The Journal of Experimental Biology 212, 542-549
Published by The Company of Biologists 2009
doi:10.1242/jeb.022889

The specific binding sites of eyestalk- and pericardial organ-crustacean hyperglycaemic hormones (CHHs) in multiple tissues of the blue crab, *Callinectes sapidus*

Hidekazu Katayama* and J. Sook Chung†

University of Maryland Biotechnology Institute, 701 E. Pratt Street, Columbus Center, Suite 236, Baltimore, MD 21202, USA

*Present address: Department of Applied Biochemistry, Institute of Glycotechnology, Tokai University, 1117 Kitakaname, Hiratsuka, Kanagawa 259-1292, Japan

†Author for correspondence (e-mail: chung@comb.umbi.umd.edu)

Accepted 18 November 2008

SUMMARY

Crustacean hyperglycaemic hormone from the pericardial organ (PO-CHH) is a CHH-related neuropeptide but its function and target tissues are not known in crustaceans. To investigate this issue, we employed radiolabelled ligand binding and cGMP assays, using eyestalk-CHH (ES-CHH) as a reference neuropeptide. The membranes were prepared from various tissues of *Callinectes sapidus*: hepatopancreas, hindgut, midgut, gills, heart, abdominal muscles and scaphognathites. Like ES-CHH, recombinant PO-CHH (rPO-CHH) specifically bound to the membranes of scaphognathites=abdominal muscles>midgut>gills>heart>hindgut and hepatopancreas (list order corresponds to the number of binding sites). The specific binding sites of ^{125}I-ES-CHH in hepatopancreas and gills were saturable and displaceable. The abdominal muscle membrane binding sites were specific and saturable to both CHHs. These binding sites were displaced by homologous neuropeptides, but poorly displaced by the heterologous counterpart. As for the second messenger, the expected increment (3- to >20-fold) in the amount of cGMP produced by ES-CHH was noted in most tissues tested except midgut. Recombinant PO-CHH increased cGMP production 1.5- to 4-fold in scaphognathites, heart, midgut, hindgut and abdominal muscles. The results obtained from the binding study suggest that PO-CHH also has multiple target tissues of which abdominal muscles and scaphognathites are the primary ones. The differences in the primary amino acid sequences of PO-CHH and ES-CHH, particularly in the C-terminal region and in the amidation at C-terminus, may contribute to the truncated responses of hyperglycaemia, cGMP stimulation and binding affinity.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/212/4/542/DC1

Key words: receptor binding assay, crustacean hyperglycaemic hormone, eyestalk, pericardial organ, hyperglycaemia.

INTRODUCTION

The occurrence of the family of crustacean hyperglycaemic hormone (CHH) neuropeptides is common in the phylum arthropoda (Phillips et al., 1998; Webster, 1998; Lacombe et al., 1999; Böcking et al., 2002; Chan et al., 2003; Chen et al., 2005; Fanjul-Moles, 2006; Drexler et al., 2007). CHH in many crustacean species is associated with multiple functions and tissue-specific structural isoforms (Yasuda et al., 1994; Aguilar et al., 1995; Chung and Webster, 1996; Chang et al., 1999; Ollivaux and Soyez, 2000; Chung and Webster, 2004). Eyestalk-CHH (ES-CHH), present in a moult stage-independent manner, is associated with hyperglycaemia, osmoregulation and the inhibition of ecdysteroid and methyl farnesoate synthesis (Webster and Keller, 1986; Liu et al., 1997; Khayat et al., 1998; Chung et al., 1999; Webster et al., 2000; Spanings-Pierrot et al., 2000; Tsutsui et al., 2005). The presence of CHH in the brain (eyestalk)–gut axis has also been reported in *Carcinus maenas* (Kegel et al., 1989; Weidmann et al., 1989; Chung et al., 1999). In contrast, insect ion transport peptide (ITP), a CHH-related neuropeptide mainly produced in the corpora cardiaca, has a specific action on Cl^- transport in the insect ileum (Phillips et al., 1998).

Multiple sequence analysis of the family of CHH neuropeptides generally shows that the first 40 amino acid residues of these neuropeptides are well conserved, compared with the C-terminal portion (Böcking et al., 2002; Chan et al., 2003; Chen et al., 2005; Fanjul-Moles, 2006). This structural variation of CHHs is reflected in the glucose responses in heterologous assays (Chung et al., 1998; Chung and Zmora, 2008; Mosco et al., 2008), indicating the importance of the C-terminal portion in bioactivity. More specifically, amidation at the C-terminus plays a crucial role in CHH-induced hyperglycaemia (Katayama et al., 2002; Mosco et al., 2008). In insects, however, the biological importance in the N-terminal domain (6–7 amino acids) was shown by expression of chimeric ITP in *Drosophila* Kc1 cells that contained the N-terminal domain of *Penaeus japonicus* CHH, and *Schistocerca gregaria* ITP sequence with the N-terminus removed was inactive in ion transport in the locust ileum (Zhao et al., 2005).

Tissue-specific CHHs may be the products of alternative splicing of multiple CHH genes (Dircksen et al., 2001; Gu and Chan, 1998; Chen et al., 2004), while cDNAs of *PO-CHH* isoforms have been reported from various non-neuronal tissues (Chen et al., 2004; Choi et al., 2006; Lee et al., 2007; Tiu et al., 2007). However, the neuropeptide form of PO-CHH that is most structurally related to insect ITP (Phillips et al., 1998; Drexler et al., 2007) has so far been found only in the intrinsic multipolar cells in the POs of *C. maenas* and *Callinectes sapidus* (Dircksen et al., 2001; Chung and Zmora, 2008).

ES-CHH secretion from the sinus gland in response to stress has been shown in many crustacean species (Chang et al., 1998; Chung

and Webster, 2005; Chung and Zmora, 2008; Kou and Yang, 1999; Webster, 1996). In *C. sapidus*, hypoxia induced increases in the expression of *PO-CHH* and its neuropeptide concentration in haemolymph, suggesting a putative role in the stress response (Chung and Zmora, 2008). Despite the observed increase in *C. sapidus* PO-CHH concentration in the haemolymph during hypoxia, injection of native PO-CHH (10–20 pmol) into *C. maenas*, *C. sapidus* or *Machrobrachium rosenbergii* (350 pmol of recombinant PO-CHH, rPO-CHH) did not produce hyperglycaemia, leaving its physiological function and target tissues as yet unidentified (Chung and Zmora, 2008; Dircksen et al., 2001; Ohira et al., 2006).

As alluded to earlier, our previous findings of an increase in *PO-CHH* expression and, moreover, the release of PO-CHH from animals experiencing hypoxia indicates that it meets the crucial criteria of a hormone, thus implying that there must be a target tissue(s). We used a cGMP radioimmunoassay (cGMP RIA) *in vitro* and a traditional radioligand binding assay with a combination of ^{125}I-rPO-CHH and the membranes of various tissues in order to identify putative target tissue(s). Moreover, we investigated a possible *in vivo* role of PO-CHH in hyperglycaemia by using 150–200 pmol (~30–50 nmol l^{-1}) of rPO-CHH, albeit a much higher concentration than the physiological levels observed by Chung and Zmora (Chung and Zmora, 2008). To validate these results, we tested native ES-CHH as a reference neuropeptide.

