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REGULAR ARTICLE

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Is crustacean hyperglycaemic hormone precursor-related peptide a circulating neurohormone in crabs?

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Abstract Sites of synthesis and release patterns of crustacean hyperglycaemic hormone precursor-related peptide (CPRP) were investigated with those of crustacean hyperglycaemic hormone (cHH), in order to determine whether this precursor-related peptide satisfies certain criteria necessary for its definition as a secretable, circulating hormone. Using the edible crab, *Cancer pagurus*, sites of CPRP synthesis were determined by immunohistochemistry and release patterns of both peptides were determined in vivo and in vitro by radioimmunoassay of haemolymph and eyestalk superfusates. Both peptides were co-released from sinus glands (SGs) following potassium-evoked depolarization of isolated eyestalk preparations. However, stress-evoked in vivo release resulted in apparent non-stoichiometric circulating peptide profiles. This phenomenon is explained by notable differences in clearance rates of the peptides in haemolymph. In contrast to cHH, CPRP is very slowly degraded in vivo. Although CPRP is clearly a circulating peptide, whose release is concomitant with that of cHH, physiologically pertinent roles for this molecule remain to be discovered.

Keywords Crustacean · Precursor · Peptide · Neurohormone · *Cancer pagurus* (Crustacea)

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Introduction

Neurosecretory neurons in the eyestalks of decapod crustaceans synthesize and secrete a variety of neuropeptides, the most prominent of which (both in terms of its abundance and the voluminous literature) is the crustacean hyperglycaemic hormone (cHH) (reviews: Keller 1992; Van Herp 1998; Webster 1998). cHH is a member of a rapidly growing family of structurally related neuropeptides, members of which include the moult-inhibiting hormone (MIH), gonad-inhibiting hormone (GIH) and mandibular organ-inhibiting hormone (MOIH) (Lacombe et al. 1999). In contrast to other members of the group, two features of cHH are striking: Firstly, it is a pleiotropic hormone – numerous actions of this peptide have been described, not only in terms of its (defining) action in blood glucose regulation (review: Van Herp 1998), but also inhibition of ecdysteroid and/or methyl farnesoate production (review: Webster 1998), stimulation of amylase release (Sedlmeier 1988), lipid regulation (Santos et al. 1997), ovarian protein synthesis (Khayat et al. 1998) and hydromineral regulation (Chung et al. 1999; Spanings-Pierrot et al. 2000). Secondly, cHH preprohormones always contain a precursor-related peptide which is C-terminally flanked by cHH (De Kleijn and Van Herp 1995; De Kleijn et al. 1994, 1995; Tensen et al. 1991; Weidemann et al. 1989). These so-called crustacean hyperglycaemic hormone precursor-related peptides (CPRPs) are between 33 and 38 residues long in lobsters and crayfish (De Kleijn et al. 1994, 1995; Tensen et al. 1991) or crabs (Chung et al. 1998; Newcombe 1987; Weidemann et al. 1989) and show rather limited identity, whilst in penaeid prawns a number of much shorter, seemingly unrelated CPRPs have been found (Davey et al. 2000; Gu and Chan 1998; Gu et al. 2000). The functions of CPRP have not been determined. Indeed this is also the case for other invertebrate neuropeptide precursor-related peptides such as adipokinetic hormone precursor-related peptides (APRPs) (Fischer-Lougheed et al. 1993; Hekimi and O'Shea 1989), and for vertebrate examples such as the neurophysins (Ando et al. 1988;

Shin et al. 1995) and insulin intrachain connecting peptide (Wahren et al. 1996). Accordingly, a first step towards identification of possible functions for CPRP would be to describe neuroanatomical architecture, in comparison with cHH, and release patterns of both peptides, in vitro and in vivo, to determine: (a) whether both peptides are co-released, (b) that CPRP circulates in the haemolymph, and (c) the clearance dynamics of CPRP in relation to cHH. The present paper describes the localization and quantification of CPRP and cHH in the crab *Cancer pagurus* and the release and circulation of both peptides using highly sensitive and specific immunochemical methods.

Materials and methods

Animals

Adult *C. pagurus* were obtained from around the coast of Anglesey, North Wales, and held in a re-circulating seawater system under ambient temperature and photoperiod. All animals used were intermoult and were unfed for 48 h before use.

Peptide purification

Sinus glands were dissected from eyestalks removed from ice-anaesthetized crabs, in batches of 100, immediately snap-frozen in liquid nitrogen and stored at –80°C. These were subsequently extracted and peptides purified by high-performance liquid chromatography (HPLC) as described previously (Webster 1998). Peak fractions previously identified as those containing CPRP and cHH (Chung et al. 1998) were collected manually and immediately dried by vacuum centrifugation. Peptides were quantified by gas-phase hydrolysis in azetropic 6 M HCl (Tarr 1986), followed by *o*-phthaldialdehyde derivatization and HPLC as previously described (Webster 1991).

Antiserum production

CPRP (25 nmol) was conjugated to bovine thyroglobulin (bTg) using either glutaraldehyde at a molar ratio of 75:1:3030 (CPRP:bTg:glutaraldehyde) (Dircksen and Keller 1988) or 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl (EDC) at a molar ratio of 118:1:200 (CPRP:bTg:EDC) (Skowsky and Fisher 1972). Two New Zealand White rabbits were immunized subcutaneously with either conjugate, with initial injection of 15 nmol peptide emulsified in complete Freund's adjuvant, and at 4-weekly intervals with 10 nmol and 5 nmol peptide in incomplete Freund's adjuvant. Four weeks after final immunization, blood was collected by terminal exsanguination under anaesthesia. Serum from retracted clots was stored at –20°C or lyophilized. Principles of laboratory animal care and specific national laws were followed.

The *C. pagurus* cHH antiserum used has been previously characterized (Webster 1996).

Immunocytochemistry (ICC) and specificity controls

Eyestalk ganglia were dissected, fixed immediately in Bouin's fluid and embedded in Paraplast after dehydration in a graded ethanol series via methyl benzoate-toluene. Sections (7 µm) were incubated overnight at 4°C in primary antisera (anti-CPRP 1/20,000 or anti-cHH 1/5,000) and immunostained by the peroxidase anti-peroxidase technique (Sternberger 1979). Sinus glands were prepared for electron microscopy by fixation in 2% paraformaldehyde, 2% glutaraldehyde, 0.1% picric acid in 0.1 M sodium cacodylate, pH 7.4, supplemented with 0.5 M sucrose and 5 mM $CaCl_2$ for 4 h at 4°C (Dircksen 1992). Fixed tissues were extensively washed in cacodylate buffer, dehydrated in graded ethanol, and embedded in Spurr's resin, cured at 65°C overnight. Ultrathin sections (80–100 nm) were mounted on Piolioform-F-coated 200-mesh nickel grids. Double immunogold labelling of ultrathin sections was done according to Dircksen et al. (1988) using primary antisera dilutions of 1/30,000 (anti-CPRP) and 1/10,000 (anti-cHH). Immunogold labelling was performed using goat anti-rabbit 30-nm colloidal gold, 1/20 (British Biocell), and protein A colloidal gold, 1/20 (Sigma). After immunolabelling, ultrathin sections were contrasted in 2% aqueous uranyl acetate and 0.15% aqueous lead citrate, viewed and photographed on a Philips EM301 electron microscope, operating at 60 kV. For whole-mount immunohistochemistry, thoracic, cerebral and stomatogastric ganglia and pericardial organs (POs) were dissected and fixed in Stephanini's fixative (Stefanini et al. 1967) and incubated for 72 h in primary antisera. Immunostaining was performed by the peroxidase antiperoxidase method and preparations dehydrated through graded ethanol and mounted in DPX via methyl benzoate.

