

Binding sites of crustacean hyperglycemic hormone and its second messengers on gills and hindgut of the green shore crab, *Carcinus maenas*: A possible osmoregulatory role

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Abstract

To determine the possible involvement of crustacean hyperglycemic hormone (CHH) in osmoregulation in crustaceans, ligand binding and second messenger assays were performed on gills and hindgut preparations of the green shore crab *Carcinus maenas*, whilst midgut gland, previously known as one of the target tissues of CHH served as a control tissue. Classical receptor binding analyses using [¹²⁵I]CHH by saturation and displacement experiments from membrane preparations from gills, hindgut, and midgut glands demonstrated that CHH binding characteristics involved one site, highly specific, saturable, and displaceable kinetics: (gills: K_D $5.87 \pm 2.05 \times 10^{-10}$ and B_{MAX} $6.50 \pm 1.15 \times 10^{-10}$, hindgut: K_D $3.54 \pm 1.49 \times 10^{-10}$ and B_{MAX} $2.31 \pm 0.44 \times 10^{-10}$, and midgut gland: K_D $7.28 \pm 0.9 \times 10^{-10}$ and B_{MAX} $3.28 \pm 0.25 \times 10^{-10}$) all expressed as M/mg protein. No differences, in terms of displacement were observed between the two CHH isoforms (N-terminally blocked pGlu and unblocked Gln) variants. CHH binding sites appeared to be coupled to a second messenger system involving cGMP in all the tissues examined. Exposure of crabs to dilute seawater increased levels of cGMP, glucose in gills and circulating CHH levels. Other crustacean neuropeptides including crustacean cardioactive peptide, molt inhibiting hormone, L-enkephalin, FMRF-amide, proctolin, and crustacean hyperglycemic hormone precursor-related peptide were tested with regard to possible osmoregulatory roles with reference to changes in second messenger (cAMP and cGMP) concentrations in gill, hindgut, and midgut tissues in vitro, following application at 2×10^{-8} M but all were found to be inactive. Thus, it seems likely that CHH is a pertinent neurohormone involved in osmoregulation, thus expanding its many functions as a pleiotropic hormone in crustaceans. © 2006 Elsevier Inc. All rights reserved.

Keywords: Crustacean hyperglycemic hormone; Binding sites; Hyperglycemia; Cyclic GMP; Cyclic AMP; Second messenger; Osmoregulation

1. Introduction

During the past decade, it has become apparent that several members of the structurally related crustacean hyperglycemic hormone (CHH) family have roles other than those originally described in many crustaceans (Böcking et al., 2002; Chan et al., 2003). The prototype—crustacean hyperglycemic hormone (CHH) illustrates this point; while CHH has a principal role as an adaptive hormone involved in energy

mobilization, several other physiologically relevant functions have been defined, including inhibition of ecdysteroid (Chung and Webster, 2003; Webster, 1993; Webster and Keller, 1986) and juvenoid synthesis (Liu et al., 1997), lipid metabolism (Santos et al., 1997), reproduction (Van Herp, 1998), and water uptake (Chung et al., 1999). With regard to the possible influence of CHH (and other neuropeptides) upon this process, rather little is known. Early studies, often using in vivo approaches on whole organisms, or in vitro studies, on isolated gill preparations, have indicated that a wide variety of putative neuroendocrine tissues, including the eyestalks, pericardial organs, thoracic, or cerebral ganglia might be impor-

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tant in hydromineral control (Mantel and Farmer, 1983) and that several putative neurotransmitter candidates might be centrally acting neurohormones (Zatta, 1987; Kamemoto, 1991). However, despite many reports suggesting that CNS tissues may be important in hydromineral regulation, the current consensus heavily influenced by “classical endocrinology” removal and replacement type experiments suggest that eyestalk removal or injection of eyestalk extracts implicates the eyestalk neurosecretory tissues as having a major role in osmoregulation (Eckhardt et al., 1995; Pierrot et al., 1995).

Contemporary studies have implicated eyestalk neuropeptides as key candidates in osmoregulation. Perfused gills of the strong hyper-hyporegulator, *Pachygrapsus marmoratus* show that sinus gland (SG) extracts significantly increase Na^+ and transepithelial potential (TEP) in posterior (ionoregulatory gills) in this crab (Eckhardt et al., 1995; Spanings-Pierrot et al., 2000). In eyestalkless juvenile lobsters (*Homarus americanus*), injection of SG extract rescues the loss of “osmoregulatory capacity” only following molting, suggesting that a putative candidate might be CHH (Charmantier-Daures et al., 1994). Using a similar approach in the crayfish, *Astacus leptodactylus*, injection of the D-Phe³ isoform of CHH is particularly potent in restoring hemolymph osmolarity via Na^+ uptake in postmolt crayfish, albeit at very large, non-physiological doses (Serrano et al., 2003). With regard to other roles for CHH osmoregulation, we have also shown that water uptake associated with ecdysis in *Carcinus maenas* is caused by a massive release of CHH from endocrine cells in the fore and hindgut that express CHH only during premolt. A rapid release of CHH from the gut endocrine cells just before ecdysis leads to pronounced dipsogenesis and water uptake by these tissues, leading to increase in post-molt volume (Chung et al., 1999). In this context, it should be noted that water uptake in the ileum of locusts is stimulated by ion transport peptide (ITP) which increases Cl^- transport across the rectal pads leading to water reabsorption (Phillips and Audsley, 1995; Phillips et al., 1998). This peptide is highly structurally related to CHH and appears to be widespread in insects: *Schistocerca gregaria* (gi 1233522), *Locust migratoria* (gi 4433632), *Drosophila melanogaster* (gi 21626755), *Anopheles gambiae* (gi 5838541), and *Bombyx mori* (gi 70074510). Thus, it seems likely that CHH-like peptides not only have a wide occurrence in arthropods, but may also have homologous roles (in the sense of osmoregulation) in this phylum.

