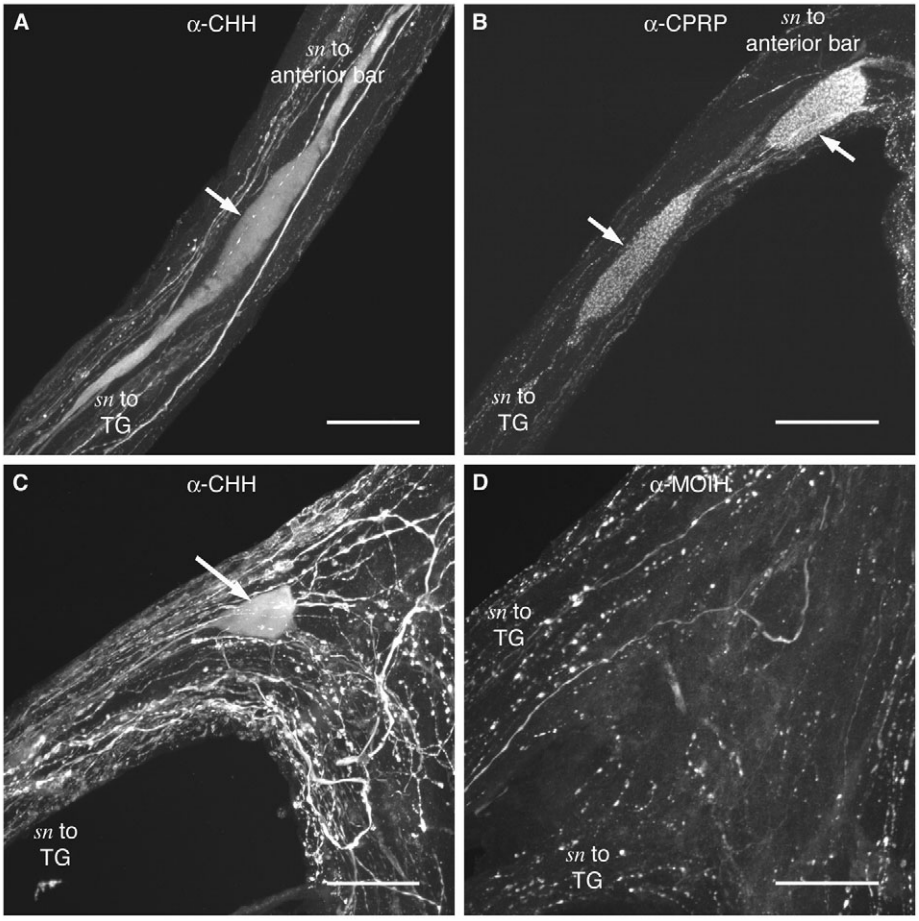


Fig. 6. Crustacean hyperglycemic hormone (CHH)-like, CHH precursor-related peptide (CPRP)-like and mandibular organ-inhibiting hormone (MOIH)-like labeling in the segmental nerves (*sn*) and pericardial organ (PO) of *Cancer productus*. (A) CHH-like labeling in the *sn* anterior to the PO. Two bipolar somata were labeled with the CHH antibody in the anterior bar of the PO or in the *sn*s just anterior to it (one stained soma shown; arrow). Staining in these somata was uniform in its distribution, with the label filling the axons emanating from the cell bodies. As this micrograph shows, many small diameter axons and superficially located nerve terminals were also labeled by the CHH antibody in the *sn*. This micrograph is a brightest pixel projection of 34 optical sections taken at 1.95 μm intervals. (B) CPRP-like labeling in the *sn* anterior to the PO. As with the CHH antibody, two bipolar somata were labeled in the *sn*/anterior bar area of the PO by the CPRP antibody (two shown; arrows). Here, labeling within the somata was granular in appearance, and little immunoreactivity was present in the axons emanating from the cell bodies. Similar to the CHH label, many small diameter axons and superficially located nerve terminals in the *sn* were stained by the CPRP antibody. This micrograph is a brightest pixel projection of 21 optical sections taken at 1.95 μm intervals. (C) CHH-like labeling in the anterior bar of the PO. In addition to the bipolar neurons seen in the *sn*, a single multipolar soma was routinely labeled by the CHH antibody in the anterior bar of the PO (arrow). As with the other PO somata labeled by this antiserum, staining in this cell body was uniform and diffuse in appearance. In addition to the soma, an extensive network of fine fibers, as well as superficial nerve terminals, were labeled by the CHH antibody within the anterior bar of the PO. Although not shown, the CPRP antibody produced an identical pattern of labeling in this portion of the PO, with the exception that labeling in the multipolar soma was granular in appearance rather than uniformly diffuse. This micrograph is a brightest pixel projection of 37 optical sections taken at 1.95 μm intervals. (D) MOIH-like labeling in the anterior bar of the PO. As with the CHH and CPRP labels, the MOIH antibody stained a network of fine fibers and superficially located nerve terminals in the anterior bar of the PO, though the immunoreactivity was less extensive and weaker than with the other sera. However, no somata were labeled by the MOIH antiserum, nor were any labeled in the *sn*s near the anterior bar. This micrograph is a brightest pixel projection of 26 optical sections taken at 1.95 μm intervals. All scale bars, 100 μm.

