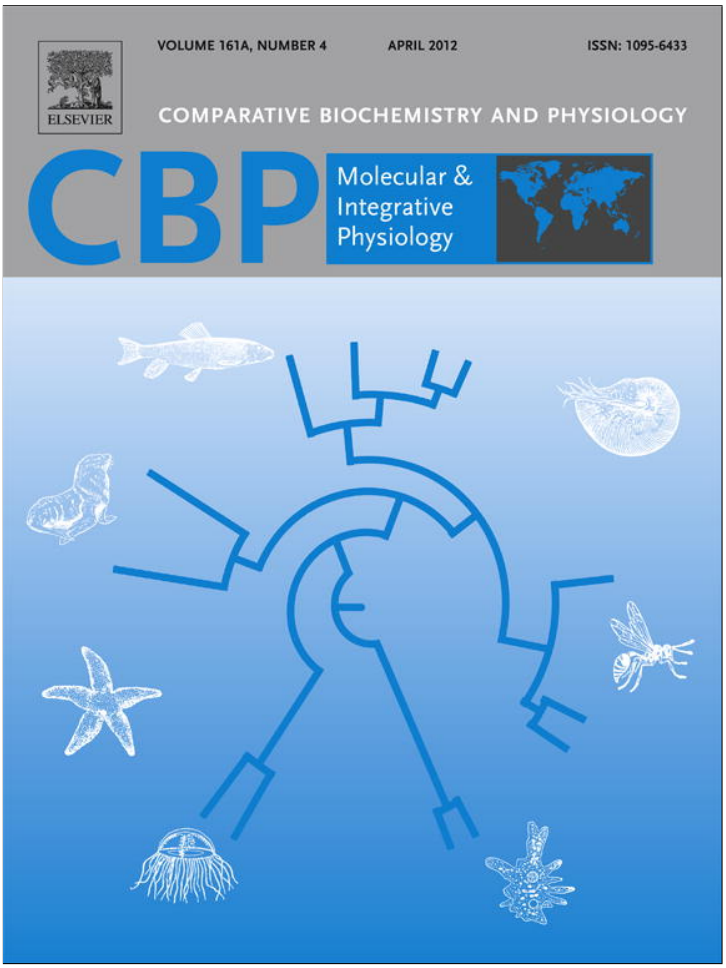

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Demonstration of expression of a neuropeptide-encoding gene in crustacean hemocytes

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ABSTRACT

Crustacean hyperglycemic hormone (CHH) was originally identified in a neuroendocrine system-the X-organ/sinus gland complex. In this study, a cDNA (*Prc-CHH*) encoding CHH precursor was cloned from the hemocyte of the crayfish *Procambarus clarkii*. Analysis of tissues by a CHH-specific enzyme-linked immunosorbent assay (ELISA) confirmed the presence of CHH in hemocytes, the levels of which were much lower than those in the sinus gland, but 2 to 10 times higher than those in the thoracic and cerebral ganglia. Total hemocytes were separated by density gradient centrifugation into layers of hyaline cell (HC), semi-granular cell (SGC), and granular cell (GC). Analysis of extracts of each layer using ELISA revealed that CHH is present in GCs (202.8 ± 86.7 fmol/mg protein) and SGCs (497.8 ± 49.4 fmol/mg protein), but not in HCs. Finally, CHH stimulated the membrane-bound guanylyl cyclase (GC) activity of hemocytes in a dose-dependent manner. These data for the first time confirm that a crustacean neuropeptide-encoding gene is expressed in cells essential for immunity and its expression in hemocytes is cell type-specific. Effect of CHH on the membrane-bound GC activity of hemocyte suggests that hemocyte is a target site of CHH. Possible functions of the hemocyte-derived CHH are discussed.