As alluded to earlier, many contemporary studies suggesting that CHH might be involved in osmoregulation in crustaceans have used in vivo bioassays. Excepting the studies on perfused gills, which showed that changes in Na^+ influx and TEP occurred very rapidly following perfusion with SG extract (Eckhardt et al., 1995), no others have addressed the immediate hormonally mediated responses which would be expected following exposure to diluted medium in crustaceans (i.e., receptor binding, initiation of second messenger pathways). Since CHH is essentially an adaptive hormone, we propose that any perceived stressful change in the external environment will be immediately accounted for release of

CHH, to meet increased energy demands, as has been shown following emersion, temperature stress (Chang et al., 1998; Webster, 1996) and hypoxia (Chung and Webster, 2005). Since osmoregulatory tissues in all animals are well known to be metabolically highly active, it seems plausible to suggest that CHH might have a dual role in tissues known to be involved in osmoregulation: not only should CHH act homeostatically in regulating the ionic milieu, but there will also be a parallel role in supply of glucose to run metabolically expensive mechanisms involved in ion pumps.

To address these issues, we have firstly determined the distribution, abundance, and affinity of binding sites for CHH on proposed osmoregulatory tissues (gills and hindgut), in comparison with midgut gland, the prototypical target tissue of CHH (Kummer and Keller, 1993). Secondly, we have identified the downstream effects of CHH binding on the relevant second messengers involved in CHH signal transduction (principally cGMP). We have shown that downstream events (increases in cGMP and intracellular glucose) can be closely correlated with hypoosmotic stress, within a few minutes of exposure to diluted seawater. We have also shown that cGMP increases in target tissues are probably due to CHH. Thus, it further strengthens the developing view that CHH is an important molecule in osmoregulation in crustaceans and that its established role in energy mobilization is probably of considerable importance in energy metabolism of these tissues.

2. Materials and methods

2.1. Animals and hormone purification

Carcinus maenas were captured using baited traps from the Menai Strait and maintained in a re-circulating seawater system with ambient temperature, photoperiodic regimes and ad libitum feeding. Intermolt crabs (C_4) were used in all experiments. The procedures of native CHH isolation, purification, and quantification were described previously (Chung and Webster, 1996). In experiments using eyestalkless crabs, eyestalks were ablated bilaterally 48 h prior to use.

2.2. Receptor binding assays

Plasma membranes were prepared from gills (anterior and posterior gills), hindgut and midgut glands of male intermolt crabs ($n=10-30$). Tissues were dissected out, rinsed in ice-cold extraction buffer (140 mM NaCl, 300 mM sucrose, 10 mM Hepes, and 10 mM benzamidine (Sigma), pH 7.4) and homogenized in the same buffer using a Polytron. The homogenates were centrifuged at 1000g for 10 min at 4 °C and supernatants were re-centrifuged at 30,000g for 40 min at 4 °C. Pellets were re-suspended in incubation buffer (140 mM NaCl and 10 mM Hepes, pH 7.4) without bovine serum albumin (BSA) estimated by using a modified micro-Lowry method (Lowry et al., 1951) and membranes stored at -20°C . [¹²⁵I]CHH was prepared using a chloramine-T labelling procedure, as detailed previously (Webster, 1993). Specific activities were approximately 37 TBq/mmol. [¹²⁵I]CHH for binding assays was used within one month after iodination. The binding conditions for receptor assays were as described in Chung and Webster (1996).

2.3. Radioimmunoassays of cGMP and cAMP

Iodination procedures for [¹²⁵I]cGMP and [¹²⁵I]cAMP were described as in Goldsworthy et al. (2003). To determine the effect of CHH on cyclic nucleotide production, pieces of hindgut, gills, or midgut glands were incu-

bated for 30 min at 15 °C with 400 μ l of crustacean saline containing various concentrations of CHH (or saline alone for controls), in the presence of 0.25 mM isobutylmethylxanthine (IBMX). Following incubation, the tissues were collected in 200 μ l of ice-cold 0.1 M acetate buffer (pH 4.5), disrupted by sonication, and centrifuged at 14,000 rpm for 10 min at 4 °C. Supernatants (100 μ l) were transferred into tubes containing 900 μ l of 0.1 M acetate buffer and immediately acetylated by the addition of 20 μ l of triethylamine and 10 μ l of acetic anhydride. Fifty or one hundred microliters of acetylated samples were assayed for the estimation of cGMP and cAMP by following the method described in Goldsworthy et al. (2003).