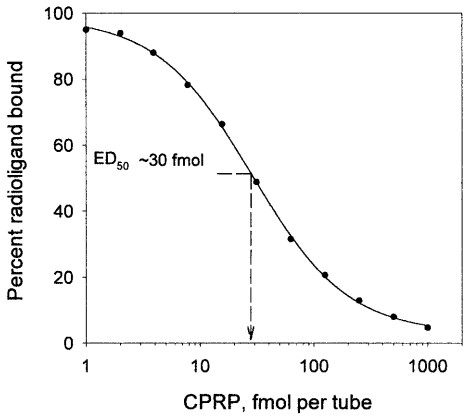


Fig. 1 Typical standard curve for the *C. pagurus* CPRP RIA. The ED_{50} (the quantity of peptide required to displace 50% of the corresponding ligand) was about 30 fmol/tube. The working range (limits of the assay) is between 5 and 250 fmol/tube

Radioimmunoassay

Iodination of CPRP with [^{125}I]NaI was performed according to Webster (1996). Labelled CPRP was separated from unreacted iodide using a Sephadex G15 column eluted with 0.2 M phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). This protocol typically resulted in labelled CPRP with specific activities of about 25 TBq/mmol. Iodinated peptide was used in radioimmunoassay (Webster 1996) using unlabelled peptide standards in the range 1.0 pmol to 1.0 fmol/tube and an antiserum (R136) dilution of 1/6,000. The standard curve gave a working range from 250 fmol to 5 fmol with an ED_{50} of approximately 30 fmol (Fig. 1). The cHH RIA was performed as previously described (Webster 1996).

Sample preparation

Haemolymph samples were subjected to crude purification on Sep-Pak C_{18} cartridges eluted with 40% isopropanol, dried in a vacuum centrifuge and resuspended in assay buffer for radioimmunoassay (RIA). CNS ganglia, eyestalk nervous tissues, POs and stomatogastric ganglia were dissected, snap frozen in liquid nitrogen and stored at –20°C. Tissues were subsequently extracted in 2 N acetic acid and supernatants dried in a vacuum centrifuge before being resuspended in assay buffer for RIA. For HPLC, acetic acid extracted tissues were injected without further purification.

HPLC of blood samples

Twenty milliliters of blood was drawn from thermally stressed crabs held in seawater maintained at 30°C for 5 min. Blood was then immediately Sep-Pak purified, pooled, dried, resuspended in 2 N acetic acid and separated by HPLC (see conditions above) on a Phenomonex Jupiter C_{18} column (250×4.6 mm) dedicated to haemolymph samples. Standards of authentic CPRP (10 pmol) were run in the same manner immediately following the samples.

Measurement of CPRP and cHH half-lives

The longevity of CPRP in vivo was compared with previously established estimates for cHH using spike-recovery experiments (Webster 1996). Crabs were bilaterally eyestalk ablated 72 h prior to experimentation and injected through the hypobranchial sinus with 10 pmol CPRP and cHH in 100 µl saline. Blood samples were subsequently withdrawn after 2, 5, 10, 20, 30, 60, 90 and 120 min, snap frozen in liquid nitrogen and prepared for RIA. To establish the longevity of CPRP in the haemolymph ex vivo, intact crabs were thermally stressed at 30°C for 5 min and 20-ml samples taken. These were kept in glass containers at ambient seawater temperature (11°C) and 2-ml subsamples taken at 60-min intervals for 5 h and prepared for RIA (see above).

In vivo half-life estimates were validated by injecting adult crabs with 10 pmol CPRP and cHH and withdrawing blood at 60 min, corresponding to the calculated half-life of the peptide. These samples were separated by HPLC and the fractions assayed by RIA.

In vitro release of peptides

In vitro release of SG peptides was evoked by potassium depolarization of isolated eyestalk ganglia. Complete eyestalk ganglia were dissected under ice-chilled low-K^+ saline (see below) and washed for 3 h in a continuous flow (1 ml/min) of ice-chilled low-K^+ saline delivered by a peristaltic pump. Tissues were then subjected to sequential, 10-min, incubations in 50 µl of the saline solutions: low-K^+ saline (containing 10 mM KCl); high-K^+ saline (containing 100 mM KCl); high-K^+ saline without calcium (containing 20 mM EDTA); and low-K^+ saline. Between incubations the saline media were removed, frozen in liquid nitrogen, subsequently diluted 1/20 in RIA buffer and assayed as above. Control ganglia were incubated at each step in low-K^+ saline.

In vivo release of peptides

In vivo release of peptides was evoked by subjecting intact crabs to increased water temperatures. Five groups of animals were held at 5, 11, 18, 25 and 30°C. Blood samples (2 ml) were taken after 5 min and prepared for RIA. Control animals were kept at ambient seawater temperature (11°C). In addition, eyestalk-ablated control animals were subjected to identical regimes as the treatment groups.

Results

Both rabbits produced antisera of high titre and specificity although the glutaraldehyde conjugate resulted in an antiserum (code R136) most suitable for background-free immunocytochemical staining and immunoassay. Figure 2 shows that immunoreactivity of HPLC fractions of *C. pagurus* SGs by CPRP-RIA using antiserum R136 is confined to fraction 38, consistent with the retention time of authentic CPRP.