1. Introduction

Crustacean hyperglycemic hormone (CHH) is a polypeptide hormone originally identified in a crustacean neuroendocrine complex, the X-organ/sinus gland (XO/SG) in the eyestalk (Keller, 1992; Soyez, 1997). The presence of CHH-related peptides in the extra-eyestalk tissues was proposed more than a decade ago based on molecular cloning and immunological data (de Kleijn et al., 1995; Chang et al., 1998). It was subsequently reported that a CHH identical to that originally found in the XO/SG complex is expressed in the gut of *C. maenas*, and that its release into hemolymph during late premolt and during ecdysis is involved in ecdysis-related water and ion uptake (Chung et al., 1999). Further, a CHH structural variant, CHH-like peptide (CHH-L), has been purified from the pericardial organs of 3 brachyurans (Dircksen et al., 2001; Chung and Zmora, 2008; Tsai et al., 2008) and recently from the thoracic ganglia of an astacidean (Wu et al., in press). CHH and CHH-L are products of alternatively spliced transcripts; they share an identical N-terminal sequence (residues 1–40), but differ considerably in the remaining sequence (Dircksen et al., 2001; Chen et al., 2004; Lee et al., 2007; Chung and Zmora, 2008; Tsai et al., 2008).

Studies of *CHH* gene expression suggested that CHH/CHH-L peptides are probably more widely expressed; transcript encoding CHH or CHH-L peptide have been found in several tissues other than those mentioned above, including the brain, ventral nerve cord, gill, antennal glands, testes, Y-organs, hindgut, and spermatophore sac (Chen et al., 2004; Lee et al., 2007; Tsai et al., 2008; Li et al., 2010; Zheng et al., 2010).

Functionally, it is in general accepted that the SG-derived CHH is involved in carbohydrate metabolism and in stress-induced hyperglycemia (Santos and Keller, 1993; Webster, 1996; Chang et al., 1998; Chang et al., 1999; Zou et al., 2003; Lorenzon et al., 2004). Other physiological processes, including molting, osmoregulation, and reproduction (Chang et al., 1990; Yasuda et al., 1994; Khayat et al., 1998; Spanings-Pierrot et al., 2000; Serrano et al., 2003; Tsutsui et al., 2005; Chung and Webster, 2006) may also be regulated by CHH. As for CHH-L, its biological function(s) have yet to be established (Dircksen et al., 2001; Ohira et al., 2006; Chung and Zmora, 2008; Chang et al., 2010).

Crustacean hemocytes are essential for immunity, carrying out important immune functions such as phagocytosis, encapsulation, melanization, cytotoxic activity, etc. (see Söderhäll and Thörnqvist, 1997; Johansson et al., 2000; Cerenius et al., 2011). In pilot experiments, we had obtained data showing that circulating hemocytes express CHH-encoding transcript, prompting us to inquire in greater depth the expression of the neuropeptide gene in crustacean immune

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cells. The aims of the present study were to determine whether *CHH* gene is expressed in crustacean circulating hemocytes and whether hemocyte is a target site for the actions of CHH. We report data confirming the expression of *CHH* gene in the hemocytes of a crustacean species (the red swamp crayfish *Procambarus clarkii*), revealing cell type-specific expression of CHH in hemocytes, and implicating the hemocyte as a possible target for the actions of CHH. Discussion is made regarding possible roles of the hemocyte-derived CHH.

2. Materials and methods

2.1. Animals and preparation of tissue samples

Animals (*P. clarkii*) were purchased from local fisherman and maintained in the laboratory as previously described (Zou et al., 2003). Intermolt adults of both sexes were used in the present study.

Hemolymph was withdrawn from animals using a syringe coupled to a 26-G needle, diluted 3:1 (V:V) with an anti-coagulant (0.15 M NaCl, 0. 1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, 10 mM EDTA, pH 4.6, Söderhäll and Smith, 1983), centrifuged at 800 g for 10 min, and washed with crayfish saline (Van Harreveld, 1936). The resulting preparations of hemocyte pellet were immediately used for total RNA extraction (see Section 2.2) and for guanylyl cyclase activity assay (see Section 2.5).