2.4. Effects of CHH and 8-bromo cGMP on glucose production *in vitro*

Following incubation (30 min, 15 °C) of gills, hindgut, and midgut glands in crustacean saline containing 20 nM CHH and 0.25 mM IBMX, tissues were homogenized in 200 μ l of 0.1 M sodium acetate buffer (pH 4.5) and centrifuged at 14,000 rpm for 10 min at 4 °C. Glucose in incubation medium and in tissues was estimated using a glucose oxidase assay as described in Webster (1996). Further aliquots of the tissue extract were used for cGMP and protein determination as mentioned above. Possible direct effects of cGMP on the glucose increase in gills, hindgut, and midgut glands (3–7 mg wet weight) were also tested by incubating these tissues with a membrane permeable analogue, 8-bromo-cGMP (8-br-cGMP) (10^{-5} M) for 40 min at 15 °C prior to glucose estimation.

2.5. The effect of exposure to diluted seawater on cGMP production, hyperglycemia, and circulating CHH levels

Crabs ($n=6$) were immersed in diluted seawater (6 ppt) for 30 min, while control groups remained in normal seawater. Measurement of CHH in hemolymph by RIA was as described by Webster (1996). The amounts of glucose in hemolymph and gills and the level of cGMP produced in this tissue were measured.

2.6. The effect of various crustacean neuropeptides on cAMP and cGMP in gills, hindgut, and midgut glands

Possible influences of several crustacean neuropeptides on osmoregulatory processes, as measured by changes in second messenger content (cAMP and cGMP) in gills, hindgut, and midgut glands were investigated by exposure of these tissues (30 min) to 2×10^{-8} M of crustacean cardioactive peptide (CCAP, Stangier et al., 1987), L-enkephalin (Lüschen et al., 1991), FMRF-amide, proctolin (Stangier et al., 1986), crustacean hyperglycemic hormone precursor-related peptide (CPRP) (Chung et al., 1998; Wilcockson et al., 2002), and molt-inhibiting hormone (MIH) (Webster, 1991), and CHH. Tissues were assayed for cAMP and cGMP using the procedure as described above.

2.7. Data analysis and statistics

Binding data were analyzed using a radioligand binding analysis program (EBDA, Elsevier-BIOSOFT). Statistical significance was tested by Student's *t* test using InStat (Graphpad Software Inc.).

3. Results

3.1. The effect of CHH *in vitro* on the production of glucose and cGMP production

Incubation of gills, hindgut, and midgut glands in 20 nM CHH in the presence of 0.25 mM IBMX resulted in 3–10-fold increases in cGMP levels after 30 min (Fig. 1A). This related well with the significant increases in glucose concentrations in these tissues (Fig. 1B). The greatest

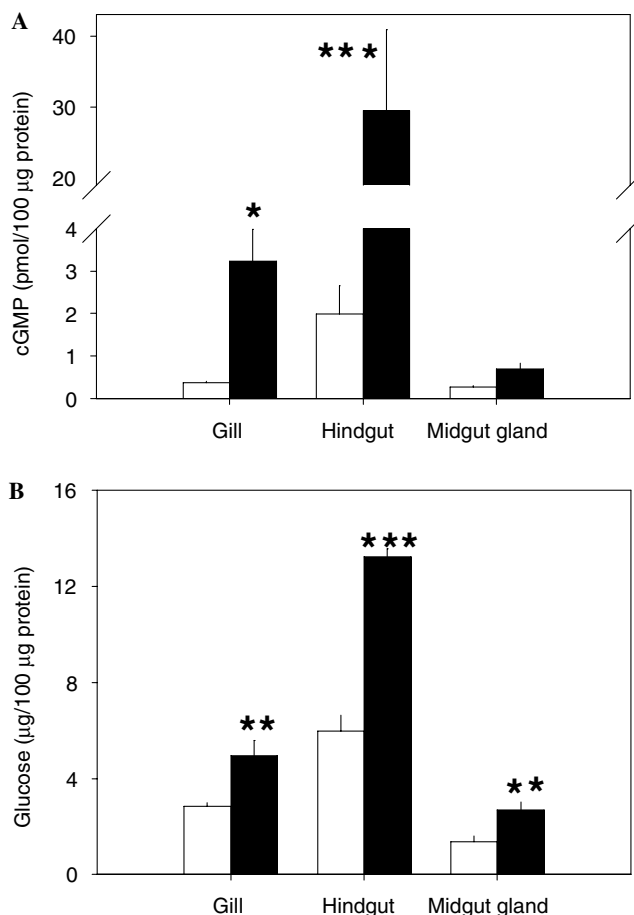


Fig. 1. Levels of cGMP (A) and glucose (B) in gill, hindgut, and midgut gland tissues *in vitro*, following 30 min incubation in saline (open bars), or 20 nM CHH (filled bars). Mean \pm 1 SE ($n=9$). Asterisks show significant differences between saline and hormone treated tissues at $p < 0.05$ (*), $P < 0.005$ (**), $P < 0.0005$ (***), and Student's *t* test.

increase in cGMP in hindgut was associated with the highest glucose levels in this tissue. Further, 8-br-cGMP at 10^{-5} M was tested on these tissues to determine whether cGMP induced by CHH is directly responsible for glucose increases. As shown in Table 1, following exposure to 8-br-cGMP, increased levels of glucose in midgut glands and hindgut were quite modest but significant ($P < 0.05$). Surprisingly, no detectable increase in glucose occurred in gills, which was in contrast to the large increases in cGMP (10-fold) observed in this tissue following exposure to CHH.