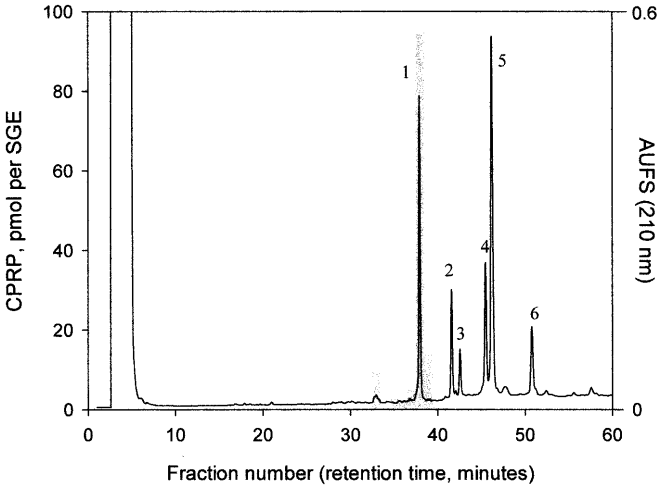
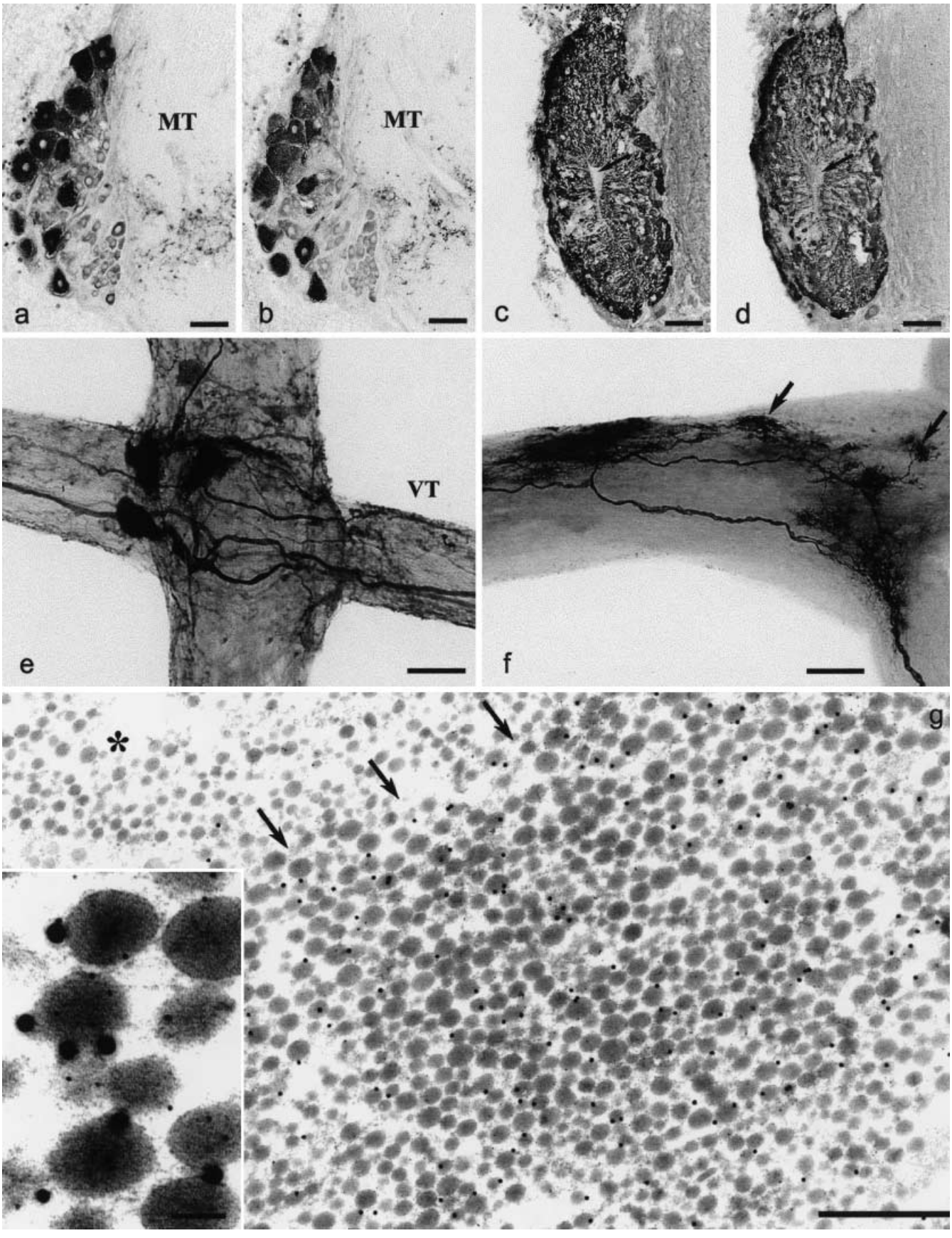


Fig. 2 Histogram showing CPRP-immunoreactive fractions of four HPLC-separated SGs. *Shaded bars* show the position of immunoreactivity and quantification of CPRP by RIA. The underlying HPLC trace indicates the corresponding CPRP UV absorbent peak eluting after 38 min. *Numbered peaks* represent previously identified and characterized neuropeptides of *C. pagurus* SGs: *1* CPRP, *2* mandibular organ-inhibiting hormone 1 (MOIH-1), *3* MOIH-2, *4* cHH-1, *5* cHH-2, *6* moult-inhibiting hormone (MIH)

Peroxidase anti-peroxidase ICC resulted in strong, background-free staining of medulla terminalis X-organ (MTXO) perikarya and SG using antisera R136. The use of anti-CPRP, in combination with existing antisera for *C. pagurus* cHH, allowed detailed, comparative localization of the two antigens. The staining patterns of the consecutive sections (Fig. 3a, b) show that both CPRP and cHH colocalize within the same perikarya of the MTXO. The morphology of these cells was consistent with those described previously as cHH cells (Dircksen et al. 1988), characterized by the large perikarya (35–50 µm across in this profile) and granular cytoplasm.

Figure 3c, d shows consecutive sections of the SG of *C. pagurus* stained for CPRP and cHH, respectively. The majority of the SG section was immunopositive for both antigens, demonstrating the predominance of cHH and CPRP within the MTXO-SG axis and suggesting that, as in the cells of the MTXO, CPRP and cHH colocalize. This was further demonstrated by electron microscopy. Double-immunogold-labelled SG sections showed binding of both 10-nm and 30-nm gold particles to the same granules within the axon terminal (Fig. 3g). The ultrastructure of the SG in many crustacean species has been described extensively (Andrew and Shivers 1976; Castany et al. 1997; Dircksen 1992; Dircksen et al. 1987; Giulianini et al. 1998; Gorgels-Kallen and Van Herp 1981; Nordmann 1977; Shivers 1976; Silverthorn 1975; Smith 1974; Weatherby 1981). Labelled granules in the present study measured approximately 150–250 nm across and correspond to type 1 granules as described by Dircksen (1992) or type 5 (Smith 1974) in the crab *Carcinus maenas*.

Preabsorbtion of antiserum R136 with native CPRP completely abolished all ICC staining of wax-sectioned eyestalk material (not shown).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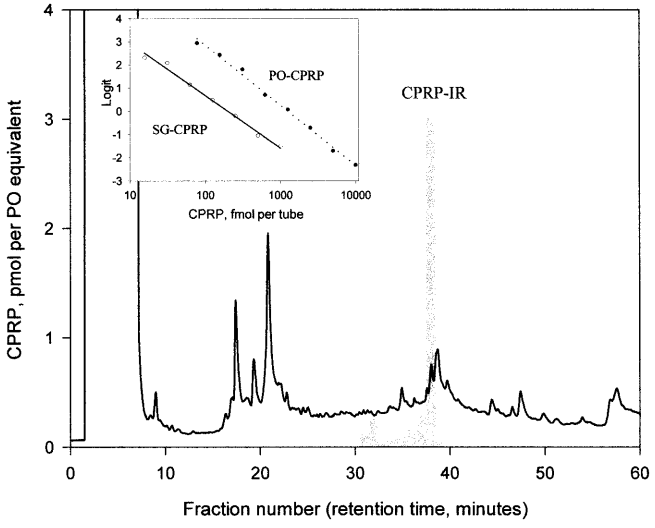

Fig. 4 Histogram showing CPRP immunoreactivity of ten HPLC-separated POs by RIA. The position of CPRP (*grey bars*)-immunoreactive fractions (CPRP-IR) is shown overlaying the HPLC trace. Note that the retention time for CPRP is identical to that for SG-CPRP. Verification of the PO-CPRP as authentic peptide was achieved by comparing the parallelism of Logit-log transformed plots of immunoreactive fraction from both tissues (*inset*)