MATERIALS AND METHODS

Animals

Juvenile blue crabs (*Callinectes sapidus* Rathbun; <30 mm carapace width) were initially obtained from the blue crab hatchery programme at the Center of Marine Biotechnology, University of Maryland Biotechnology Institute (Baltimore, MD, USA). The crabs were cultured in individual compartments to grow to the size of 70–90 mm (carapace width) using re-circulating artificial seawater under the same rearing conditions as described previously (Chung and Zmora, 2008). Animals at intermoult were used for these experiments (Drach and Tchenigovtzeff, 1967).

Purification of native CHH from extracts of the sinus gland of *C. sapidus* and *C. maenas*

The sinus glands were dissected out from ice-cold adult males and females of *C. sapidus* and kept at –80°C. Prior to purification, the sinus gland (a batch of 50–100) was extracted in 2 mol l^{-1} acetic acid by sonication (Branson, Danbury, CT, USA). After centrifugation at 12,800 ***g*** for 10 min the supernatant was separated by a two-step purification method as described elsewhere (Webster, 1991). ES-CHH and oxidized ES-CHH were identified by dot-blot analysis using *C. sapidus* ES-CHH antiserum followed by mass determination (Chung and Zmora, 2008). The procedures for *C. maenas* were as stated in Webster (Webster, 1991).

Recombinant rPO-CHH

Construction of expression plasmids

Two primers were designed based on the nucleotide sequence of the cDNA encoding PO-CHH (DQ667141) (Chung and Zmora, 2008). The construction of expression plasmids was followed as described by Ohira and colleagues (Ohira et al., 1999). In brief, the forward primer (5′-AT*CCATGG***CC**CAGATTTACGACTCCTC-CTGT-3′, Invitrogen, Carlsbad, CA, USA) contained 14 nucleotide residues encoding QIYDS (the first five amino acid residues of mature PO-CHH). Two additional nucleotides (AT and ***CC***) were added: at the beginning and the end of the *Nco*I site (italic) to prevent the removal of the terminal nucleotide due to exonuclease activity of Taq DNA polymerase and to adjust the reading frame, respectively. The reverse primer (5′-AT*GAATTC*<u>TTA</u>TCCTCT-GATAGCATCCCTG-3′, Invitrogen) also contained 14 nucleotide residues encoding DAIRG (the last five amino acid residues of mature PO-CHH) and two additional residues: an *Eco*RI site (italic) and a stop codon (underlined). PCR was conducted with these primers using a plasmid containing the PO-CHH cDNA as a template. The amplified cDNA was subcloned into a pGEM T-Easy vector (Promega, Madison, WI, USA). After release from the vector by *Nco*I/*Eco*RI digestion, the PO-CHH insert was ligated into *Nco*I/*Eco*RI sites of a pET-Duet expression vector (Novagen, Gibbstown, NJ, USA).

Expression of recombinant PO-CHH

The procedure for the expression of rPO-CHH in *E. coli* Rosetta-gami(DE3)pLysS competent cells (Novagen) was as described (Ohira et al., 1999). The supernatant and the re-suspended insoluble material were separated by 18% SDS-PAGE, and the rPO-CHH was found in both fractions (supplementary material Fig. S1A). The soluble fraction was then purified by RP-HPLC (Waters, Milford, MA, USA) on a C18 column (Gemini, 4.6 mm×150 mm, Phenomenex, Torrence, CA, USA) with the following gradient conditions: 30–80% B over 30 min (A: 0.11% TFA in water, B: 0.1% TFA in 60% acetonitrile and 40% water) at a flow rate of 0.5 ml min^{-1}. The peak was further examined by dot-blot analysis using PO-CHH antiserum (Chung and Zmora, 2008) (supplementary material Fig. S1B).

The quantification of rPO-CHH was carried out using a Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA, USA) and a molecular mass of 8447.35 Da was determined by MALDI-TOF, which differed by 4.5 Da from the calculated mass of rPO-CHH (8443.2 Da, ABI Voyager DE Pro, Institute of Glycotechnology, Tokai University, Japan).

Iodination of CHH neuropeptides

Using chloramine T, 300 pmol of rPO-CHH or ES-CHH was iodinated with 300 μCi of ^{125}I-Na (Amersham, Pittsburgh, PA, USA). ^{125}I-rPO or ^{125}I-ES-CHH was eluted on a pre-conditioned PD-10 column (Bio-Rad) as described previously (Chung and Webster, 1996) and kept in a 1:1 ratio of glycerol at 4°C. The specific activity of both CHHs was approximately 500–700 Ci $mmol^{-1}$.

Membrane preparations

Plasma membranes of various tissues were prepared by following the protocol described previously (Chung and Webster, 1996). In detail, after dissection of the midgut, hindgut, scaphognathites, gills, hepatopancreas and heart from anaesthetized ice-cold juvenile crabs, the tissues were briefly rinsed in ice-cold crustacean saline and homogenized in extraction buffer (300 mmol l^{-1} sucrose, 140 mmol l^{-1} NaCl, 10 mmol l^{-1} benzamidine, and 10 mmol l^{-1} Hepes, pH 7.4) using a Polytron homogenizer (IKA, Staufen, Germany). The homogenates were centrifuged at 500 ***g*** for 10 min at 4°C and the supernatants were re-centrifuged at 24,000 ***g*** for 45 min at 4°C (Jouan, Waltham, MA, USA). The resulting microsomal pellets were re-suspended in the binding assay buffer without BSA (140 mmol l^{-1} NaCl and 10 mmol l^{-1} Hepes, pH 7.4) and protein concentrations were estimated as stated above. Membranes were aliquoted into 0.5 ml tubes and stored at –80°C until further use.

Binding assays

Binding assays were carried out in 100 μl final volume following the procedure described (Chung and Webster, 1996). Non-specific

binding ranged from 20% to 30% of the total binding. All triplicate assays were repeated at least twice.

cGMP measurements

Iodination of the cGMP analogue and the method of cGMP RIA are given (Chung and Webster, 2006). In brief, midgut, hindgut, scaphognathites, gills, hepatopancreas and heart tissues were dissected from intermoult crabs and incubated for 45 min at room temperature with 200 µl of Medium 199 at 900 mosmol l^{-1} adjusted with NaCl containing various concentrations of rPO-CHH (or medium alone for control) in the presence of 0.25 mmol l^{-1} isobutylmethylxanthine. A set of tissues was also treated with 50 nmol l^{-1} ES-CHH. After incubation, the tissues were collected in 100 µl of ice-cold 0.1 mol l^{-1} acetate buffer (pH 4.5) and disrupted by brief sonication (Bronson, Danbury, CT, USA) then centrifuged for 10 min at 12,800 ***g*** at 4°C. The protein concentrations were estimated as described above. RIA was used to determine cGMP levels of 25–50 µl of each acetylated sample as described previously (Chung and Webster, 2006). The data are given as pmol mg^{-1} protein.

In vivo glucose bioassay

Juvenile blue crabs at intermoult were injected with 100 µl of rPO-CHH (150–200 pmol) or native ES-CHH (10 pmol), while control animals received equal volumes of crustacean saline. At time t=0 (prior to injection), 100 µl of haemolymph was withdrawn in a marine anticoagulant at ratio of 1:1 as described before (Chung and Zmora, 2008). At time intervals of 30, 60, 90 and 120 min, the haemolymph was collected and glucose levels were determined using a glucose oxidase assay (Webster, 1996). Due to high individual variation in the level of initial glucose, the data were converted into percentage increment and the results were pooled from three separate experiments.

Statistical analysis

Statistical significance was tested using the GraphPad InStat program (GraphPad Software, San Diego, CA, USA).