Table 1
In vitro effect of 8-br-cGMP on tissue hyperglycemia

	Glucose (μ g/100 μ g protein)	
	Control (saline)	8-br-cGMP
Midgut gland	6.59 \pm 0.41	8.42 \pm 0.53*
Gill	3.33 \pm 0.41	3.33 \pm 0.38
Hindgut	4.99 \pm 0.80	8.34 \pm 0.68*

Values are shown as means \pm 1 SE ($n=9$).

* Statistical significance by Student's *t* test at $P < 0.05$.

3.2. Analysis of CHH binding sites on gill and hindgut

To determine whether any topographical gradient existed in the gills regarding the number of binding sites for CHH per gill, membrane preparations from both anterior gills (1–4) and posterior gills (5–8) were used to determine the binding of 40 fmol [125 I]CHH (ca. 100,000 cpm) to gill membranes (100 μ g). A 100 nM excess of unlabelled CHH was used to estimate the non-specific binding. The number of specific binding sites was similar between anterior and posterior gill preparations [anterior: $3567 \pm 340/100 \mu$ g membrane protein, posterior: 3661 ± 282 cpm/100 μ g membrane protein (mean \pm 1 SE, $n = 6$)]. These results suggested that CHH binding sites were ubiquitous with regard to gill position, therefore all gills were subsequently used for membrane preparation.

Experiments to determine maximal CHH binding sites were performed using the membranes prepared from gill, hindgut, and midgut glands with concentrations of [125 I]CHH between 43 and 1869 pM. These experiments were repeated three times. All tissues exhibited saturable binding kinetics. The results of these experiments are shown in Figs. 2A–C. The calculated dissociation constants (K_D) were: gills ($5.87 \pm 2.05 \times 10^{-10}$ M/mg protein), hindgut ($3.54 \pm 1.49 \times 10^{-10}$ M/mg protein), and midgut glands ($7.28 \pm 0.90 \times 10^{-10}$ M/mg protein). The calculated number of binding sites (B_{MAX}) were: gills ($6.50 \pm 1.15 \times 10^{-10}$ M/mg protein), hindgut ($2.31 \pm 0.44 \times 10^{-10}$ M/mg protein), and midgut glands ($3.28 \pm 0.25 \times 10^{-10}$ M/mg protein). The binding sites of [125 I]CHH in these tissues were one type, high affinity, and saturable, as shown Scatchard plots (Figs. 2A–C and insets).

The determination of displacement of binding sites in gill, hindgut, and midgut glands was carried out using CHH as a competing ligand at concentrations ranging from 6×10^{-12} to 8×10^{-8} M. Additionally, the minor isoform of CHH (CHH-I), which has glutamine at the N-terminus, rather than the blocked glutamate (pyroglutamate) of the major CHH isoform (Chung and Webster, 1996) was used at the same range of concentrations. The competition curves and IC_{50} values determined in these experiments are shown in Figs. 3A and B. The results suggest that there was little difference in the binding affinities of either isoform to the receptors in tissues.

3.3. The effect of CHH on levels of cGMP production

The effects of CHH on levels of cGMP were tested in gill, hindgut, and midgut glands in vitro, the results are shown in Fig. 4. The responses of cGMP production by gills and hindgut were very much larger (ca. 3-fold) than that of the midgut glands. In the hindgut, the dose–response relationship appeared to be quite limited with regard to dose range. Additionally, this tissue displayed much higher basal levels of cGMP (350 ± 70 fmol/100 μ g protein) compared with that of gills and midgut glands (180 ± 10 and 120 ± 10 fmol/100 μ g protein, respectively).