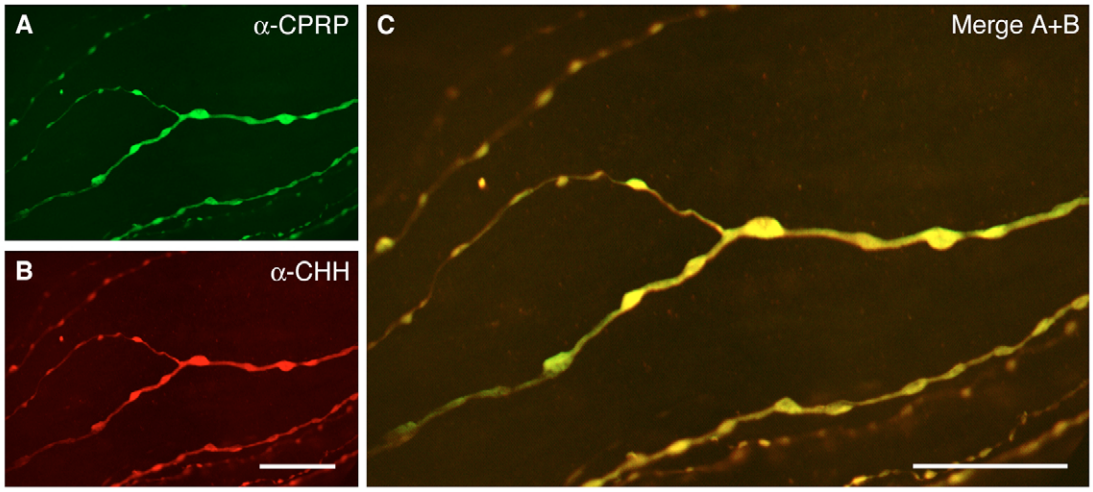


Fig. 7. Co-localization of crustacean hyperglycemic hormone (CHH)-like and CHH precursor-related peptide (CPRP)-like immunoreactivity in the pericardial organ (PO) of *Cancer productus*. This series of micrographs consists of pseudo-colored single optical sections collected from a single focal plane from a PO labeled with both anti-CPRP (A, pseudo-colored green) and anti-CHH (B, pseudo-colored red). When A and B are merged (C), complete overlap in the labeled structures was revealed (i.e. yellow, but not green or red, coloration is seen in all immunopositive processes in the micrograph), suggesting that the CPRP- and CHH-like labels are contained in a common set of structures in the PO. A and B are shown at the same magnification. Scale bars in B and C, 25 μm.

any of the CHH-related or CPRP-like peptides, we immunoprocessed the STNSs from each *Cancer* species with the same complement of antisera used to map the SG and PO.

Immunohistochemical detection of MOIH, but not CHH, MIH or CPRP in the ACP

In each of the investigated species, no immunoreactivity was seen anywhere in the STNSs processed with the CHH, CPRP or MIH antisera ($N \geqslant 3$ preparations for each antibody in each species). By contrast, in all crabs, extensive labeling was produced throughout using the MOIH antibody ($N \geqslant 3$; Figs 8–10), including staining of the ACP (Fig. 8) but not the ACO (data not shown).

Regardless of species and in all preparations examined, extensive MOIH-like immunoreactivity was seen in the nerve terminals constituting the ACP (Fig. 8A). In each ACP, the immunopositive terminals originated from four axons projecting from the stomatogastric nerve (*stn*; Fig. 8B), two traveling from the right superior esophageal nerve (*son*) and two from the left *son* (Fig. 8C). Although it was not possible to follow the immunoreactivity in the axons back to their somata of origin, they probably originate from two large (approximately 100 μm in major cross-sectional diameter), weakly immunopositive cell bodies located at the junction of the son and CoG, as these somata are of the same size and location as the ACN1/2s, which are known to be the sole source of innervation to the ACP (Christie et al., 2004; Christie and Messinger, 2005; Messinger et al., 2005b).