RESULTS

Receptor binding assay

Tissue distribution of specific binding sites of ^{125}I-ES-CHH and ^{125}I-rPO-CHH

As shown in Fig. 1A,B, the spatial distribution of binding sites of ^{125}I-ES-CHH and ^{125}I-rPO-CHH was tested by the incubation of 160,000 d.p.m. (~75 fmol) of ^{125}I-ES-CHH or ^{125}I-rPO-CHH with 100 µg of membranes (final volume, 100 µl) from the following tissues: scaphognathites, abdominal muscles, heart, midgut, hindgut, gills and hepatopancreas. Non-specific binding was estimated by the addition of 10 pmol of cold ES-CHH and rPO-CHH at 10^{-7} mol l^{-1}.

Specific binding sites for ^{125}I-ES-CHH were common in the tested membranes (Fig. 1A) with the highest number of sites being $8.6(\pm0.9)\times10^{-11}$ mol l^{-1} mg^{-1} protein in hepatopancreas (N=6) and the lowest $5.3(\pm0.2)\times10^{-11}$ mol l^{-1} mg^{-1} protein in heart (N=6). Interestingly, these ^{125}I-ES-CHH binding sites in membranes of hepatopancreas, gills, midgut and heart were competitively displaced with cold ES-CHH but not with rPO-CHH. The specific ^{125}I-ES-CHH binding sites in the membranes of scaphognathites and abdominal muscles were also poorly displaced by heterologous cold rPO-CHH at 10^{-7} mol l^{-1} (Fig. 1A), yielding $6.1(\pm0.7)\times10^{-11}$ and $3.7(\pm1.0)\times10^{-11}$ mol l^{-1} mg^{-1} protein (N=6), respectively. In the hindgut, approximately 16% of the specific binding sites of ^{125}I-ES-CHH were competitively displaced by cold rPO-CHH.

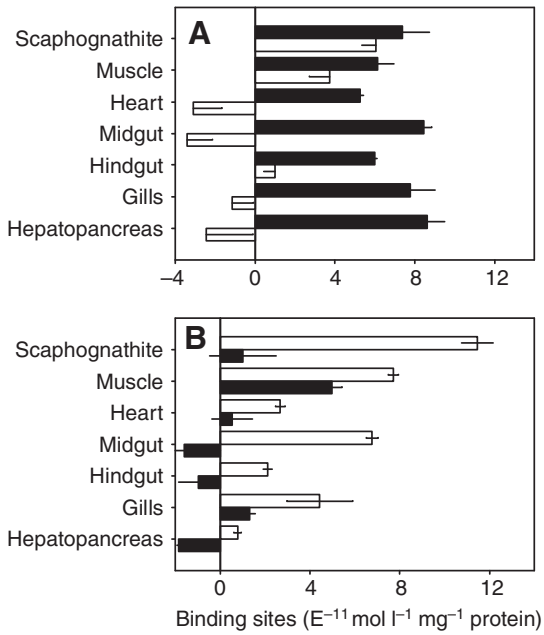

Fig. 1. Spatial distribution of specific binding sites of ^{125}I-ES-CHH (A) and ^{125}I-PO-CHH (B). (A) The specific binding sites of ^{125}I-ES-CHH were challenged with 10 pmol of cold ES-CHH (filled bars) or cold rPO-CHH (open bars). (B) The specific binding sites of ^{125}I-PO-CHH were competed for with 10 pmol of cold rPO-CHH (open bars) or cold ES-CHH (filled bars). Data are presented as means ±1 s.e.m. (N=6).

Fig. 1B shows the number of specific binding sites of ^{125}I-rPO-CHH. All tissue membranes tested except hepatopancreas contained specific binding sites for ^{125}I-rPO-CHH, with varying concentrations from the highest in scaphognathites, $11.5(\pm0.7)\times10^{-11}$ mol l^{-1} mg^{-1} protein (N=6), to the lowest in gills, $2.12(\pm0.50)\times10^{-11}$ mol l^{-1} mg^{-1} protein (N=6). These specific ^{125}I-rPO-CHH binding sites in the membranes of abdominal muscles and gills were also partially displaced by the addition of cold ES-CHH at 10^{-7} mol l^{-1}, giving ~65% and ~30% of the specific binding, compared with that of cold rPO-CHH, respectively.

Binding characteristics of ^{125}I-ES-CHH in the membranes of hepatopancreas and gills

The specific binding sites of ^{125}I-ES-CHH in the membranes of hepatopancreas and gills were further characterized in terms of saturation and displacement (Figs 2A,B, respectively). These membrane sites were tested for saturation with increasing concentrations of ^{125}I-ES-CHH from 5.5×10^{-11} to 8.1×10^{-10} mol l^{-1}, whereas values for non-specific binding were obtained by the addition of cold ES-CHH at 10^{-7} mol l^{-1}. The K_D values were $1.68(\pm0.34)\times10^{-10}$ mol l^{-1} for hepatopancreas and $2.40(\pm0.42)\times10^{-10}$ mol l^{-1} for gills. The calculated number of maximal binding sites (B_{max}) was $3.8(\pm0.5)\times10^{-11}$ mol l^{-1} mg^{-1} protein for hepatopancreas and $1.2(\pm0.2)\times10^{-10}$ mol l^{-1} mg^{-1} protein for gills, giving a similar Hill co-efficient of ~1.0 for the two.

Displacement of the specific binding of ^{125}I-ES-CHH was tested by incubating the membranes of gills and hepatopancreas with increasing concentrations of cold ES-CHH from 6.1×10^{-11} to 10^{-6} mol l^{-1}. The binding sites in gill membranes were further tested with oxidized ES-CHH, C-terminal synthetic peptides of ES-CHH or *C. maenas* ES-CHH. As shown in Fig. 2C, the addition of cold ES-CHH displaced the specific binding of ^{125}I-ES-CHH equally in

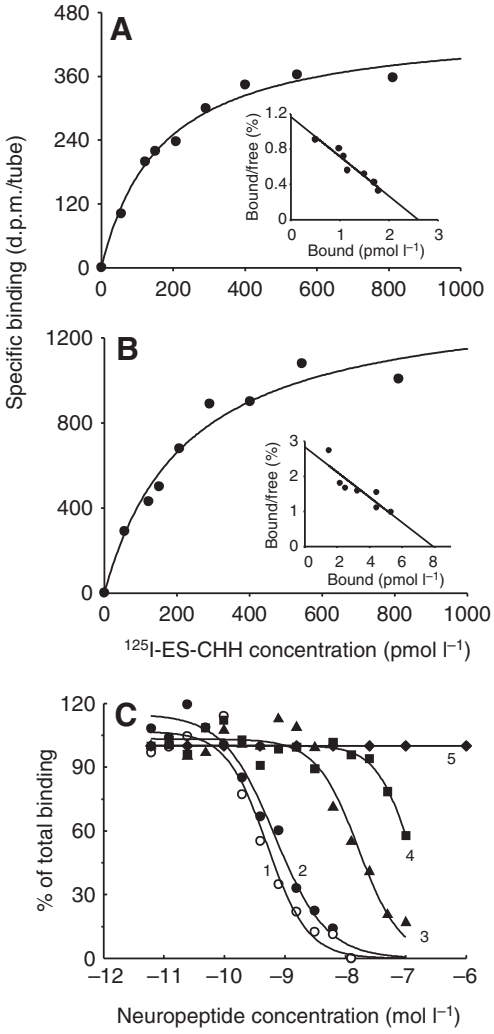

Fig. 2. Saturation curves and Scatchard plots (insets) of the specific binding of ^{125}I-ES-CHH in the membranes of hepatopancreas (A) and gills (B). Displacement curves (C) of specific binding in gills by ES-CHH (1) and in hepatopancreas by ES-CHH (2), oxidized ES-CHH (4), *C. maenas* ES-CHH (3) and C-terminal synthetic peptide of ES-CHH (5).