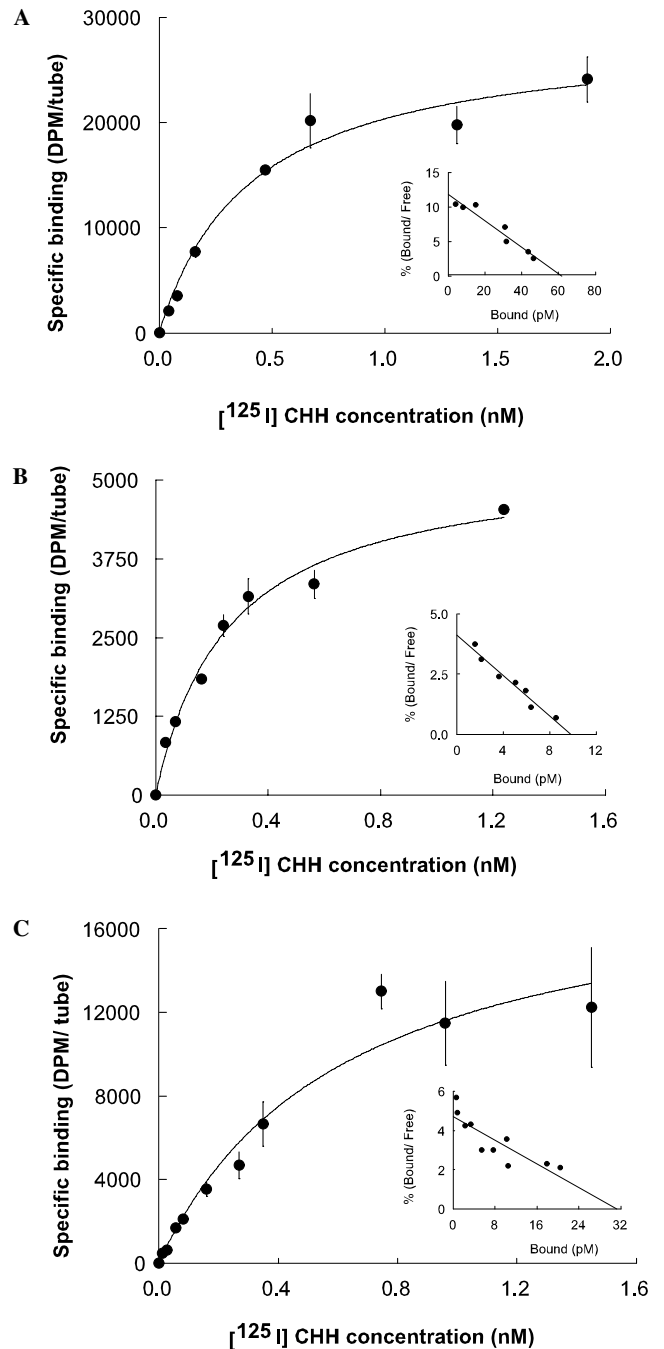


Fig. 2. Saturation curves and Scatchard plots of [125 I]CHH binding to gill (A), midgut (B), and hindgut membranes (C). (A) Specific binding is saturable with the values of K_D , 5.87 ± 2.05 and B_{MAX} , 6.50 ± 1.15 (10^{-10} M/mg protein). (B) Specific binding is saturable with the values of K_D , 3.54 ± 1.49 and B_{MAX} , 2.31 ± 0.44 (10^{-10} M/mg protein). (C). Specific binding is saturable with the values of K_D , 7.28 ± 0.90 and B_{MAX} , 3.28 ± 0.25 (10^{-10} M/mg protein). Data points are presented in A–C, as means \pm 1 SE ($n = 4$).

3.4. The effect of low salinity on levels of glucose, CHH, and cGMP from normal and eyestalk ablated animals

To establish CHH response to change in salinity, intact crabs were immersed in diluted seawater (6 ppt) for 30 min, while control groups remained in normal seawater. At the

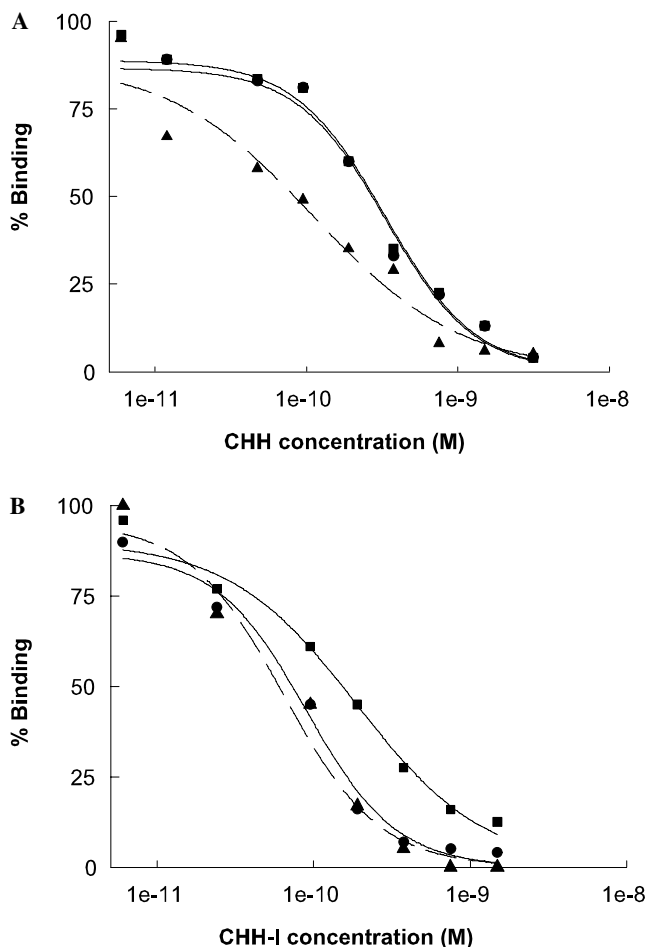


Fig. 3. Displacement curve of specific ligand binding by CHH (A) and CHH-I (B). Membranes prepared from gills (\blacktriangle), hindgut (\bullet), and midgut glands (\blacksquare) were incubated with [125 I]CHH (100,000 cpm per assay tube) and different concentrations of either CHH (A) or CHH-I (B). CHH-I differs from CHH at N-terminus, starting with glutamine, instead of pyroglutamate. Data points are the mean of four determinations. Binding sites of each different tissue were displaced with unlabeled CHH (A) and CHH-I (B).