Whole-mount immunocytochemistry of the major ganglia failed to reveal the presence of cHH or CPRP in all but the eyestalk tissues and the POs. In the POs three or sometimes four large multipolar cells were found in the ventral side of the anterior bar (Fig. 3e), giving rise to local fine arborizations which terminated in putative neurosecretory endings abutting the nerve surface. In addition, longer axonal projections from the perikarya terminated in an umbellate form at the inner surface of the posterior bar (Fig. 3f). Despite repeated attempts, no CPRP-immunopositive axons could be traced back to the TG via the PO and it appears that the perikarya in the anterior bar are the exclusive contributors to CPRP-immunoreactive material in the POs of this species. Whole-mount ICC of POs using the anti-cHH

◀ **Fig. 3a–g** Immunocytochemical localization of CPRP and cHH in the eyestalk ganglia and CPRP localization in the POs of *C. pagurus*. **a** CPRP-immunoreactive cells of the MTXO; **b** cHH immunoreactivity in a consecutive section. Note the granular staining patterns within the cell cytoplasm. **c, d** Adjacent section of SG stained for CPRP **(c)** and cHH **(d)**. *Arrows* indicate the haemolymph lacunae. **e** Anterior bar of *C. pagurus* PO showing the CPRP-immunoreactive neuroarchitecture. Three conspicuous CPRP-containing multipolar neurons give rise to prominent axonal projections which ramify throughout the ventral trunk (*VT*) of the PO and into the posterior bar **(f)**. Putative neurosecretory terminals (*arrows*) on the posterior bar surface are visible as umbellate arborizations of the axons emanating from the cells of the anterior bar. **g** TEM of SG material from *C. pagurus*. Large gold particles (30 nm) show the localization of CPRP and small (10-nm) particles localize cHH. Both sizes of gold can be seen binding to the same electron-dense neurosecretory granules (*inset*) within the same axon terminal. *Arrows* indicate the position of basement membrane, separating two adjacent terminals, which has been lost during fixation. No labelling is seen in the adjacent terminal (*), which contains morphologically dissimilar granules. *Scale bars* 50 µm **(a–d)**, 100 µm **(e–f)**, 500 nm **(g)**, 100 nm (**g** *inset*)

Table 1 Between and within individual variation (left and right eyestalks) of CPRP and cHH in the SGs of *C. pagurus* determined by RIA of crude tissue extracts; *n*=5

CPRP (pmol/SG)		CHH (pmol/SG)	
Left	Right	Left	Right
97	87	142	91
144	135	130	130
88	98	85	118
112	116	110	93
148	198	202	203
Mean 117.8	126.8	133.8	127
(±SEM) 12.15	19.6	19.6	20.4

No statistical difference was found in the CPRP or cHH content between the left and right eyestalk SGs (P=0.46, CPRP; and P=0.65, cHH, t-test) or between cHH and CPRP content (P=0.40, t-test)

serum resulted in very weak staining, possibly due to molecular differences between the cHH of the PO and the cHH of the eyestalk against which the antiserum was raised. However, whilst the staining of cHH-immunoreactive structures was inadequate for photography, careful observation suggested that the neural architecture for cHH- and CPRP-stained structures was strikingly similar.

Immunoreactivity to CPRP was restricted to the eyestalk ganglia and POs. However, in contrast to the SGs, which had a store of about 100 pmol CPRP per SGE (Fig. 2), PO extracts contained only between 2 and 4 pmol CPRP per organ equivalent. The authenticity of the PO CPRP was verified by RIA of HPLC fractions of PO acetic acid extracts (Fig. 4). The retention time of the CPRP-immunoreactive material and the dilution characteristics (Fig. 4, inset) of this fraction corresponded to those from authentic CPRP. The cHH RIA failed to convincingly detect immunoreactivity in the POs.

Quantification of cHH and CPRP from eyestalk extracts showed also that, despite considerable individual variation, the ratio of CPRP and cHH was approximately 1:1. Moreover, the left and right SGs contained approximately equal quantities of each peptide (Table 1).

Incubation of eyestalk ganglia in saline containing an elevated concentration of potassium ions resulted in marked increases in release of CPRP and cHH ($P<0.05$ and 0.02, respectively, t-test, Fig. 5).

Whilst considerable variation occurred between ganglia, the release of CPRP and cHH appeared to conform to a 1:1 stoichiometry. In the calcium-free conditions the release of CPRP and cHH was markedly reduced. A statistically significant increase in peptide release was observed in the "Ca^{2+}-free" and "normal 2" conditions, although this is likely to be attributable to slow exchange of media. Furthermore, the amount of peptide detected was approaching the lower limits of the assay and differences measured may be due to assay variability.

In view of the abundance of CPRP in the SG, experiments were performed in order to establish whether CPRP is released into circulation. Given that cHH is released in response to stresses such as hypoxia and emersion (Keller and Orth 1990; Webster 1996), a suitable ex-

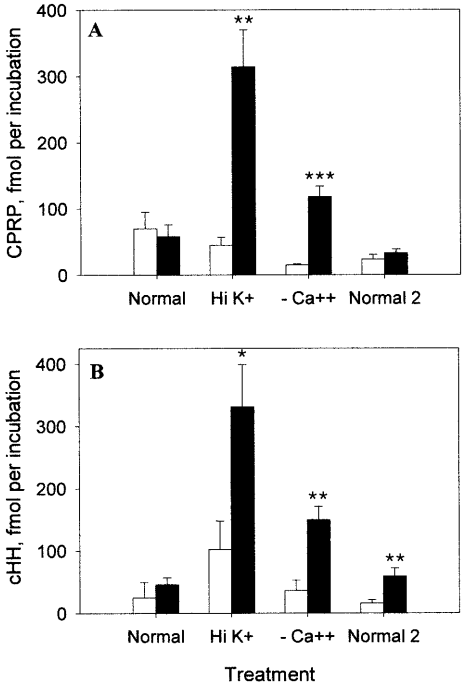

Fig. 5 Bar graphs to show the release of **A** CPRP and **B** cHH from isolated eyestalk ganglia depolarized with elevated potassium ion concentrations. Eyestalks were incubated sequentially in normal saline (low K^+, 10 mM), Hi K^+ (100 mM), Hi K^+, calcium free (-Ca^{2+}) (containing 20 mM EDTA) and the media assayed by RIA. Treatment with Hi K^+ resulted in a statistically significant release of both peptides compared with control superfusates taken from ganglia incubated in normal saline. Calcium-free, and a second incubation in low-K^+, saline ("normal 2") resulted in release of CPRP and cHH, and whilst statistically significant the concentration of peptides in the media was approaching the lower sensitivity levels of the assay (*filled bars* treatment, *open bars* control, **P*<0.05, ***P*<0.02, ****P*<0.001, *bars* SEMs); *n*=9