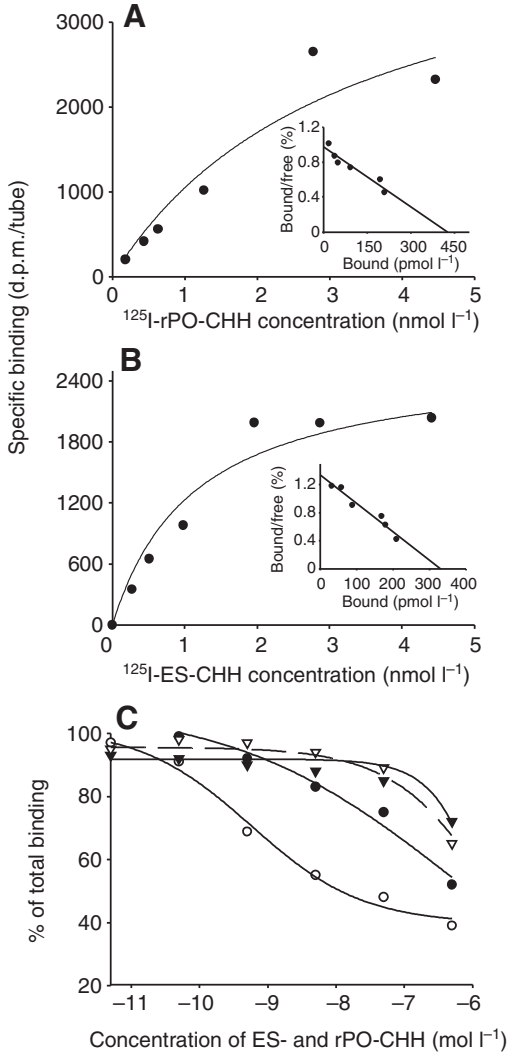

Fig. 3. Saturation curves and Scatchard plots (insets) of the specific binding of ^{125}I-rPO-CHH (A) or ^{125}I-ES-CHH (B) in the abdominal muscle membranes. Displacement curves (C) of specific binding of ^{125}I-rPO-CHH by rPO-CHH (open circles), and by ES-CHH (filled circles); displacement curves of specific binding of ^{125}I-ES-CHH by ES-CHH (filled triangles) and by rPO-CHH (open triangles).

the membranes of gills and hepatopancreas (lines 1 and 2, respectively), giving IC_{50} values of $7.0(\pm1.1)\times10^{-10}$ and $9.5(\pm1.0)\times10^{-10}$ mol l^{-1}, respectively. *C. maenas* ES-CHH displaced ^{125}I-ES-CHH from the specific binding site in the gill membranes at an IC_{50} of 3.0×10^{-8} mol l^{-1} (line 3), whereas oxidized ES-CHH gave an IC_{50} value of 1.0×10^{-7} mol l^{-1} (line 4). The synthetic peptide of the C-terminal fragment of ES-CHH (1 μmol l^{-1}) consisting of 19 amino acid residues [EDLLI(norL)DNFEEYARKIQVV-NH_2] did not compete with ^{125}I-ES-CHH for its binding sites in the gill membranes (line 5).

Binding characteristics of ^{125}I-rPO-CHH in the membranes of abdominal muscles

Based on the results shown in Fig. 1A and B, the membrane of abdominal muscles was chosen for further analysis of the binding characteristics of ^{125}I-rPO-CHH and ^{125}I-ES-CHH, as it was easy to collect a large amount compared with scaphagonathites. Fig. 3A,B shows the saturation of the specific binding sites of ^{125}I-rPO-CHH (Fig. 3A) and ^{125}I-ES-CHH (Fig. 3B) with B_{max} values of $1.3(\pm0.2)\times10^{-10}$ and $7.8(\pm0.1)\times10^{-11}$ mol l^{-1} mg protein, respectively. The calculated K_D was similar at $1.3(\pm0.2)\times10^{-9}$ mol l^{-1} for ^{125}I-rPO-CHH and $1.1(\pm0.2)\times10^{-9}$ mol l^{-1} for ^{125}I-ES-CHH, while the Hill co-efficient of both CHHs was ~1.0.

The competitiveness of the specific binding sites of ^{125}I-rPO-CHH or ^{125}I-ES-CHH was assayed by adding cold rPO-CHH and/or ES-CHH at concentrations from 5×10^{-11} to 5×10^{-7} mol l^{-1} (Fig. 3C). The calculated IC_{50} values were $2.8(\pm0.3)\times10^{-9}$ and $5.0(\pm0.9)\times10^{-7}$ mol l^{-1} for the homologous competition of rPO-CHH and ES-CHH, respectively. With heterologous neuropeptides, the IC_{50} values were estimated at >1 μmol l^{-1} for both CHHs.

cGMP measurements

The effect of ES-CHH (50 nmol l^{-1}) and rPO-CHH (100 nmol l^{-1}) on cGMP production was examined in the following tissues: Y-

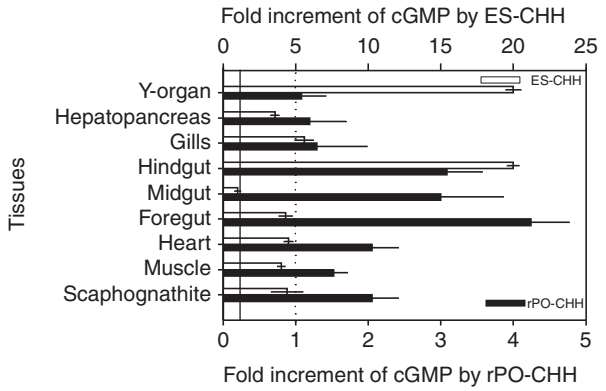

Fig. 4. The effects of ES-CHH (50 nmol l^{-1}) and rPO-CHH (100 nmol l^{-1}) incubation on cGMP production in various tissues: open bars, ES-CHH; filled bars, rPO-CHH. The data were converted into fold increase over control that was only treated with saline, and are presented as means ±1 s.e.m. (N=6). Solid line represents the level of control for ES-CHH and dotted line that for rPO-CHH.

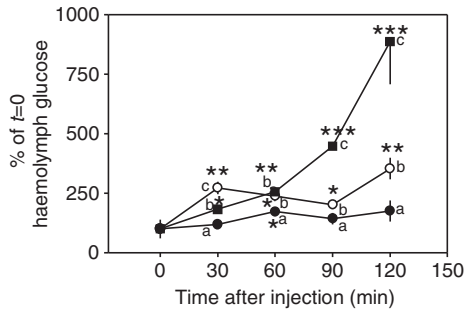

Fig. 5. Time course effects of CHH neuropeptides on the level of glucose in haemolymph *in vivo*: (open circles) rPO-CHH at 150–200 pmol (filled squares) native ES-CHH at 10 pmol, and (filled circles) saline control. The data are presented as means ±1 s.e.m. (N=18) of the percentage increase over control at t=0 min. Student's t-test was used for statistical analysis of each experiment. *P<0.05, **P<0.01, ***P<0.001. The data obtained from each time point of three experimental groups were tested using one-way ANOVA at P<0.05. Statistical significance was shown in lowercase letters.

organs, foregut, midgut, hindgut, scaphognathites, abdominal muscles, gills, hepatopancreas and heart. The results were converted into fold increment of control. As shown in Fig. 4, ES-CHH (open bars) produced varying degrees of stimulation of cGMP from 3.5- to >20-fold in all tissues tested except midgut, with the lowest in hepatopancreas (~3.5-fold) and the greatest in Y-organ and hindgut (>20-fold). The incubation of rPO-CHH with foregut, midgut, hindgut, scaphognathites, abdominal muscles and heart did produce a 1.5- to 4.0-fold increase in the level of cGMP (Fig. 4, filled bars), whereas gut tissues produced the greatest response of 3- to 4-fold. Abdominal muscles had the lowest response of only 1.5-fold. The level of cGMP in gills, Y-organs and hepatopancreas was not affected by incubation with rPO-CHH at 100 nmol l^{-1}.