end of exposure, hemolymph and gills were collected for estimation of the levels of CHH and glucose in hemolymph and cGMP and glucose levels in gills. Hemolymph glucose after exposure to diluted seawater for 30 min did not change; however, the amounts of both glucose and cGMP in gills increased dramatically (Table 2). In contrast to this result, exposure to dilute seawater (6 ppt, 30 min) did not increase cGMP in gills of eyestalk ablated crabs. Gills from ablated animals in normal seawater contained 124.58 ± 62.31 fmol cGMP/100 μ g protein, while those from animals in diluted seawater (6 ppt) contained 128.94 ± 31.85 fmol cGMP/100 μ g protein.

Estimation of circulating CHH levels in hemolymph exposed to dilute seawater showed that there was a slight increase in the treated group (17.5 ± 2.9 fmol/ml hemolymph, $n=6$) compared to the controls (13.5 ± 2.1 fmol/ml hemolymph, $n=6$), which was statistically insignificant. However, crabs acclimatized to 9 ppt seawater for 1 week had a higher level of CHH (26.0 ± 5.2 fmol/ml hemolymph,

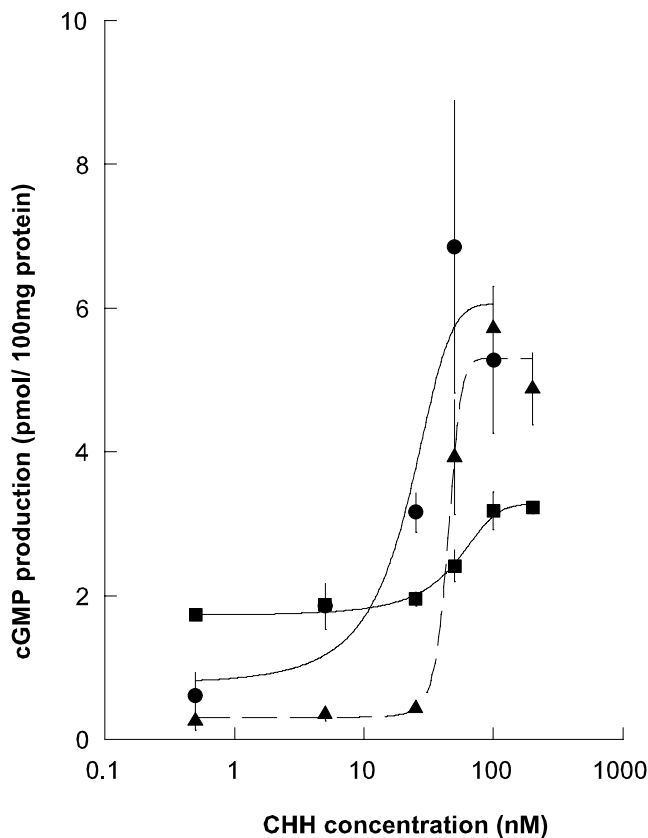


Fig. 4. Dose-response curves of the CHH effect on cGMP production by gills (\blacktriangle), hindgut (\bullet), and midgut glands (\blacksquare). Data points are expressed as pmol/100 μ g protein are shown with means \pm 1 SE ($n=4-6$).

Table 2

Effect of low salinity (6 ppt, 30 min exposure) on levels of glucose and cGMP in gills of normal and eyestalk ablated crabs

	Glucose in the gills of normal crabs (μ g/100 μ g protein)	cGMP in gills of normal crabs (fmol/100 μ g protein)	cGMP in gills of eyestalk ablated crabs (fmol/100 μ g protein)
Control	2.19 ± 1.10	66.50 ± 6.00	124.58 ± 62.31
6 ppt seawater	$5.90 \pm 0.85^*$	$154.00 \pm 2.31^{***}$	128.94 ± 31.85

Data points show means \pm 1 SE ($n=4$ or 9) for normal and ablated crabs, respectively.

* Student's t test was applied at $P < 0.05$.

*** Student's t test was applied at $P < 0.0005$.

$n=6$) than those exposed to normal seawater (11.3 ± 0.3 fmol/ml hemolymph, $n=4$).

3.5. Effects of various crustacean neuropeptides on stimulation of cAMP and cGMP in gills, hindgut, and midgut glands

Among all tested crustacean neuropeptides including CCAP, L-enkephalin, FMRF-amide, proctolin, CPRP, MIH, and CHH, CHH was the only neuropeptide to stimulate production of both cAMP and cGMP (Figs. 5A–C). In the hindgut, CHH increased the amounts of both cGMP and cAMP. However, the degree of stimulation of cAMP production was rather modest compared with that of cGMP (Fig. 5B).