In addition to the staining just described, in all species the MOIH antiserum also labeled an extensive neuropil in the stomatogastric ganglion that appeared to originate from both the arborizations of two intrinsic immunopositive somata and from the arborizations of projection neurons traveling to the ganglion *via* the *stn* (Fig. 9). This, and all other MOIH-like labeling in the STNS, is summarized schematically in Fig. 10.

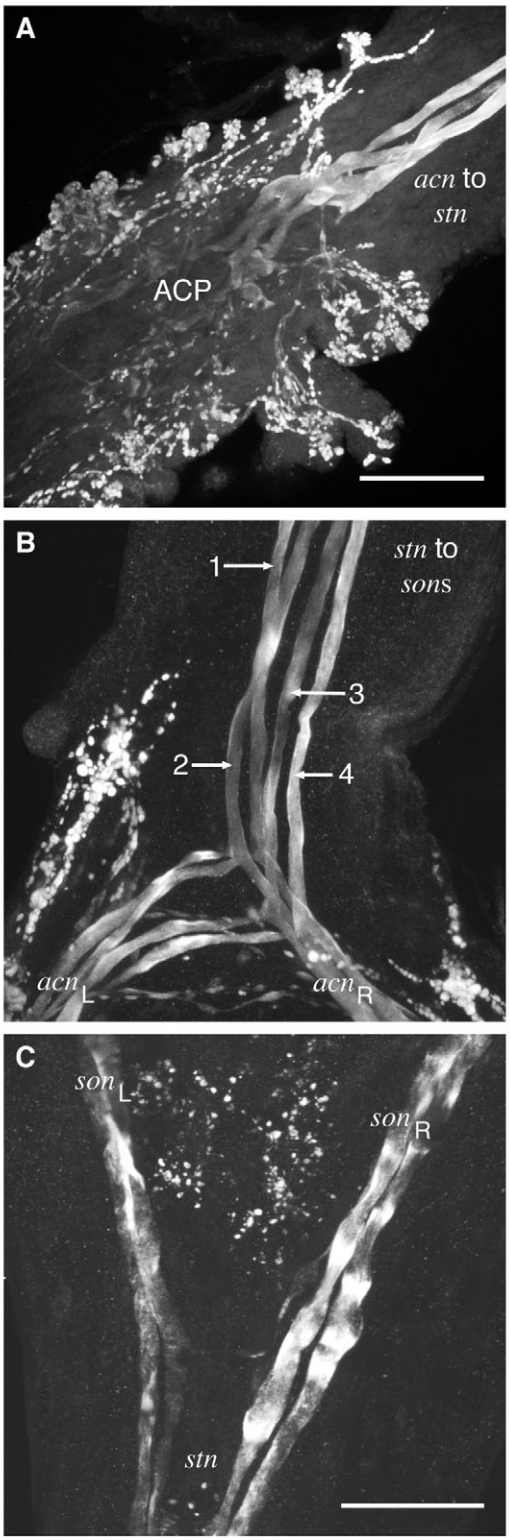
Discussion

Members of the CHH family and CPRP are differentially distributed between the neuroendocrine organs of Cancer species

In this study we used antisera generated against Capa-CHH II, Capa-MIH, Capa-MOIH I and Capa-CPRP to assay the neuroendocrine organs of the crabs *C. antennarius*, *C. anthonyi*, *C. borealis*, *C. gracilis*, *C. irroratus*, *C. magister* and *C. productus* for the presence of isoforms of these peptides. Our results show that in all species, moieties immunologically related to the peptides used for antibody production are present in the SG, PO and ACP, but not in the ACO. Moreover, the patterns of immunoreactivity revealed by these antisera suggest that the native CHH family members and CPRPs are differentially distributed between the neuroendocrine organs of these species, with all members of the CHH family and CPRP present in the SG; CHH, MOIH and CPRP, but not MIH, present in the PO; and only MOIH present in the ACP.

As are all antibodies, the sera used in our study are biological and not chemical reagents and thus are subject to potential cross-reactions with antigens other than that to which they were raised (Saper and Sawchenko, 2003). As Table 1 shows, significant sequence homology is present between the *C. pagurus* CHH-family members used for antibody production. Despite the sequence similarity of the antigens, the distinct patterns of labeling seen with each antibody strongly suggest that they are detecting different complements of peptides.