For ELISA analysis (see Section 2.3), hemocytes were collected as described above, and the sinus gland, thoracic ganglia, cerebral ganglia and abdominal muscle dissected out from ice-anesthetized animals; these tissues were separately homogenized in saline, and the homogenates centrifuged at 12,500 g for 20 min at 4 °C. Aliquots of the supernatants were determined for the amount of total protein using a commercially available assay kit and bovine serum globulin (Bio-Rad) as standard; the rest of the supernatants were saved at −20 °C until ELISA analysis.

2.2. Polymerase chain reaction (PCR) cloning of CHH transcripts

Total RNA was extracted from freshly prepared hemocytes samples (TRIzol® Reagent, Invitrogen), treated with RQ1 RNase-free DNase (Promega), and reverse-transcribed into cDNA according to previously described protocols (Tsai et al., 2008).

For PCR amplification of crayfish hemocyte cDNA samples, the primers used were SP-F1 (5′-GATGTGGTCGTTGGTGGTGGT-3′) and CHH-mr (5′-CAGATCTGTCACCTTACTTGCC-3′) designed based on a transcript (GenBank accession no. **AB027291**) encoding a crayfish CHH precursor. PCR reactions were performed using a DNA Thermal Cycler (MJ Research) under the following conditions: an initial denaturation (5 min, 94 °C), 35 cycles of denaturation (30 s, 94 °C), annealing (30 s, 60 °C), and extension (1 min, 72 °C), followed by a final extension (7 min, 72 °C).

After PCR or RACE amplification, an aliquot of the reaction was separated on a 1.2% agarose gel and visualized with GelStar® (Cambrex). Reagents and procedures used for purification, cloning, and auto-sequencing of the PCR products, as well as software resources for sequence analyses, were as described by Tsai et al. (2008).

2.3. Sandwich enzyme-linked immunosorbent assay (ELISA)

A sandwich ELISA using two CHH-specific antibodies was developed in order to quantify CHH levels in crayfish tissues. One of the antibodies, the capture antibody (anti-CHH), was raised against high performance liquid chromatograph (HPLC)-purified CHH and its specificity for CHH has been reported (Lee et al., 2001). A second anti-CHH antibody, anti-CHH (59–72), was raised in rabbits against a synthetic peptide (IDVVDEYISGVQTV-NH_2) that corresponds in sequence to a C-terminal stretch (from the 59th to the 72nd residues) of CHH (Yasuda et al., 1994) and is N-terminally coupled to keyhole limpet hemocyanin; specificity of anti-CHH (59–72) for CHH has been demonstrated (Wu et al., in press). Immunoglobulin G (IgG) fraction was purified from the antisera by a protein G column and the IgG fraction of anti-CHH (59–72) further biotinylated using protocols as previously described (Zou et al., 2003).

The ELISA reagents, procedure, and data processing were as described previously (Zou et al., 2003). Standard curve for CHH was generated by assaying a serial dilution of HPLC-purified CHH (Wu et al., in press); sample titration curve was also generated by assaying a serial dilution of each sample homogenates (sinus gland, cerebral ganglia, thoracic ganglia, or hemocyte). CHH and tissue homogenates were assayed concurrently. These sample titration curves were found to be parallel to CHH standard curve over a range of 10 to 100× (depending on the tested tissue) sample dilution (data not shown). Only data obtained from the parallel region of the sample titration curves were used for determining tissue CHH levels.

Wells ($n=3$ per plate) serving as backgrounds were assayed using the ELISA procedure mentioned above with the exception that blocking buffer (2% bovine serum albumin in 0.01 M phosphate buffered saline) was added instead of standard or tissue sample. The assay sensitivity (*i.e.*, the lowest amounts of CHH giving an optical density significantly greater than background) was approximately 1 fmol/well.