In vivo glucose bioassay

The injection of 20 pmol of native PO-CHH into the juvenile crabs (60–90 mm carapace width, weighing between 30 and 60 g) gave calculated *in vivo* concentrations of ~3–7 nmol l^{-1}, providing the haemolymph volume is 10% of bodyweight. This concentration of 3–7 nmol l^{-1} did not produce hyperglycaemia in haemolymph of *C. sapidus* (Chung and Zmora, 2008). Based upon the results obtained in the current study (binding assays and cGMP response), we conducted further *in vivo* assays using a higher dose at a final concentration of 30–50 nmol l^{-1} by injecting 150–200 pmol of rPO-CHH, 10 pmol of ES-CHH or crustacean saline as a control. The results obtained from the time course response of CHH injections on the glucose level in haemolymph are shown in Fig. 5: rPO-CHH induced hyperglycaemia by significantly elevating glucose to 270±26% (N=18) after 30 min compared with that at t=0 (P<0.01) and at 30 min of ES-CHH injection (P<0.05). This elevated level of glucose by rPO-CHH was maintained to the end of the incubation (120 min, 353±46%; N=18; P<0.01). The hyperglycaemic response of ES-CHH was positively related to incubation time, as the level of glucose was significantly and steadily raised from 182±15% (N=18, P<0.05) at 30 min to 850±180% (N=18, P<0.001) at 120 min. At 90 and 120 min, ES-CHH produced much higher hyperglycaemia than rPO-CHH. Animals that received crustacean saline showed 120–170% increases throughout the experimental period, with the highest level of 172±15% (N=15, P<0.05) at 60 min, compared with that of control at t=0.

DISCUSSION

Using a radioligand binding assay, we have demonstrated the co-presence of specific binding sites for PO-CHH and ES-CHH in the membranes of scaphognathites, abdominal muscles, heart, midgut, hindgut and gills. These ^{125}I-rPO-CHH binding sites were specific, saturable and displaceable in the abdominal muscle membranes. Our results suggest that the difference in the C-terminal portion of CHHs of PO and ES plays a crucial role in the truncated responses of glucose and cGMP stimulation.

We produced rPO-CHH in *E. coli* for the binding assay, as it readily generates a large amount of protein. We reasoned that rPO-CHH with an extended Ala residue at the N-terminus binds its binding sites and is bioactive, as a previous report has shown that recombinant moult-inhibiting hormone (rMIH) produced using the same procedure was biologically active and retained binding capacity to its receptors (Okumura et al., 2005; Asazuma et al., 2005). The study of the structure and activity of CHH-related neuropeptides revealed that the C-terminus is more critical for bioactivity than the N-terminus, as the cyclization of Gln to pyroglutamate at the N-terminus or the extended Ala at the N-terminus does not affect the secondary structure, binding capacity or bioactivity (Chung and Webster, 1996; Katayama et al., 2002; Katayama et al., 2003; Katayama et al., 2004; Okumura et al., 2005; Asazuma et al., 2005).

To our surprise, approximately half of rPO-CH was recovered in the supernatant, suggesting that the protein was properly folded in its native structure (Anfinsen, 1973). The dot-blot analysis showed that the protein purified by an RP-HPLC that was present in the supernatant was recognized by PO-CHH antiserum (Chung and Zmora, 2008). The 4.2 Da difference by MALDI-TOF between the calculated and estimated mass is acceptable, as it lies within a 0.05% margin of error.

Specific binding sites for rPO-CHH and ES-CHH were commonly found in the membranes of various tissues of *C. sapidus*: scaphognathites, abdominal muscles, heart, midgut, hindgut, gills and hepatopancreas, most of which were considered to be target tissues of ES-CHH (Webster, 1993; Kummer and Keller, 1993; Chung and Webster, 1996; Chung and Webster, 2003; Chung and Webster, 2006). We proposed that scaphognathites may be the target tissues of PO-CHH (Chung and Zmora, 2008) after considering the

anatomical structure and location of POs in animals (Maynard, 1961). Interestingly, it appeared that cold rPO-CHH partially competed to displace ^{125}I-ES-CHH from 50–60% of the binding sites that are present in membranes of scaphognathites and abdominal muscles, while it did not compete for specific binding sites in the other tissues.

As for ^{125}I-rPO-CHH, all the membranes tested exhibited specific binding sites, with the greatest number occurring in the scaphognathites and the least in the hepatopancreas. Based on the number of binding sites in the tissues, scaphognathites, abdominal muscles and midgut are considered to be the major target tissues for rPO-CHH. Interestingly, the specific binding sites for ^{125}I-rPO-CHH in the membranes of scaphognathites were displaced with homologous neuropeptide but not with ES-CHH. In the abdominal muscles, 65% of specific binding sites of ^{125}I-rPO- CHH and of ^{125}I-ES-CHH were competed out by adding cold ES-CHH and rPO-CHH, respectively. Hepatopancreas, gills, hindgut, heart and midgut are the major target tissues of ES-CHH of *C. sapdius*, as reported for *C. maenas* (Webster, 1993; Kummer and Keller, 1993; Chung and Webster, 2006).

All tissues except abdominal muscles showed two types of CHH binding site with a varying degree of compatibility to the heterologous counterpart. Those present in heart, gut and hepatopancreas are not compatible with heterologous CHH, but are specific to the homologous neuropeptide. Gills and scaphognathites possess the major receptor types of ES-CHH and PO-CHH, respectively, while their minor forms, the sites of PO-CHH binding in gills and ES-CHH binding in scaphognathites, are likely to be compatible with heterologous neuropeptides. Interestingly the abdominal muscle that was considered to be one of the major target tissues of ES-CHH (Keller and Sedlmeier, 1988) contains binding sites compatible with both CHHs, as similar levels of binding in the presence of heterologous neuropeptides were found. It is reported that tissues or cells co-express two similar receptors for follicle stimulating hormone (FSH): one is specific to FSH, while the other is promiscuous, binding to either human chorionic gonadotropin or thyrotropin stimulating hormone (Costagliola et al., 2005). In this case, the sequence similarity between these ligands is only ~40% (Costagliola et al., 2005). Thus, considering the sequence identity between two CHHs (>60%), the CHH receptor in abdominal muscles may be promiscuous by binding to both neuropeptides, but with different affinities.

The specific binding sites of ES-CHH in the membranes of hepatopancreas and gills of *C. sapidus* showed two typical binding characteristics: saturation and displacement, which is similar to those found in the hepatopancreas of *Orconectes limosus* and *C. maenas* and the gills and Y-organs of *C. maenas* in terms of the values of B_{max} and K_D (Kummer and Keller, 1993; Webster, 1993; Chung and Webster, 2006). It appears that the receptors for ES-CHH present in the gills and hepatopancreas of these species are rather similar in terms of affinity, despite the possible difference in the receptor density of each tissue.