Furthermore, in all tissues, only adsorption of an antibody by the peptide used for its production completely eliminated labeling. Thus, we feel confident that the staining presented here is an accurate reflection of the distributions of CHH-family members and CPRPs in the neuroendocrine organs of *Cancer* species.



Members of the CHH family and CPRP are differentially distributed within the XO–SG system as well as within the PO

In addition to observing a differential distribution of CHH-family peptides and CPRP among the neuroendocrine organs of various *Cancer* species, we also found that these peptides are differentially distributed within both the XO–SG and PO of at least *C. productus*. Using a protocol that allowed double labeling of whole-mount tissue with primary antibodies generated in the same host species, we found complete overlap of labeling for CHH and CPRP in both the XO–SG and the PO. Similarly, a complete overlap of labeling was seen for MIH and MOIH in the XO–SG, but in a different set of neurons than those containing CHH and CPRP. MOIH was also present in the PO, again in a set of structures distinct from those possessing CHH and CPRP.

Ours is the first study to show co-localization of CHH- and CPRP-like immunoreactivities in either the XO–SG or the PO, but the association of these two peptides is not surprising given that both peptides are produced from a common preprohormone in *C. productus* (de la Iglesia et al., 2005; Hsu et al., 2005a). More interesting is the complete overlap of the MIH and MOIH labels in the XO–SG, which are encoded by different genes (Lu et al., 2000). As stated earlier, the peptides used for the production of the MIH and MOIH antibodies exhibit significant amino acid sequence identity (approximately 61%; Table 1), and thus it is possible that each serum was cross-reacting with a common peptide. However, we were unable to completely block the MIH staining in the XO–SG with any peptide other than Capa-MIH, including Capa-MOIH I. Similarly, the XO–SG label produced by the MOIH antibody was suppressed only after adsorption by Capa-MOIH and not by Capa-MIH. These results suggest that the MIH- and MOIH-like labelings were specific for their respective antigens. Clearly, molecular and/or biochemical characterization of the

Fig. 8. Mandibular organ-inhibiting hormone (MOIH)-like labeling in the anterior cardiac plexus (ACP) of *Cancer productus*. (A) MOIH-like labeling in the ACP. Immunoprocessing of the stomatogastric nervous system with the MOIH antibody consistently produced labeling in the ACP. This micrograph is a brightest pixel projection of 16 optical sections taken at 1.95 μm intervals. (B) MOIH-like labeling at the junction of the anterior cardiac (*acn*) and stomatogastric (*stn*) nerves. Labeling in each ACP arose from four axons that projected into each *acn* from the *stn* (axons in the *stn* denoted by arrows). This micrograph is a brightest pixel projection of 14 optical sections taken at 1.95 μm intervals. (C) MOIH-like labeling at the junction of the *stn* and the superior esophageal (*son*) nerves. The four axons that are the source of the MOIH-like labeling in the ACP can be traced through the *stn* to its junction with the paired *son*s. Here, two of the four axons enter the left *son* and two the right *son*. Within each *son*, MOIH-like labeling in the axons became weak near the junction of the nerve with the commissural ganglion, and further tracing was not possible from immunoreactivity alone (not shown). This micrograph is a brightest pixel projection of 17 optical sections taken at 1.95 μm intervals. Both B and C are shown at the same magnification. Scale bars in A and C, 75 μm.