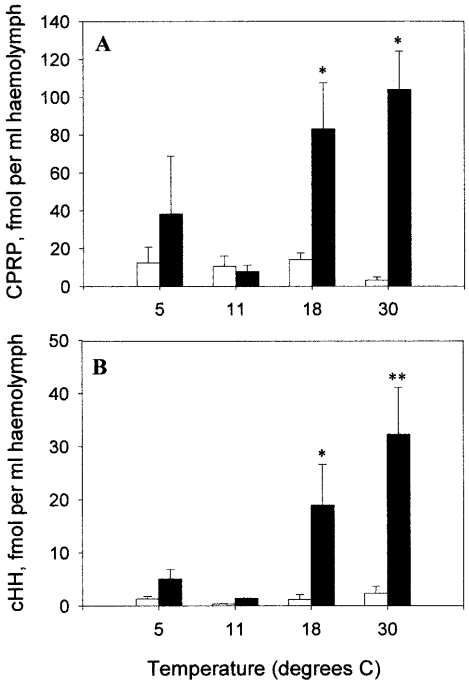

Fig. 6 Bar graphs to show the haemolymph titres of **A** CPRP and **B** cHH in *C. pagurus* following emersion in water held at different temperatures. Increases in water temperature above ambient (11°C) resulted in marked increases in titres of both peptides measured by RIA. Note that cHH titres were consistently lower than CPRP (*filled bars* treatment, *open bars* control, **P*<0.05, ***P*<0.01, *bars* SEM); *n*=6

perimental stress was required to evoke the release of the peptides. Increased water temperature proved highly effective as a stressor and crabs subjected to elevated water temperatures showed dramatic increases in the blood titres of both CPRP and cHH (Fig. 6). The release of CPRP and cHH occurred in a temperature-dependent fashion. Typically, the titres of CPRP at ambient seawater temperature were in the range 20–30 fmol/ml blood, increasing to a maximal response of 100–150 fmol/ml during thermal stress. Interestingly, the amount of cHH measured was much lower, approximately by a factor of 5.

Blood taken from thermally stressed crabs and separated by HPLC showed CPRP- and cHH-immunoreactive fractions at 38 and 51 min, respectively, consistent with the retention times of SG CPRP and cHH (Fig. 7). Dilution curves of blood CPRP showed parallelism to authentic SG-CPRP (Fig. 7, inset).

Injection-recovery experiments have previously shown the half-life of circulating cHH in *C. pagurus* to be approximately 5–10 min (Webster 1996). Using an identical approach in the present study, estimates of CPRP longevity in circulation indicated a half-life of around 60 min for this peptide (Fig. 8).

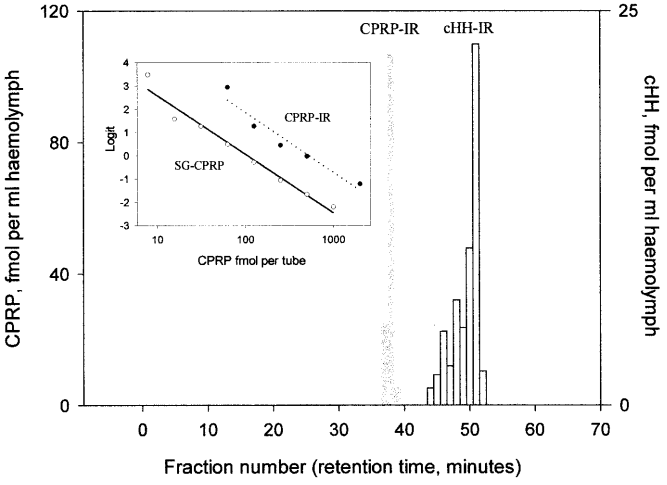

Fig. 7 HPLC separation of a large haemolymph sample taken from a thermally stressed crab. *Bars* show fractions immunoreactive to CPRP (*filled bars*) and cHH (*open bars*) at 38 and 51 min, respectively. The dilution characteristics of the major CPRP-IR fraction show parallelism to the SG-CPRP standard (*inset*)

Table 2 shows RIA measurements of CPRP from blood held ex vivo during which peptide titres remained unchanged for a 5-h period, providing further evidence that the peptide is refractory to degradation by other blood factors.

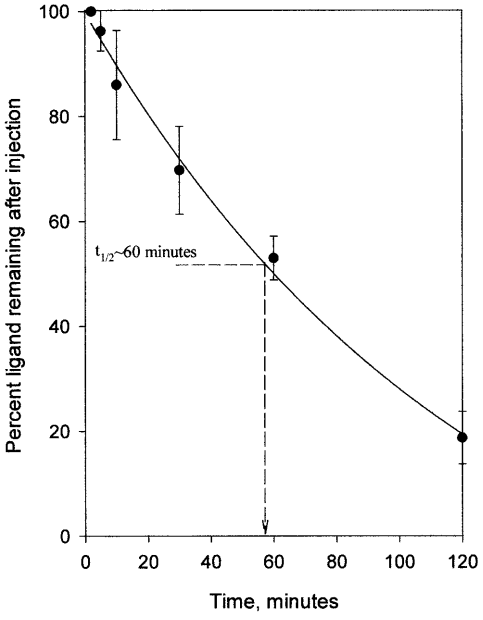

Fig. 8 CPRP immunoreactivity recovered from the blood of eyestalk-ablated *C. pagurus* following injection of 10 pmol CPRP and cHH. Recovery is expressed as a percentage of the initial level. The first sample was taken 2 min after injection to ensure even dispersal of the peptide bolus. These data suggest that the half-life ($t_{1/2}$) of CPRP in the circulating blood of *C. pagurus* is approximately 60 min (*bars* SEMs); *n*=5

Table 2 Experiment to determine possible degradation of CPRP and cHH in haemolymph

Sample time (h)	Concentration of peptide (fmol/ml)					
	Crab 1		Crab 2		Crab 3	
	CPRP	cHH	CPRP	cHH	CPRP	cHH
0	136	11	59	5	100	5
1	108	17	77	13	130	8
2	112	20	73	14	137	11
3	102	19	67	15	144	12
4	112	18	76	15	132	11
5	100	14	88	16	130	11
Mean	112	15	128	11.3	129	9.7
±SEM	5.3	2.1	4.0	2.3	6.2	1.1

However, it was recognized that antiserum R136 may recognize epitopes presented on fragmented degradation products of the injected peptide. Radioimmunoassay of HPLC fractions of blood samples taken from crabs following injection of 10 pmol CPRP and cHH showed peak immunoreactivity at 38 and 51 min, respectively (Fig. 9). Since no difference in elution time between the immunoreactive material and the known retention times for the peptides was observed, it is likely that the materials recovered were intact cHH and CPRP.

No CPRP was detected in blood taken from eyestalk-ablated control animals (not shown).

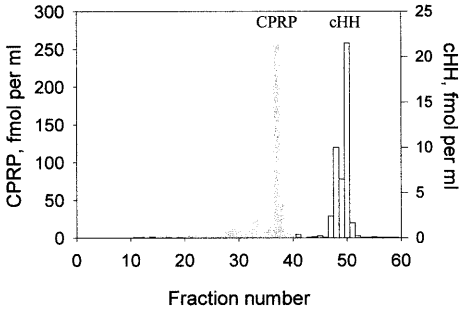

Fig. 9 Histogram to show CPRP and cHH immunoreactivity in HPLC fractions of haemolymph from a crab injected with CPRP and cHH 60 min prior to sampling. Immunoreactivity is restricted to single fractions consistent with authentic CPRP and cHH, suggesting that recovered peptides were intact

Discussion

In the classical sense, a hormone is a molecule secreted from a gland into the circulation and transported to a target organ, where it has a physiological effect. The present study represents the first attempt to define a CPRP as a neurohormone. Results reported here show that CPRP is synthesized in the MTXO, is secreted from the neurohemal SG and subsequently circulates in the haemolymph. At least in these respects, CPRP fulfils important criteria for hormone nature.