The IC_{50} values of *C. maenas* ES-CHH and oxidized ES-CHH were interesting, as they were one or two orders of magnitude lower than that of *C. sapidus* ES-CHH. The greatest variation in the sequence of CHHs of *C. sapidus* and *C. maenas*, which is a common feature among the family of CHH neuropeptides, lies in the C-terminal tail (Phillips et al., 1998; Webster, 1998; Lacombe et al., 1999; Böcking et al., 2002; Chan et al., 2003; Chen et al., 2005; Fanjul-Moles, 2006), thus implying the importance of this region in species-specific receptor recognition and, thus, for bioactivity. However, the C-terminal synthetic peptide of *C. sapidus* ES-CHH did not compete with ^{125}I-ES-CHH, suggesting that the C-terminal fragment alone is not structurally sufficient for binding. As for the oxidized *C. sapidus* ES-CHH with an additional mass of 16 Da (Chung and Zmora, 2008), the exact position of oxidation at the Met residue is unknown, since three Met residues are present in the primary amino acid sequence, at positions 47, 53 and 59. The Met residue at position 47 resides within a globular structure that is formed by three intradisulphide bridges (Katayama et al., 2003). However, Met positions 53 and 59 which are conserved in most crab species are located in the C-terminal tail; thus one of these residues may be the target of oxidation. Nonetheless, it seems that the oxidation of a single Met residue interferes with binding to its receptors, which results in a low value of IC_{50} as shown in Fig. 2C. This fact may further imply the importance of the C-terminus in binding as well as the bioactivity of CHHs (Chung et al., 1998; Katayama et al., 2002; Mosco et al., 2008). Such differences in the values of IC_{50} support our previous observation that injection of *C. maenas* ES-CHH or oxidized *C. sapidus* ES-CHH induced only 20–30% hyperglycaemia of ES-CHH (Chung and Zmora, 2008).

As shown in Fig. 1A,B, the abdominal muscle membrane contained specific binding sites for both rPO-CHH and ES-CHH with comparable K_D values. However, they show differences in B_{max} and IC_{50} values of the displacement: two times more and 50 times higher ^{125}I-rPO-CHH values than those of ^{125}I-ES-CHH, respectively. As shown in the insets of Fig. 3A,B, the Hill coefficient (~1.0) indicates that binding of each neuropeptide to these sites occurs in a non-cooperative manner. Interestingly, the membrane of scaphognathites (shown in supplementary material Fig. S2) revealed IC_{50} values of $4.5\,E^{-10}$ mol l^{-1} for rPO-CHH, but >1 μmol l^{-1} for ES-CHH, suggesting that this respiratory tissue may be the primary target tissue of PO-CHH, as proposed previously (Chung and Zmora, 2008). The binding sites of each neuropeptide were poorly displaced by their heterologous counterpart.

Compared with the values of B_{max} and K_D for ^{125}I-ES-CHH binding in the membranes of hepatopancreas and gills as shown in Fig. 2, the specific binding sites of this neuropeptide in the abdominal muscles exhibited one order of magnitude less affinity but were 4–7 times greater in number. Low affinity but great binding capacity suggests that hepatopancreas and gills have a similar type of receptor to that in the major target tissues for ES-CHH of *C. sapidus*. However, the CHH receptor present in the abdominal muscles appears to be capable of binding to both CHHs, but with a preference for PO-CHH over ES-CHH.

The cGMP response to ES-CHH and rPO-CHH was similar as reported in *C. maenas* (Dircksen et al., 2001; Chung and Webster, 2006). In particular rPO-CHH had no effect on cGMP production in the Y-organ, gills and hepatopancreas of *C. sapidus*. The lack of cGMP response in hepatopancreas was anticipated, in that it showed the least number of specific binding sites for rPO-CHH (Fig. 1B). The abdominal muscles contain the specific binding sites of rPO-CHH, yet the stimulation of cGMP production was only 1.5-fold. The second messenger of rPO-CHH is not known in these muscles, but cGMP is not likely to be the second messenger of this CHH. ES-CHH of *C. sapidus*, on the other hand, stimulated an ~7-fold increase in cGMP in this tissue, and a similar result was reported in *Homarus americanus* (Goy, 1990), therefore indicating that it may be the second messenger.

Previously, 10–20 pmol injections of native PO-CHH (3–5 nmol l^{-1}) lacked hyperglycaemic activity (Dircksen et al., 2001; Chung and Zmora, 2008). However, we reasoned that injection of a higher concentration of PO-CHH, although not its physiological level, may be required to induce hyperglycaemia in

haemolymph due to the following findings: (1) the limited specific binding sites found in the hepatopancreas (Fig. 1B); (2) the relatively small cGMP response of PO-CHH, compared with that of ES-CHH; and (3) the structural differences in the C-terminal tail. As stated above, the Met of ES-CHH at positions 53 and 59 that is required for high affinity binding to receptors and bioactivity (Chung and Zmora, 2008) is replaced by Val and Pro, respectively, in PO-CHH. Moreover, the C-terminus of PO-CHH is not amidated, as its importance in hyperglycaemia has previously shown (Katayama et al., 2002; Mosco et al., 2008). Therefore, we pursued an *in vivo* time course study of haemolymph glucose by the injection of rPO-CHH at 30–50 nmol l^{-1}. The hyperglycaemia caused by the injection of a high concentration of rPO-CHH was immediate, as the elevated level of glucose at 30 min after injection was significantly greater than that of ES-CHH. Overall the hyperglycaemic response by rPO-CHH, like cGMP production, was truncated compared with that caused by ES-CHH. We were not able to use native PO-CHH as a control for rPO-CHH due to the lack of material, but ES-CHH elevated the glucose to the highest level at 90–120 min after injection, similar to earlier reports (Chung and Webster, 1996; Chang et al., 1998; Chung et al., 1998; Dircksen et al., 2001; Chung and Zmora, 2008).

These truncated responses of cGMP and hyperglycaemia to PO-CHH are comparable with the levels of hyperglyacemia caused by the injection of *C. meanas* CHH and oxidized ES-CHH (Chung and Zmora, 2008). The difference in IC_{50} values of *C. meanas* CHH, oxidized ES-CH and rPO-CHH (as shown in Fig. 2C, Fig. 3C), and their truncated hyperglycaemia response suggest that the structural difference in the C-terminus may affect the efficacy and potency of CHH molecules. This is in accordance with previous reports of the importance of the C-terminal portion of the family of CHH neuropeptides in binding to specific receptors, as shown in the structure–activity of the moult-inhibiting hormone and CHH (Katayama et al., 2002; Katayama et al., 2004; Mosco et al., 2008).

In this paper, we have shown the co-presence of the specific binding sites of ES- and PO-CHH in multiple tissues of *C. sapidus*. As stated above, the tested tissues of *C. sapidus* probably contain at least three types of CHH receptor: specific receptors for (1) ES-CHH and (2) PO-CHH, and (3) one promiscuous receptor. Thus, the tissues which contain the promiscuous receptor bind to each CHH and also displace homologous and heterologous CHH. As for the second messenger, in contrast to ES-CHH which utilizes cGMP in most tissues where its binding sites were found (Chung and Webster, 2006), PO-CHH appears to modulate a different second messenger(s), particularly in muscles. It will be interesting to examine in future studies how the structures of these receptors differ from each other within a species as well as among different species, as it is reported that there is a co-evolution of binding specificity in families of homologous ligands and their receptors (Moyle et al., 1994; Costagliola et al., 2005). Based on our previous observation (Chung and Zmora, 2008) and the current binding study, we infer that the co-presence of ES- and PO-CHH receptors in these tissues may be a common feature in crustaceans.