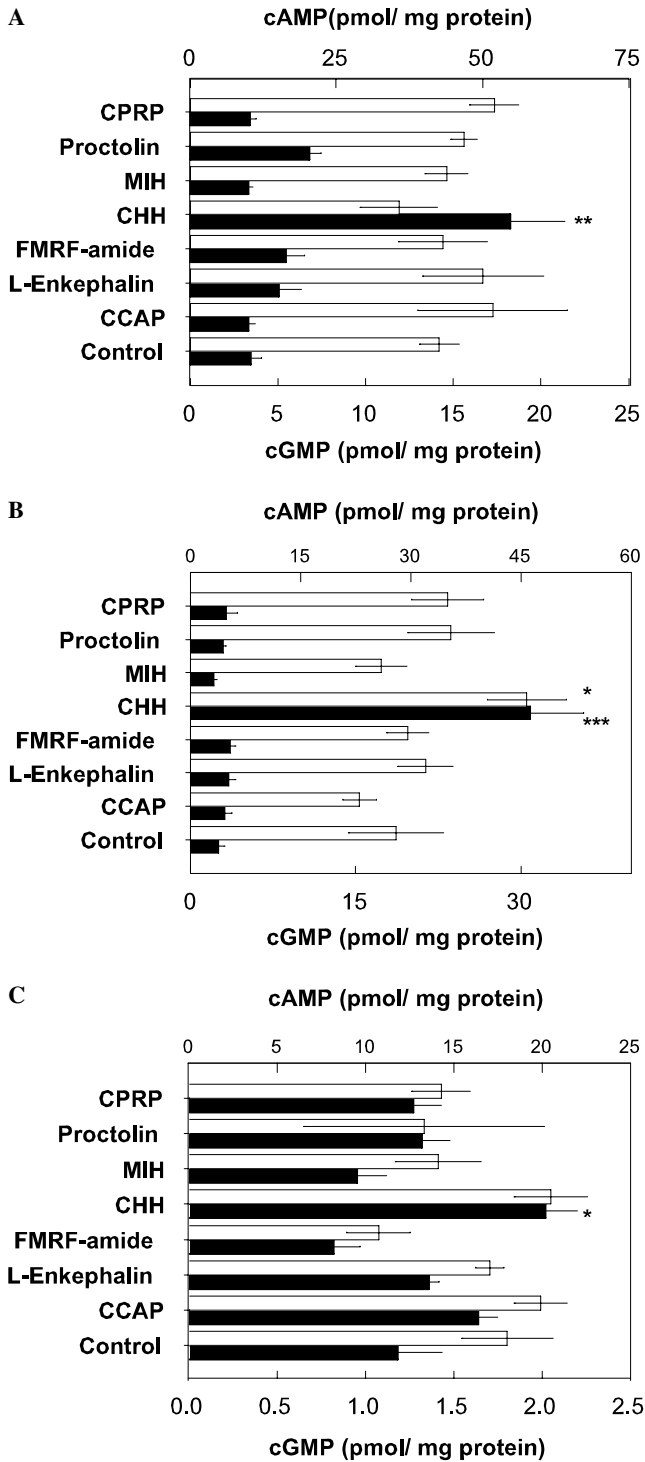


Fig. 5. Effect of various neuropeptides on cGMP (■) and cAMP (□) production by gills (A), hindgut (B), and midgut glands (C). Each peptide was tested at the concentration of 2×10^{-8} M. Data points are expressed as pmol/mg protein and shown with means \pm 1 SE ($n = 4-6$). Asterisks show significant differences between saline and hormone treated tissues at $P < 0.05$ (*), $P < 0.005$ (**), $P < 0.0005$ (***), Student's t test.

4. Discussion

Receptor binding experiments using CHH as the relevant ligand have shown for the first time that gills and hind-

gut may be important in osmoregulation in crustaceans. Binding characteristics of [125 I]CHH are indicative of high affinity single-site receptors (and will subsequently be referred to as thus). The values for B_{MAX} and K_D obtained in this study are comparable with those for midgut tissues in *C. maenas* and *Orconectes limosus* (Kummer and Keller, 1993) and those reported for CHH binding to the plasma membrane preparations of Y-organs in *C. maenas* (Webster, 1993). Thus, it seems likely that a major component of receptor populations for CHH consists of similar, if not identical, receptors. With regard to binding site density (B_{MAX}), gill membrane preparations exhibited the highest receptor density, but affinities were similar in all tissues. With regard to topographical differences in receptor site density, across anterior to posterior gills, it was notable that no anterior–posterior gradient was observed. This was possibly surprising with regard to the proposed role of CHH in osmoregulation, since it is well established that the anterior gills of brachyurans are adapted for respiratory functions, whereas posterior gills are (for crabs) involved in osmoregulation (Gilles and Pequeux, 1983; Lucu and Towle, 2003; Mantel and Farmer, 1983). Despite suggesting the spatial distribution of these functions, it has also been reported that there is no gradient of active ammonia excretion between anterior and posterior gills (Weihrrauch et al., 1999). Thus, it could be argued that the lack of such a gradient, with respect to receptor number in gills might not be unexpected, as they are multifunctional tissues, consisting of at least 6 functionally different cell types in the posterior gill alone (Lawson et al., 1994). This aspect of the ubiquity and action of CHH has not been given due attention—all tissues of crustaceans require a regulated supply of glucose, and highly metabolically active tissues, such as the gill also have this requirement, notwithstanding any additional role of CHH in osmoregulation.