Specificity of the ELISA for CHH was tested in two experiments. First, the ELISA procedure was carried out as described (Zou et al., 2003) except that HPLC-purified CHH-L (Wu et al., in press), instead of CHH, was assayed. Second, CHH or tissue samples (sinus gland, thoracic ganglia, or hemocyte) were serially diluted and assayed using the procedure except that the biotinylated antibody was preadsorbed with the synthetic peptide (100 μg/mL) that was used for raising anti-CHH (59–72).

2.4. Separation of hemocytes by density gradient centrifugation

Hemolymph was withdrawn from *P. clarkii* and mixed 3:1 (V:V) with the anticoagulant (see Section 2.1). Separation of hemocytes was achieved by centrifugation using Percoll (Amersham Biosciences) in a continuous density gradient according to Söderhäll and Smith (1983). Separated cell layers were collected from the centrifuge tube using a 23-G syringe and washed with 0.1 M phosphate-buffered saline. Cell type of each separated hemocyte layer was identified under a microscope (Axioskop-2, Zeiss). Photomicrographs of hemocytes were taken using a digital camera system (Penguin 600 CL, Pixera®) and the morphometric measurements (cell length, cell width, nuclear area, and cytoplasmic area) of hemocytes determined using computer software (In Studio, Pixera®).

Hemocytes of each layer were also processed using methods mentioned above (see Section 2.1.) for determination of total protein and ELISA analysis.

Fig. 1. Sequences of CHH and CHH-like peptide (CHH-L) in the crayfish *Procambarus clarkii*. The mature CHH peptide encoded *by Prc-CHH* (GenBank accession no. **JQ060964**) cloned in the present study and the mature CHH-L encoded by a previously cloned cDNA (GenBank accession no. **AF474408**) are aligned. Note that CHH and CHH-L share an identical N-terminal sequence (residues 1–40).

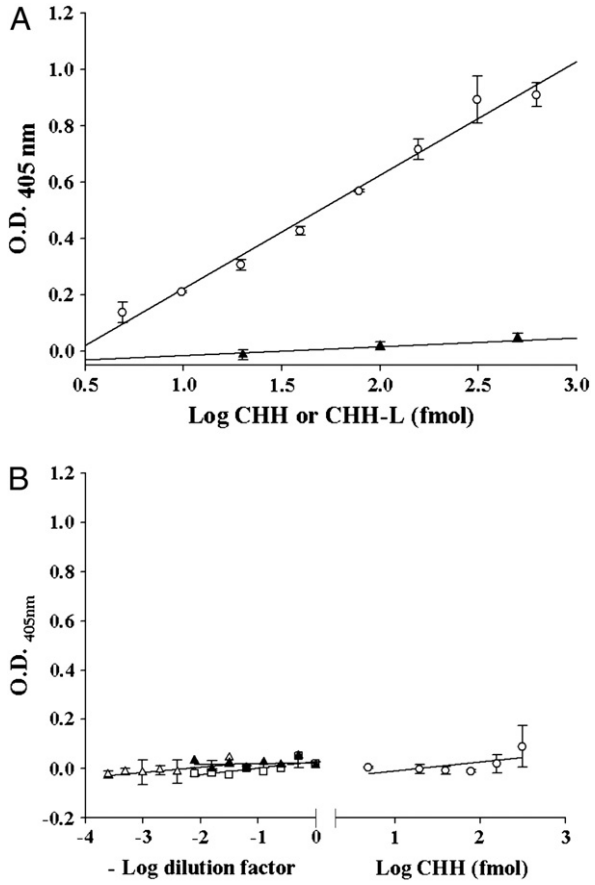

Fig. 2. Characterization of the CHH-specific enzyme-linked immunosorbent assay. (A) HPLC-purified CHH (○, 4.88–625.00 fmol/well) or CHH-L (▲, (20.00–500.00 fmol/well) was serially diluted and assayed using the assay procedure mentioned in the Materials and methods. The 2 antibodies used in the assay were anti-CHH and anti-CHH (59–72) (Lee et al., 2001; Wu et al., in press). (B) Serially diluted tissue (Δ: sinus gland, ▲: hemocyte, □: thoracic ganglia) or purified CHH (○, 4.88–312.50 fmol/well) samples were assayed using the same procedure except that the anti-CHH (59–72) was preadsorbed with the synthetic peptide (100 μg/mL) that was used for raising the antibody. Data are presented as a mean ± S.D. ($n = 3$).