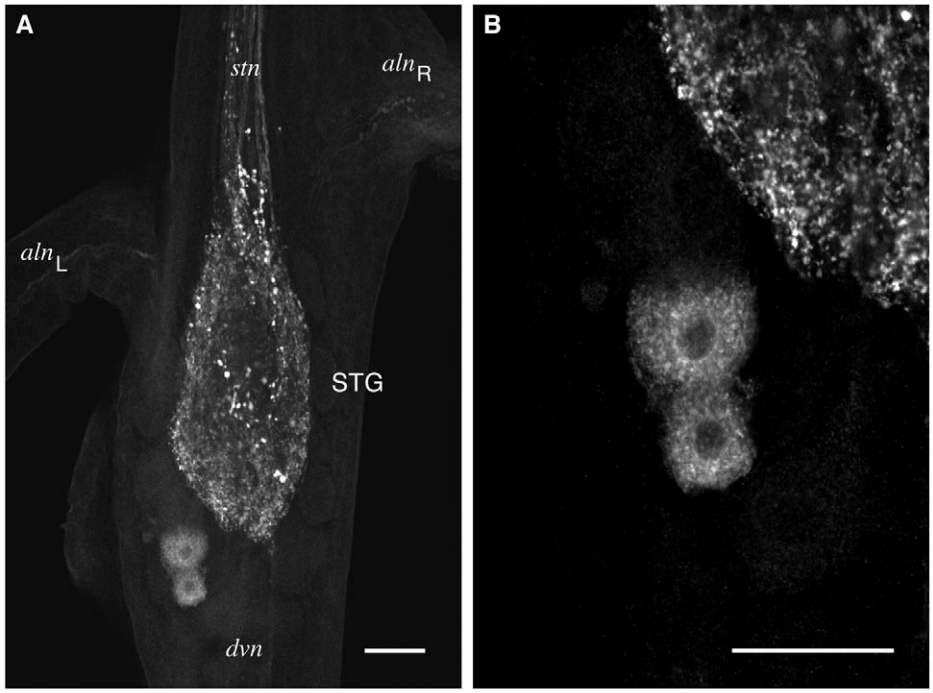


Fig. 9. Mandibular organ-inhibiting hormone (MOIH)-like labeling in the stomatogastric ganglion (STG) of *Cancer productus*. (A) MOIH-like labeling in the STG. Among the regions of the stomatogastric nervous system labeled by the MOIH antibody was the STG. Here, staining was present in two intrinsic somata as well as in the neuropil within the ganglion. The immunopositive neuropil appears to originate from both the arborizations of the intrinsic immunopositive somata as well as from the arborizations of projection neurons, which send axons to the STG *via* the stomatogastric nerve (*stn*). (B) Higher magnification image of the MOIH-immunopositive somata shown in A. Note that the staining in these cell bodies is cytoplasmic and distinctly punctate. The micrographs shown in both A and B are single optical sections. Scale bars in A and B, 75 μm.

complement of native *C. productus* CHH-family peptides will be necessary to resolve this issue unambiguously.

Biologically, it is interesting to postulate why MIH and MOIH are present in the same set of XO somata. If MIH and MOIH are contained in the same secretory vesicles, then all signals triggering their release would probably result in the coordinated inhibition of both somatic growth (*via* the inhibition of steroid production in the Y-organ by MIH) and gonadal growth (*via* the inhibition of methyl farnesoate production in the mandibular organ by MOIH). If, however, the two peptides are contained in distinct sets of vesicles, then the two peptides might well be released in response to distinct physiological cues. Clearly, the former versus the latter