Antisera raised against HPLC purified CPRP conjugated to bTg proved suitable for use in immunochemical techniques including ICC and RIA. Comparative immunocytochemical studies showed that CPRP and cHH are colocalized throughout the MTXO-SG system. This conclusion was based on the identical staining patterns of cHH- and CPRP-immunopositive structures identified in alternate sections of eyestalk ganglia. The morphology of CPRP- and cHH-immunopositive cells was consistent with previously described cHH cells in other brachyuran and astacuran species (Dircksen et al. 1988; Gorgels-Kallen 1985; Gorgels-Kallen and Van Herp 1981; Gorgels-Kallen and Voorter 1984; Keller 1992; Marco and Gäde 1999). It was observed also that CPRP and cHH immunostaining of the cytoplasm appeared granular. It has been suggested previously that these staining patterns indicate aggregations of immunopositive material around the trans-Golgi and reflect a state of high synthetic activity (Gorgels-Kallen and Voorter 1984; Kallen et al. 1988). In this respect, the granular staining pattern of the cHH-immunoreactive cells in *C. pagurus* is consistent with the notion that this peptide plays a critical role in many aspects of crustacean physiology and reflects the importance of cHH within the eyestalk neuropeptide inventory. The CPRP staining in the same cells also displayed granularity, suggesting that cHH and CPRP colocalize at the site of packaging and transport from the perikarya. Furthermore, staining of the SG indicates that CPRP, as with cHH, appears to be transported through the axon tract for storage and release at the SG. This result is consistent with the findings of Stuenkel

(1983), who used pulse-chase analysis to show that cHH and peptide H (CPRP) undergo axonal transport to the SG from the MTXO.

Electron-microscopic double-immunogold staining of SG material provided conclusive evidence of the complete colocalization of CPRP and cHH within the axon terminals. The labelling of identical granules with both gold conjugates demonstrated that both antigens were contained within the same granules. The packaging and transport of cHH and CPRP within the same neurosecretory granules suggests that, following release of the vesicle content, both peptides would circulate in the haemolymph.

Whilst the results of the ICC may appear facile (considering previous studies on cHH localization and the fact that CPRP is synthesized as part of the cHH preprohormone), it was considered necessary to identify the spatial relationship of cHH and CPRP throughout the MTXO-SG axis. This is particularly relevant considering the potential for differential packaging and transportation of peptide hormone precursor products as has been shown in other invertebrate neurohemal systems (Fisher et al. 1988; Sossin et al. 1990b). The present ICC study has shown that CPRP persists throughout the MTXO-SG axis and apparently is not degraded or differentially packaged following synthesis.

Whole-mount ICC of *C. pagurus* nervous tissues indicated that CPRP immunoreactivity is restricted to the eyestalk ganglia and the POs. Assuming that cHH and CPRP invariantly occur together throughout the nervous system of crabs, the CPRP immunoreactivity detected in the POs vindicates previous findings of cHH in these organs (Dircksen and Heyn 1998; Keller et al. 1992). The synthesis of PO-CPRP appears to occur in three or four large (80 μm) somata located in the ventral side of the anterior bar. These cells give rise to an extensive network of axonal arborizations, which ramify throughout the organs and terminate distally in putative neurosecretory endings at the surface of the posterior bar and locally in the anterior bar. Whilst CPRP staining intensity permitted clear representation of the CPRP-immunoreactive neuroarchitecture, cHH staining was very poor. However, in view of the colocalization of CPRP and cHH in the MTXO-SG axis, it is not unreasonable to assume that cHH and CPRP are colocalized in the PO.

Interestingly, in *C. pagurus*, immunoreactivity was not detected by ICC or RIA in the thoracic ganglia. Moreover, the segmental nerves appeared free of CPRP immunoreactivity. Previous reports of cHH and CPRP immunoreactivity in the POs of *C. maenas* suggest that about 24 cHH producing perikarya in the POs project axons into the dorsolateral edge of the thoracic ganglia via the segmental nerves leading into the POs (Dircksen 1998; Dircksen et al. 2001). In *C. maenas*, up to 24 cHH-immunoreactive neurons have been found in the POs. Clearly, the morphology of CPRP/cHH neuroarchitecture in extra-eyestalk nerves differs between these species.

Quantification of cHH and CPRP indicated that both peptides occur in the left and right sides of the MTXO-SG axes in approximately equal amounts. This result may seem unsurprising considering evidence from cDNA cloning studies of *C. maenas* SG cHH that suggest the preprohormone transcript encodes for CPRP and cHH in a 1:1 ratio (Weidemann et al. 1989). However, as alluded to earlier, many invertebrate preprohormone products undergo post-translational modification such as differential degradation and differential packaging which leads to alterations in the ratio of precursor products, perhaps as a means by which hormonal effects are modulated, or in order to allow the differential release of peptides liberated from a common precursor (Fisher et al. 1988; Sossin et al. 1990a; Sossin et al. 1990b). As such, it was considered important to establish the quantitative relationship between cHH and CPRP in the neurohaemal SG since changes in the ratio or release of cHH to CPRP may have provided insights as to the respective roles of each prepro-cHH product. However, in vitro depolarization of eyestalk ganglia showed a calcium-dependent release of both peptides in an approximately 1:1 ratio. These data, taken together with the electron-microscopic study and RIA quantification of the peptides in the SG, confirm that differential degradation and packaging of cHH and CPRP does not occur in the MTXO-SG axis of *C. pagurus*.

In attempting to define CPRP as a neurohormone, the demonstration of its circulation is of primary importance and in this regard the measurement of authentic CPRP in the blood and its release during periods of thermal stress represent critical findings. Measurements of blood CPRP and cHH following thermal stress showed that CPRP and cHH blood titres increase in a temperature-dependent fashion. Interestingly, whilst the in vitro release of cHH and CPRP from the SGs occurred in approximately equal quantities, the blood titres of cHH were in five-fold deficit to those of CPRP. Further investigation of the half-life of CPRP revealed that the peptide has remarkable longevity in circulation (approximately 60 min) when compared with cHH. It was shown also that, in terms of their HPLC retention times and RIA Logit-log dilution curve parallelism, the recovered peptides were intact cHH and CPRP and not immunoreactive fragments of degraded peptide. Furthermore, ex vivo pools of blood drawn from thermally stressed crabs showed consistent levels of CPRP throughout a 5-h period, indicating that degradation of the peptides was not occurring as a result of blood-borne peptidase activity. The resistance of cHH to peptidase activity in the blood has been remarked upon previously (Webster 1996). However, that CPRP appears to be refractory to degradation in the haemolymph is surprising. The major isoform of cHH in brachyurans is blocked by pyroglutamate and amidation at the amino and carboxyl termini, respectively (Chung et al. 1998), features that are thought to confer resistance to exopeptidase activity. In contrast, CPRP, which lacks terminal blockade (Chung et al. 1998), would appear susceptible to degradation.