2.5. Membrane-bound guanylyl cyclase (EC 4.6.1.2) activity in crayfish hemocytes

Crayfish hemocyte samples, prepared from 15-mL hemolymph as described above (see Section 2.1), were disrupted by sonication in 1 mL homogenization buffer containing 10% glycerol, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 μg/mL leupeptin, 10 μg/mL aprotinin and centrifuged at 100,000 g for 20 min at 4 °C. The resulting pellets were washed with 1 mL homogenization buffer and centrifuged at 100,000 g for 20 min for 2 times. The final pellets were re-suspended in 500 μL of homogenization buffer and sonicated; the supernatant was collected by centrifugation at 12,500 g for 20 min at 4 °C. An aliquot of the supernatants was determined for the amount of total protein. To initiate cyclase activity, another aliquot of the supernatant (containing 15 μg of membrane protein) was mixed with 50 μL of 2× reaction buffer containing 50 mM HEPES, pH7.4, 4 mM GTP, 2 mM 3-isobutyl-1-methylxanthine (IBMX), 4 mM ATP, 20 mM sodium azide, 1 mg/mL bovine serum albumin, 240 mM NaCl, 10 mM $MgCl_2$ (Chao et al., 2010) in the absence or presence of HPLC-purified CHH (Wu et al., in press) in a final reaction volume of 100 μL. After incubation for 30 min at 37 °C, the reaction was stopped by adding 250 μL of ice-cold 100% ethanol. After centrifugation (3000 g, 5 min, 4 °C), the supernatant was dried in a speed-vacuum concentrator, and cyclic guanosine 3′,5′-monophosphate (cGMP) content was measured by a competitive cGMP assay kit (CatchPoint cGMP fluorescent assay kit, Molecular Devices) according to the manufacturer's instruction. cGMP levels were calculated and expressed as pmol/min/mg protein and taken as an indicator of guanylyl cyclase activity (as the incubation was performed in the presence of IBMX, a phosphodiesterase inhibitor). Effect of CHH on the guanylyl cyclase activity was analyzed by one-way analysis of variance followed by Tukey's HSD test (SPSS Manager, SPSS Inc.).

Table 1
CHH levels in the tissues of the crayfish *Procambarus clarkii*.

Tissue	CHH levels[a]	
	fmol/mg protein	fmol/tissue
Sinus gland	$310.1 \times 10^4 \pm 7.0 \times 10^4$	$2.2 \times 10^4 \pm 0.5 \times 10^4$
Thoracic ganglia	116.1 ± 26.8	67.8 ± 15.7
Cerebral ganglia	31.0 ± 11.7	5.4 ± 3.0
Hemocyte	316.1 ± 26.8	174.3 ± 74.6 (fmol/mL)[b]
Muscle	ND	ND

ND: not detectable.
[a] Data are presented as a mean ± S.D.; $n = 5$ for each tissue.
[b] Value is obtained for hemocytes derived from 1 mL of hemolymph.