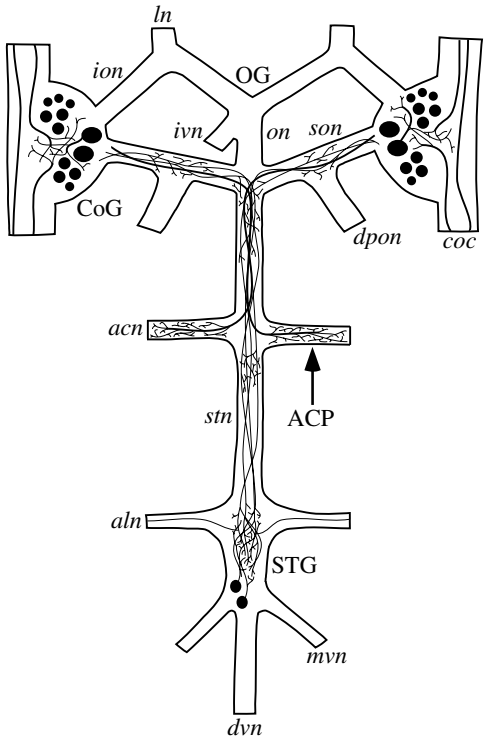


Fig. 10. Schematic representation of mandibular organ-inhibiting hormone (MOIH)-like immunoreactivity in the stomatogastric nervous system (STNS) of *Cancer* species. In this diagram, filled circles represent immunopositive somata, thick lines within nerves represent immunopositive axons and tangles of thin lines represent regions of immunopositive neuropil or neuroendocrine release sites. In addition to labeling the anterior cardiac plexus (ACP) and the axons innervating it, MOIH-like labeling was also evident in other regions of the STNS. In brief, approximately a dozen somata were labeled in each commissural ganglion (CoG), as were two in the stomatogastric ganglion (STG). No immunopositive somata were present in the esophageal ganglion (OG) nor were any seen in the nerves of the STNS. Immunopositive neuropil was present in the CoGs and the STG. Extraganglionic neuropil was present in the superior esophageal nerves (*son*s; most commonly in the vicinity of the dorsal posterior esophageal nerve (*dpon*)], at the junction of the *son*s, the esophageal nerve (*on*) and the stomatogastric nerve (*stn*), as well as in the *stn* proper [commonly near the insertion point of the anterior cardiac nerves (*acn*s)]. The immunopositive neuropil in the STG probably originates from both the arborizations of the intrinsic somata as well as from the arborizations of approximately six axons projecting from the *stn* (approximately three from each *son*). Immunopositive axons were also present in the circumesophageal connectives (*coc*s), which link the STNS to the supraesophageal and thoracic ganglia, as well as occasionally in the anterior lateral nerves (*aln*s; one axon in each *aln*) that emanate from the STG and innervate muscles of the gastric mill region of the foregut.

situation would have profound physiological consequences for an animal, and with future studies it will be interesting to determine the physiological significance of the co-localized peptides.

Do multiple tissue localizations imply pleiotropic functions for the CHH family members and CPRP in Cancer species?

As just discussed, we have found that the CHH, MOIH and CPRP are each present in at least two neuroendocrine sites. With such multiple tissue localizations, the potential clearly exists for distinct cues triggering the release of a given hormone from each site. In addition, distinct isoforms of the CHH, MOIH and CPRP may be present in each tissue, as has been shown for CHH in several crab species (Dircksen et al., 2001; Toullec et al., 2006). In *Carcinus maenas*, the CHH of the PO, unlike its SG counterpart, does not possess hyperglycemic activity nor does it appear to inhibit ecdysteroid synthesis (Dircksen et al., 2001). Instead, this CHH isoform is proposed to serve either a sensory or local modulatory role in this species (Dircksen et al., 2001). Although unproven, the differential distributions of CHH, CPRP and MOIH revealed in our study suggest the possibility of pleiotropic functions for these peptides in *Cancer* species (e.g. their classically ascribed functions versus possible roles as locally released neuromodulators). As additional studies are undertaken, it will be interesting to see if this hypothesis is borne out.

Conclusions

In conclusion, the data we present show that members of the CHH family and CPRPs are differentially distributed both between and within the neuroendocrine organs of crabs of the genus *Cancer*. These results represent the most complete immunohistochemical survey of CHH-family peptides and CPRP in the neuroendocrine organs of crustaceans and provide a foundation for future studies directed at the identification of the native isoforms present in each species and tissue, as well as the elucidation of their physiological roles in members of this genus. The finding of CHH, CPRP and MOIH-like labeling in multiple neuroendocrine tissues suggest that these peptides may be released into the circulatory system in response to varied cues and that some of their isoforms may possess distinct tissue-specific functions.

List of abbreviations

acn	anterior cardiac nerve
ACO	anterior commissural organ
ACP	anterior cardiac plexus
CHH	crustacean hyperglycemic hormone
CoG	commissural ganglion
CPRP	crustacean hyperglycemic hormone precursor-related peptide
GIH	gonad-inhibiting hormone
MIH	moult-inhibiting hormone
MOIH	mandibular organ-inhibiting hormone
PO	pericardial organ
SG	sinus gland
sn	segmental nerve
son	superior esophageal nerve
STG	stomatogastric ganglion
stn	stomatogastric nerve
STNS	stomatogastric nervous system
VIH	vitellogenesis-inhibiting hormone
XO–SG	X-organ–sinus gland system

John Edwards, Lilliam Ambroggio, Rachel Latham, Minhui Lin, Christina Ngo and Trendafil Halatchev are thanked for their help with some of the immunohistochemistry and confocal microscopy. John Weller, Curtis Easton and Christopher Goiney are thanked for their assistance in animal collection. Dr William Stegeman (Jackson ImmunoResearch Laboratories) is thanked for his help in designing and trouble-shooting the double-immunohistochemical studies as well as providing the reagents necessary for conducting them. Y.A.H. gratefully acknowledges financial support from the University of Washington Graduate Opportunities & Minority Advancement Program for Graduate Opportunity Program Fellowship. Some of the animals used here were collected under the auspices of Washington Department of Fish and Wildlife Scientific Collection Permits #03-018a and #04-021 (to A.E.C.).

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