Detection of CPRP in nervous tissues other than the eyestalks was restricted to the POs. This was in accord with the ICC results and previous cHH localization studies in other arthropod species (Dircksen and Heyn 1998; Keller et al. 1992, 1985). However, no immunoreactivity

was seen in the thoracic or suboesophageal ganglia as has been found in lobsters (Chang et al. 1999). As alluded to earlier, the HPLC retention time of PO-CPRP-immunoreactive material corresponds exactly with SG-CPRP (Fig. 4), and Logit-log transformations of PO-CPRP dilution curves show parallelism to the SG peptide standard curves (Fig. 4, inset). These results provide strong evidence that the PO-CPRP of *C. pagurus* is very similar, if not identical, to the CPRP from the SGs as has been shown also in *C. maenas* by molecular cloning (Dircksen et al. 2001). Application of crude acetic acid extracted PO material and HPLC fractions of PO showed that each PO of a large, intermoult male crab contained approximately 2–4 pmol CPRP. As such, the amount of CPRP in the PO represents <5% of the SG-CPRP content. Results from whole-mount ICC showed putative release sites on the PO surface. However, considering the small quantity of CPRP in the POs (relative to the SG), it seems improbable that these organs have the capacity to contribute sufficient quantities of peptide into circulation to change the haemolymph CPRP titre – the amount of CPRP released from the SGs of *C. pagurus* during emersion stress represents ~10% of the total SG-stored content, which is more than 5 times the content of a single PO. Moreover, CPRP was not detected in the blood after the removal of the eyestalks, even during periods of considerable thermal stress. In *C. maenas* the release of PO-cHH has been demonstrated in vitro although a functional role of PO-cHH has yet to be defined (Dircksen et al. 2001). As has been shown here for CPRP in *C. pagurus*, the quantity of cHH in the POs of *C. maenas* is less than 5–10% that of the SGs and it is possible that these peptides act locally rather than systemically.

A recent important finding regarding the nature of PO-cHH in *C. maenas* (Dircksen et al. 2001) has shown that expression of this peptide arises from alternative splicing: whilst the first 40 residues are identical to SG-cHH, the sequence of the C-terminal portion of this molecule (residues 41–72) is largely different to that of SG-cHH. The poor recognition of the antiserum to cHH in the PO in the present study is presumably indicative of a similar splicing arrangement in *C. pagurus*.

The present paper demonstrates the localization, quantification, release and subsequent circulation of CPRP in the crab *C. pagurus*. In addition, the relative ratios of CPRP:cHH have been established throughout the MTXO-SG axis and in circulation following release from the SG. The deficit of cHH in relation to CPRP following release into the haemolymph reflects the rather rapid in vivo clearance of this peptide, relative to CPRP, which is cleared extremely slowly. Since hormone signals must be integrated to effect proper, relevant biological responses to appropriate signals, the long half-life of CPRP is rather puzzling, and despite the (presumably) considerable metabolic cost of transcription and translation of CPRP, this feature might argue against a hormonal role for this peptide: If there is no biological function, then there is little selection pressure to provide mechanisms for rapid removal and degradation, and its presence in the haemolymph only reflects a co-release with cHH! Nevertheless, the results presented in this paper represent a foundation upon which further work can be performed in an effort to establish a function of CPRP.

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References

Ando S, Murthy ASN, Eipper BA, Chaiken IM (1988) Effect of neurophysin on enzymatic maturation of oxytocin from its precursor. J Biol Chem 263:769–775

Andrew RD, Shivers RR (1976) Ultrastructure of neurosecretory granule exocytosis by crayfish sinus gland induced with ionic manipulations. J Morphol 150:253–278

Castany J, Van Herp F, Charmantier G, Trilles J-P, Charmantier-Daures M (1997) Ultrastructural study of the neurosecretory granules and crustacean hyperglycemic hormone (CHH) immunolocalization in the sinus gland of *Homarus gammarus*. J Crust Biol 17:6–12

Chang ES, Chang SA, Beltz BS, Kravitz EA (1999) Crustacean hyperglycemic hormone in the lobster nervous system: localization and release from cells in the subesophageal ganglion and thoracic second roots. J Comp Neurol 414:50–56

Chung JS, Wilkinson MC, Webster SG (1998) Amino acid sequences of both isoforms of crustacean hyperglycemic hormone (CHH) and corresponding precursor-related peptide in *Cancer pagurus*. Regul Pept 77:17–24

Chung JS, Dircksen H, Webster SG (1999) A remarkable, precisely timed release of hyperglycemic hormone from endocrine cells in the gut is associated with ecdysis in the crab *Carcinus maenas*. Proc Natl Acad Sci U S A 96:13103–13107

Davey ML, Hall MR, Willis RH, Oliver RW, Thurn MJ, Wilson KJ (2000) Five crustacean hyperglycemic family hormones of *Penaeus monodon*: complementary DNA sequence and identification in single sinus glands by electrospray ionization-Fourier transform mass spectrometry. Mar Biotech 2:80–91

De Kleijn DPV, Van Herp F (1995) Molecular biology of neurohormone precursors in the eyestalk of Crustacea. Comp Biochem Physiol 112:573–579

De Kleijn DPV, Janssen KPC, Martens GJM, Van Herp F (1994) Cloning and expression of two crustacean hyperglycemic-hormone mRNAs in the eyestalk of the crayfish *Orconectes limosus*. Eur J Biochem 224:623–629

De Kleijn DPV, De Leeuw EPH, Van den Berg MC, Martens GJM, Van Herp F (1995) Cloning and expression of two mRNAs encoding structurally different crustacean hyperglycemic hormone precursors in the lobster *Homarus americanus*. Biochem Biophys Acta 1260:62–66

Dircksen H (1992) Fine structure of the neurohemal sinus gland of the shore crab, *Carcinus maenas*, and immuno-electron-microscopic identification of neurosecretory endings according to their neuropeptide contents. Cell Tissue Res 269:249–266

Dircksen H (1998) Conserved crustacean cardioactive peptide neural networks and functions in arthropod evolution. In: Coast GM, Webster SG (eds) Recent advances in arthropod endrocrinology. Cambridge University Press, Cambridge, pp 302–333

Dircksen H, Keller R (1988) Immunocytochemical localization of CCAP, a novel crustacean cardioactive peptide, in the nervous system of the shore crab, *Carcinus maenas* L. Cell Tissue Res 254:347–360

Dircksen H, Heyn U (1998) Crustacean hyperglycemic hormone-like peptides in crab and locust peripheral intrinsic neurosecretory cells. Ann N Y Acad Sci U S A 839:392–394

Dircksen H, Zahnow CA, Gaus G, Keller R, Rao KR, Riehm JP (1987) The ultrastructure of nerve endings containing pigment-dispersing hormone (PDH) in crustacean sinus glands: identification by an antiserum against a synthetic PDH. Cell Tissue Res 250:377–387

Dircksen H, Webster SG, Keller R (1988) Immunocytochemical demonstration of the neurosecretory systems containing putative moult-inhibiting hormone and hyperglycemic hormone in the eyestalk of brachyuran crustaceans. Cell Tissue Res 251:3–12

Dircksen H, Böcking D, Heyn U, Mandel C, Chung JS, Baggerman G, Verhaert P, Daufeldt S, Plösch T, Jaros PP, Waelkens E, Keller R, Webster SG (2001) Crustacean hyperglycemic hormone (CHH)-like peptides and CHH-precursor related peptides from pericardial organ neurosecretory cells in the shore crab, *Carcinus maenas*, are putatively spliced and modified products of multiple genes. Biochem J 356:159–170

Fischer-Lougheed J, O'Shea M, Cornish I, Losberger C, Roulet E, Schulz-Aellen F (1993) AKH biosynthesis: transcriptional and translational control of two co-localised prohormones. J Exp Biol 177:223–241

Fisher JM, Sossin W, Newcombe R, Scheller RH (1988) Multiple neuropeptides derived from a common precursor are differentially packaged and transported. Cell 54:813–822