We thank O. Zmora and the personnel in the blue crab hatchery programme for the juvenile crabs and S. Rogers and the ARC personnel for maintaining the water quality of the re-circulation system. Anti-cGMP and anti-cAMP sera were generous gifts of Professor J. deVante (Maastricht University). Dr H. Katayama was sponsored by a fellowship from the Japan Society for Promotion of Science. This article is contribution no. 08-190 of the Center of Marine Biotechnology (University of Maryland Biotechnology Institute, Baltimore, MD, USA) and the work is supported by a program grant (NA17FU2841) from NOAA Chesapeake Bay Office to the Blue Crab Advanced Research Consortium.

REFERENCES

Aguilar, M. B., Soyez, D., Falchetto, R., Arnott, D., Shabanowitz, J., Hunt, D. F. and Huberman, A. (1995). Amino acid sequence of the minor isomorph of the crustacean hyperglycemic hormone (CHH-II) of the Mexican crayfish *Procambarus bouvieri* (Ortmann): presence of a D-amino acid. *Peptides* **16**, 1375-1383.

Anfinsen, C. B. (1973). Principles that govern the folding of protein chains. *Science* **181**, 223-230.

Asazuma, H., Nagata, S., Katayama, H., Ohira, T. and Nagasawa, H. (2005). Characterization of a molt-inhibiting hormone (MIH) receptor in the Y-organ of the kuruma prawn, *Marsupenaeus japonicus*. *Ann. NY Acad. Sci.* **1040**, 215-218.

Böcking, D., Dircksen, H. and Keller, R. (2002). The crustacean neuropeptides of the CHH/MIH/GIH family: structures and biological activities. In *The Crustacean Nervous System* (ed. K. Wiese), pp. 84-97. New York: Springer.

Chan, S.-M., Gu, P.-L., Chu, H.-K. and Tobe, S. S. (2003). Crustacean neuropeptide genes of the CHH/MIH/GIH family: implications from molecular studies. *Gen. Comp. Endocrinol.* **134**, 214-219.

Chang, E. S., Keller, R. and Chang, S. A. (1998). Quantification of crustacean hyperglycemic hormone by ELISA in hemolymph of the lobster, *Homarus americanus*, following various stresses. *Gen. Comp. Endocrinol.* **111**, 359-366.

Chang, E. S., Chang, S. A., Beltz, B. S. and Kravitz, E. A. (1999). Crustacean hyperglycemic hormone in the lobster nervous system: localization and release from cells in the suboesophageal ganglion and thoracic second roots. *J. Comp. Neurol.* **414**, 50-56.

Chen, S.-H., Lin, C.-Y. and Kuo, C. M. (2004). Cloning of two crustacean hyperglycemic hormone isoforms in freshwater giant prawn (*Machrobrachium rosenbergii*): evidence of alternative splicing. *Mar. Biotechnol.* **6**, 83-94.

Chen, S. H., Lin, C.-Y. and Kuo, C. M. (2005). *In silico* analysis of crustacean hyperglycemic hormone family. *Mar. Biotechnol.* **7**, 199-206.

Choi, C. Y., Zheng, J. and Watson, R. D. (2006). Molecular cloning of a cDNA encoding a crustacean hyperglycemic hormone from eyestalk ganglia of the blue crab, *Callinectes sapidus*. *Gen. Comp. Endocrinol.* **148**, 383-387.

Chung, J. S. and Webster, S. G. (1996). Does the N-terminal pyroglutamate residue have any physiological significance for crab hyperglycemic neuropeptides? *Eur. J. Biochem.* **240**, 358-364.

Chung, J. S. and Webster, S. G. (2003). Moult cycle-related changes in biological activity of moult-inhibiting hormone (MIH) and crustacean hyperglycaemic hormone (CHH) in the crab, *Carcinus maenas*. *Eur. J. Biochem.* **270**, 3280-3288.

Chung, J. S. and Webster, S. G. (2004). Expression and release patterns of neuropeptides during embryonic development and hatching of the green shore crab, *Carcinus maenas*. *Development* **131**, 4751-4761.

Chung, J. S. and Webster, S. G. (2005). Dynamics of *in vivo* release of molt-inhibiting hormone and crustacean hyperglycemic hormone in the shore crab, *Carcinus maenas*. *Endocrinology* **146**, 5545-5551.

Chung, J. S. and Webster, S. G. (2006). 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. *Gen. Comp. Endocrinol.* **147**, 206-213.

Chung, J. S. and Zmora, N. (2008). Functional studies of crustacean hyperglycemic hormone (CHHs) of the blue crab, *Callinectes sapidus*-the expression and release of CHH in eyestalk and pericardial organ in response to environmental stress. *FEBS J.* **275**, 693-704.

Chung, J. S., Wilkinson, M. C. and Webster, S. G. (1998). Amino acid sequences of both isoforms of crustacean hyperglycaemic hormones (CHH) and corresponding precursor-related peptide in *Cancer pagurus*: the complete inventory of CHH family neuropeptides now identified in a crab. *Reg. Peptides* **77**, 17-24.

Chung, J. S., Dircksen, H. and Webster, S. G. (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.* USA **96**, 13103-13107.

Costagliola, S., Urizar, E., Mendive, F. and Vassart, G. (2005). Specificity and promiscuity of gonadotropin receptors. *Reproduction* **130**, 275-281.

Dircksen, H., Böcking, D., Heyn, U., Mandel, C., Chung, J. S., Baggerman, G., Verhaert, P., Daufeldt, S., Plosch, T., Jaros, P. P. et al. (2001). Crustacean hyperglycaemic 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.

Drach, P. and Tchernigovtzeff, C. (1967). Sur la méthode de détermination des stades d'intermue et son application générale aux Crustacés. *Vie et milieu ser A Biol.* **18**, 595-610.

Drexler, A. L., Harris, C. C., Dela Pena, M. G., Asuncion-Uchi, M., Chung, J. S., Webster, S. and Fuse, M. (2007). Molecular characterization and cell-specific expression of an ion transport peptide in the tobacco hornworm, *Manduca sexta*. *Cell Tissue Res.* **319**, 391-408.

Fanjul-Moles, M. L. (2006). Biochemical and functional aspects of crustacean hyperglycemic hormone in decapod crustaceans: review and update. *Comp. Biochem. Physiol. C* **142**, 390-400.

Goy, M. F. (1990). Activation of membrane guanylyl cyclase by an invertebrate peptide hormone. *J. Biol. Chem.* **265**, 20220-20227.

Gu, P. and 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.

Katayama, H., Ohira, T., Aida, K. and Nagasawa, H. (2002). Significance of a carboxyl-terminal amide moiety in the folding and biological activity of crustacean hyperglycemic hormone. *Peptides* **23**, 1537-1546.

Katayama, H., Nagata, K., Ohira, T., Yumoto, F., Tanokura, M. and Nagasawa, H. (2003). The solution structure of molt-inhibiting hormone from the kuruma prawn, *Marsupenaeus japonicus*. *J. Biol. Chem.* **278**, 9620-9623.