Short-term exposure (30 min) of gills to diluted seawater (6 ppt) led to rapid increases in cGMP and intracellular glucose levels. This response was absent in destalked crabs, thus implicating eyestalk neuropeptide(s) in this response. Although early studies on CHH-mediated second messenger production have suggested that increases in both cAMP and cGMP are relevant to the CHH second messenger pathway (Sedlmeier, 1985), we have found that pure CHH administration, in vivo and in vitro, primarily leads to dramatic and sustained increases in cGMP, in accordance to results in lobsters (*H. americanus*), which indicates that CHH specifically activates a membrane bound guanylyl cyclase in this species (Goy, 1990). Furthermore, the expression of a membrane bound guanylyl cyclase has been observed in gills and hepatopancreas of *Procambarus clarkii* (Liu et al., 2004), which might be a candidate in the signal transduction pathway of CHH. Thus, it seems likely that any increases in cGMP are probably consequent of receptor binding and activation of this second messenger pathway in *Carcinus*. Results on dose–response relationships for levels of cGMP following incubation of gill, hindgut, and to a lesser extent midgut glands (Fig. 4) clearly

show reasonable correlation. Interestingly, application of 8-br-cGMP, increased intracellular glucose in hindgut and midgut gland tissues, but was ineffective on gill preparations. This may have been due to inadequate penetration across a primarily chitin-lined tissue, but paradoxically, exposure of this tissue to CHH always resulted in very large increases in cGMP. The specificity of second messenger responses to cGMP (and cAMP), using CCAP, L-enkephalin, proctolin, CPRP, MIH, and CHH on gills, gut, and midgut gland tissues once again highlighted the activity of CHH—none of the other peptides tested showed significant second messenger response.

These results clearly point to a central role of CHH and associated signaling pathways in a biologically relevant response to hypoosmotic conditions. However, the measurement of circulating CHH 30 min after exposure to dilute seawater did not show increased titers, although preliminary experiments on long-term acclimation of crabs to 9 ppt seawater clearly showed elevation of CHH titer. This result was initially unexpected, as it should be noted CHH release in *Carcinus* shows great dynamism, suggestive of episodic rather than sustained hormone release (Chung and Webster, 2005), and given the very short half-life of CHH in the hemolymph, of between 5 and 10 min. However, it is possible that exposure to dilute seawater initiates a very brief release of CHH, which would not be measurable after 30 min. Although exposure of the lobster (*H. americanus*) to 15 ppt seawater results in increases in CHH titer within 2 h (Chang et al., 1998), this animal is stenohaline and has a fairly limited hyperregulatory response to dilute conditions. However, for a strong hyperregulator, like *Carcinus*, rapid adaptation to dilute conditions would seem plausible. Such a response could involve a very rapid but brief release of CHH, to initiate signaling cascades, notwithstanding any long-term adaptation that might require maintenance of elevated CHH levels. Further studies, at a fine temporal scale are now needed to determine the dynamics of CHH release following exposure to dilute seawater.

Taken together, these results support the emerging view that CHH may be an important osmoregulatory neuropeptide, a view which is reinforced considering previous studies that have shown that perfusion of gills of *P. marmoratus* sinus gland extracts ($M_r > 5$ kDa) rapidly increase Na^+ influx and TEP (Eckhardt et al., 1995). Moreover, the D-Phe³ isoform of *Astacus leptodactylus* CHH increases Na^+ content of the hemolymph in postmolt crayfish, that had previously been eyestalk ablated (Serrano et al., 2003). Nevertheless, other eyestalk-derived neuropeptides may also be involved: in *Eriocheir sinensis*, split lamellae preparations have indicated that a small (2 kDa) peptide probably modulates via cAMP and stimulates transcellular conductance of Na^+ and Cl^- via stimulation of the activity of a V-ATPase (Onken et al., 2000).

The hindgut seems to be involved in osmoregulation during molting, since our previous observations showing that a brief, but dramatic release of CHH from intrinsic gut endocrine cells appear to stimulate diposgenesis and water

uptake during molting in *Carcinus* (Chung et al., 1999). Thus, we were interested to see whether this tissue might have any role in intermolt animals. As described earlier, the hindgut rapidly responds to incubation in physiologically relevant doses of CHH with a particularly impressive (but rather variable) accumulation of cGMP. Nevertheless, since midgut tissues also show this response, it cannot be said with any certainty that this response is coupled with any osmoregulatory phenomenon, and the very substantial chitinous lining of the hindgut of intermolt crabs seems to argue against an osmoregulatory role of the hindgut during intermolt.

At the present time, it is probably premature to conclude that CHH is the osmoregulatory peptide in crustaceans, without determining its direct effects on ion transport mechanisms *in vitro*. However, since the tissues examined possess the cellular machinery for transducing CHH signals, and in view of the very rapid, adaptive responses observed in these tissues following reduction in external salinity, CHH continues to be a strong candidate as a central player in osmoregulatory adaptation in this, and other strongly hyperregulating crustaceans.

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