Giulianini PG, Smullen RP, Bentley MG, Ferrero EA (1998) Cytological and immunocytochemical study of the sinus gland in the Norway lobster *Nephrops norvegicus* (L.) Invertebr Reprod Dev 33:57–68

Gorgels-Kallen JL (1985) Appearance and innervation of CHH-producing cells in the eyestalk of the crayfish *Astacus leptodactylus* examined after tracing with Lucifer yellow. Cell Tissue Res 240:385–391

Gorgels-Kallen JL, Van Herp F (1981) Localization of crustacean hyperglycemic hormone (CHH) in the X-organ sinus gland complex in the eyestalk of the crayfish, *Astacus leptodactylus* (Nordmann, 1842). J Morphol 170:347–355

Gorgels-Kallen JL, Voorter EM (1984) Secretory stages of individual CHH-producing cells in the eyestalk of the crayfish *Astacus leptodactylus*, determined by means of immunocytochemistry. Cell Tissue Res 237:291–298

Gu P-L, Chan S-M (1998) The shrimp hyperglycemic hormone-like neuropeptide is encoded by multiple copies of genes arranged in a cluster. FEBS Lett 441:397–403

Gu P-L, Yu K, Chan S-M (2000) Molecular characterization of an additional shrimp hyperglycemic hormone: cDNA cloning, gene organization, expression and biological assay of recombinant proteins. FEBS Lett 472:122–128

Hekimi S, O'Shea M (1989) Biosynthesis of adipokinetic hormones (AKHs): further characterization of precursors and identification of novel products of processing. J Neurosci 9:996–1003

Kallen JL, Rigiani NR, Trmpenaars HJAJ (1988) Aspects of entrainment of CHH cell activity and haemolymph glucose levels in crayfish. Biol Bull 175:137–143

Keller R (1992) Crustacean neuropeptides: structures, functions and comparative aspects. Experientia 48:439–448

Keller R, Orth H-P (1990) Hyperglycemic neuropeptides in crustaceans. In: Epple A, Scanes GC, Stetson MH (eds) Progress in comparative endocrinology, Wiley-Liss, New York, pp 265–271

Keller R, Jaros PP, Kegel G (1985) Crustacean hyperglycemic neuropeptides. Am Zool 25:207–221

Khayat M, Yang W-J, Aida K, Nagasawa H, Tietz A, Funkenstein B, Lubzens E (1998) Hyperglycaemic hormones inhibit protein and mRNA synthesis in *in vitro*-incubated ovarian fragments of the marine shrimp *Penaeus semisulcatus*. Gen Comp Endocrinol 110:307–318

Lacombe C, Grève P, Martin G (1999) Overview of the sub-grouping of the crustacean hyperglycemic hormone family. Neuropeptides 33:71–80

Marco HG, Gäde G (1999) A comparative immunocytochemical study of the hyperglycaemic, moult-inhibiting and vitellogenesis-inhibiting neurohormone family in three species of decapod Crustacea. Cell Tissue Res 295:171–182

Newcombe RW (1987) Amino acid sequences of neuropeptides in the sinus gland of the land crab *Cardisoma carnifex*: a novel neuropeptide proteolysis site. J Neurochem 49:1–10

Nordmann JJ (1977) Ultrastructural appearance of neurosecretory granules in the sinus gland of the crab after different axon fixation procedures. Cell Tissue Res 185:557–563

Santos EA, Nery LEM, Keller R, Gonçalves AA (1997) Evidence for the involvement of crustacean hyperglycemic hormone in the regulation of lipid metabolism. Physiol Zool 70:415–420

Sedlmeier D (1988) The crustacean hyperglycemic hormone releases amylase from the crayfish midgut gland. Regul Pept 20:91–98

Shin SH, Heisler RL, Lee CSS (1995) Neurophysin stimulates prolactin release from primary cultured rat pituitary cells. J Endocrinol 144:225–231

Shivers RR (1976) Exocytosis of neurosecretory granules from the crustacean sinus gland in freeze fracture. J Morphol 150:227–252

Silverthorn U (1975) Neurosecretion in the sinus gland of the fiddle crab, *Uca pugnax*. Cell Tissue Res 165:129–133

Skowsky WR, Fisher DA (1972) The use of thyroglobulin to induce antigenicity to small molecules. J Lab Clin Med 80:134–144

Smith G (1974) The ultrastructure of the sinus gland of *Carcinus maenas* (Crustacea: Decapoda). Cell Tissue Res 155:117–125

Sossin WS, Fisher JM, Scheller RH (1990a) Sorting within the regulated secretory pathway occurs in the *trans*-Golgi network. J Cell Biol 110:1–12

Sossin WS, Sweet-Cordero A, Scheller RH (1990b) Dale's hypothesis revisited: different neuropeptides derived from a common prohormone are targeted to different processes. Proc Natl Acad Sci U S A 87:4845–4848

Spanings-Pierrot C, Soyez D, Van Herp F, Gompel M, Skaret G, Grousset E, Charmantier G (2000) Involvement of crustacean hyperglycemic hormone in the control of gill ion transport in the crab *Pachygrapsus marmoratus*. Gen Comp Endocrinol 119:340–350

Stefanini M, DeMartino C, Zamboni L (1967) Fixation of ejaculated spermatozoa for electron microscopy. Nature 216:173–174

Sternberger LA (1979) The unlabelled antibody peroxidase-anti-peroxidase (PAP) method. Immunocytochemistry, chap 5, 2nd edn. Wiley Medical, pp 104–169

Stuenkel EL (1983) Biosynthesis and axonal transport of proteins and identified peptide hormones in the X-organ sinus gland neurosecretory system. J Comp Physiol 153:191–205

Tarr GE (1986) Manual Edman sequencing system. In: Shively JE (ed) Methods of protein microcharacterization: a practical handbook. Humana Press, New Jersey, pp 155–194

Tensen CP, De Kleijn DVP, Van Herp F (1991) Cloning and sequence analysis of cDNA encoding two crustacean hyperglycemic hormones from the lobster *Homarus americanus*. Eur J Biochem 200:103–106

Van Herp F (1998) Molecular, cytological and physiological aspects of the crustacean hyperglycemic hormone family. In: Coast GM, Webster SG (eds) Recent advances in arthropod endrocrinology. Cambridge University Press, Cambridge, pp 53–70

Wahren J, Johansson B-L, Wallberg-Henriksson H, Linde B, Fernqvist-Forbes E, Zierath JR (1996) C-peptide revisited – new physiological effects and therapeutic implications. J Int Med 240:115–124

Weatherby TM (1981) Ultrastructural study of the sinus gland of the crab, *Cardisoma carnifex*. Cell Tissue Res 220:293–312

Webster SG (1991) Amino acid sequence of putative moult-inhibiting hormone from the crab *Carcinus maenas*. Proc R Soc Lond 244:247–252

Webster SG (1996) Measurement of crustacean hyperglycemic hormone levels in the edible crab *Cancer pagurus* during emersion stress. J Exp Biol 199:1579–1585

Webster SG (1998) Neuropeptides inhibiting growth and reproduction in crustaceans. In: Coast GM, Webster SG (eds) Recent advances in arthropod endocrinology. Cambridge University Press, Cambridge, pp 33–53

Weidemann W, Grommoll J, Keller R (1989) Cloning and sequence analysis of cDNA for precursor of a crustacean hypergylcemic hormone. FEBS Lett 257:31–34