Katayama, H., Ohira, T., Nagata, S. and Nagasawa, H. (2004). Structure-activity relationship of crustacean molt-inhibiting hormone from the kuruma prawn *Marsupenaeus japonicus*. *Biochemistry* **43**, 9629-9635.

Kegel, G., Reichwein, B., Weese, S., Gaus, G., Peter-Katalinic, J. and Keller, R. (1989). Amino acid sequence of the crustacean hyperglycemic hormone (CHH) from the shore crab, *Carcinus maenas*. *FEBS Lett.* **255**, 10-14.

Keller, R. and Sedlmeier, D. (1988). A metabolic hormone in crustaceans: the hyperglycemic neuropeptide. In *Endocrinology of Selected Invertebrate Types* (ed. H. Laufer and G. H. Downer), pp. 315-326. New York: Alan R. Liss.

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

Kou, C. M. and Yang, Y. H. (1999). Hyperglycemic responses to cold shock in the freshwater giant prawn, *Macrobrachium rosenbergii*. *J. Comp. Physiol. B* **169**, 49-54.

Kummer, G. and Keller, R. (1993). High-affinity binding of crustacean hyperglycaemic hormone (CHH) to hepatopancreatic plasma membranes of the crab *Carcinus maenas* and the crayfish *Orconectes limosus*. *Peptides* **14**, 103-108.

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

Lee, K. J., Doran, R. M. and Mykles, D. L. (2007). Crustacean hyperglycemic hormone from the tropical land crab, *Gecarcinus lateralis*: cloning, isoforms, and tissue expression. *Gen. Comp. Endocrinol.* **154**, 174-183.

Liu, L., Laufer, H., Wang, Y. J. and Hayes, T. K. (1997). A neurohormone regulating both methyl farnesoate synthesis and glucose metabolism in a crustacean. *Biochem. Biophys. Res. Commun.* **237**, 694-701.

Maynard, D. M. (1961). Thoracic neurosecretory structures in Brachyura. II. Secretory neurons. *Gen. Comp. Endocrinol.* **1**, 237-263.

Moscos, A., Edomi, P., Guarnaccia, C., Lorenzon, S., Pongor, S., Ferrero, E. A. and Giulianini, P. G. (2008). Functinal aspects of cHH C-terminal amidation in crayfish species. *Reg. Peptides* **147**, 88-95.

Moyle, W. R., Campbell, R. K., Myers, R. V., Bernard, M. P., Han, Y. and Wang, X. (1994). Co-evolution of ligand-receptor pairs. *Nature* **368**, 251-255.

Ohira, T., Nishimura, T., Sonobe, H., Okuno, A., Watanabe, T., Nagasawa, H., Kawazoe, I. and Aida, K. (1999). Expression of a recombinant molt-inhibiting hormone of the kuruma prawn *Penaeus japonicus* in *Escherichia coli*. *Biosci. Biotech. Biochem.* **63**, 1576-1581.

Ohira, T., Tsutsui, N., Nagasawa, H. and Wilder, M. N. (2006). Preparation of two recombinant crustacean hyperglycemic hormones from the giant freshwater prawn, *Machrobrachium rosenbergii*, and their hyperglycemic activities. *Zool. Sci.* **23**, 383-391.

Okumura, T., Ohira, T., Katayama, H. and Nagasawa, H. (2005). *In vivo* effects of a recombinant molt-inhibiting hormone on molt interval and hemolymph ecdysteroid level in the kuruma prawn, *Marsupenaeus japonicus*. *Zool. Sci.* **22**, 317-320.

Ollivaux, C. and Soyez, D. (2000). Dynamics of biosynthesis and release of crustacean hyperglycemic hormone isoforms in the X-organ-sinus gland of the crayfish, *Orconectes limosus*. *Eur. J. Biochem.* **267**, 5106-5114.

Phillips, J. E., Meredith, J., Audsley, N., Ring, M., Macins, A., Brock, H., Theilmann, D. and Littleford, D. (1998). Locust ion transport peptide (ITP): function, structure, cDNA and expression. In *Recent Advances in Arthropod Endocrinology: (Society for Experimental Biology Seminar Series 65)* (ed. G. M. Coast and S. G. Webster), pp. 210-226. Cambridge: Cambridge University Press.

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

Tiu, S. H., He, J. G. and Chan, S. M. (2007). The LvCHH-ITP gene of the shrimp (*Litopenaeus vannamei*) produces a widely expressed putative ion transport peptide (LvITP) for osmo-regulation. *Gene* **396**, 226-235.

Toullec, J.-Y., Serrano, L., Lopez, P. and Spanings-Pierrot, C. (2006). The crustacean hyperglycemic hormones from an euryhaline crab *Pachygrapsus marmoratus* and a fresh water crab *Potamon ibericum*: Eyestalk and pericardial isoforms. *Peptides* **27**, 1269-1280.

Tsutsui, N., Katayama, H., Ohira, T., Nagasawa, H., Wilder, M. N. and Aida, K. (2005). The effects of crustacean hyperglycemic hormone-family peptides on vitellogenin gene expression in the kuruma prawn, *Marsupenaeus japonicus*. *Gen. Comp. Endocrinol.* **144**, 232-239.

Webster, S. G. (1991). Amino acid sequence of putative moult-inhibiting hormone from the crab *Carcinus maenas*. *Proc. R. Soc. Lond. B, Biol. Sci.* **244**, 247-252.

Webster, S. G. (1993). High-affinity binding of putative moult-inhibiting hormone (MIH) and crustacean hyperglycaemic hormone (CHH) to membrane bound receptors on the Y-organ of the shore crab, *Carcinus maenas*. *Proc. R. Soc. Lond. B, Biol. Sci.* **251**, 53-59.

Webster, S. G. (1996). Measurement of crustacean hyperglycaemic hormone levels in the edible crab *Cancer pagurus* during emersion stress. *J. Exp. Biol.* **199**, 1579-1585.

Webster, S. G. (1998). Neuropeptides inhibiting growth and reproduction in crustaceans. In *Recent Advances in Arthropod Endocrinology (Society for Experimental Biology Seminar Series 65)* (ed. G. M. Coast and S. G. Webster), pp. 32-52. Cambridge: Cambridge University Press.

Webster, S. G. and Keller, R. (1986). Purification, characterization and amino acid composition of the putative moult-inhibiting hormone (MIH) of *Carcinus maenas* (Crustacea, Decapoda). *J. Comp. Physiol. B* **156**, 617-624.

Webster, S. G., Dircksen, H. and Chung, J. S. (2000). Endocrine cells in the gut of the shore crab *Carcinus maenas* immunoreactive to crustacean hyperglycemic hormone and its precursor-related peptide. *Cell Tissue Res.* **300**, 193-205.

Weidemann, W., Cromoll, J. and Keller, R. (1989). Cloning and sequence analysis of cDNA for precursor of a crustacean hyperglycemic hormone. *FEBS Lett.* **257**, 31-34.

Yasuda, A., Yasuda, Y., Fujita, T. and Naya, Y. (1994). Characterization of crustacean hyperglycemic hormone from the crayfish (*Procambarus clarkii*): multiplicity of molecular forms by stereoinversion and diverse functions. *Gen. Comp. Endocrinol.* **95**, 387-398.

Zhao, Y., Meredith, J., Brock, H. W. and Phillips, J. E. (2005). Mutational analysis of the N-terminus in Schistocerca gregaria ion-transport peptide expressed in *Drosophila* Kc1 cells. *Arch. Insect Biochem. Physiol.* **58**, 27-38.