3. Results

To examine if *CHH* gene, a neuropeptide-encoding gene, is expressed in crustacean immune cells, cDNA samples derived from circulating hemocytes of the crayfish *P. clarkii* were amplified using a pair of CHH-specific primers. A cDNA (*Prc-CHH*) of 437 base pairs was thus cloned from the crayfish hemocyte. The amino acid sequence of the encoded CHH precursor is identical to that of a previously documented CHH precursor (GenBank accession no. **AB027291**) except substitution of a residue (serine to leucine)

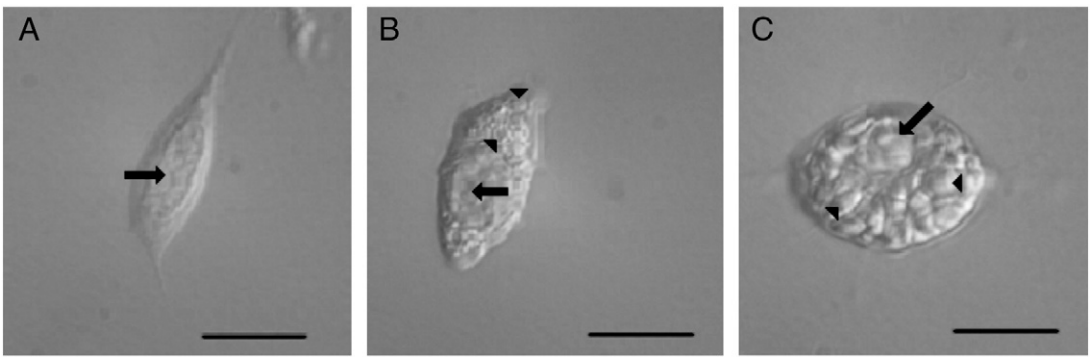

Fig. 3. Photomicrograph of the three types of hemocyte in the crayfish *Procambarus clarkii*. Hemolymph withdrawn from animals was centrifuged in a continuous density gradient of Percoll. Three distinct cell layers were resolved: (A) hyaline cell, (B) semi-granular cell, and (C) granular cell. Nucleus was indicated by an arrow and granules by arrowheads. Scale = 10 μm. See Table 2 for the morphometric data and CHH levels of each cell type.

located at the CHH precursor-related peptide of the precursor (data not shown); its mature peptide has a C-terminal amidation signal sequence (-GK) (Fig. 1).

To confirm the presence of CHH peptide and to quantify its levels in hemocytes, a sandwich enzyme-linked immunosorbent assay (ELISA) was developed, intended to specifically detect the crayfish CHH (Yasuda et al., 1994), but not an alternatively spliced *CHH* gene product—CHH-L (see Fig. 1), by including in the ELISA assay an antibody, anti-CHH (59–72), specific for the C-terminus of CHH (Wu et al., in press; also see Fig. 1). A typical CHH standard curve is shown in Fig. 2A. ELISA analysis of CHH-L, instead of CHH, resulted in low values of optical density (Fig. 2A) similar to background reading. To further evaluate specificity of the ELISA, assay procedure was carried out with anti-CHH (59–72) pre-adsorbed with the synthetic peptide (100 μg/mL) that was used for raising the antibody. CHH standards or tissue sample thus assayed yielded low values of optical density (Fig. 2B) similar to background reading.

The developed ELISA was employed to quantify CHH levels in hemocyte samples, and for comparison, in the sinus gland, thoracic ganglia, cerebral ganglia, and muscle. CHH levels are the highest in the sinus glands – $310.6\times10^4\pm7.0\times10^4$ fmol/mg protein ($2.2\times10^4\pm0.5\times10^4$ fmol/tissue), followed by the hemocyte (316.1 ± 26.8 fmol/mg protein; 174.3 ± 74.6 fmol/mL), thoracic ganglia (116.1 ± 26.8 fmol/mg protein; 67.8 ± 15.7 fmol/tissue), and cerebral ganglia (31.0 ± 11.7 fmol/mg protein; 5.4 ± 3.0 fmol/tissue) (Table 1). CHH was not detectable in the muscle when assayed by the ELISA (Table 1).

To determine and compare CHH levels among different types of hemocyte, hemolymph samples were separated using a continuous density gradient centrifugation. Three cell layers were resolved; microscopic examination of cells taken from these layers revealed these include a hyaline cell (HC) layer, a semi-granular cell (SGC) layer, and a granular cell (GC) layer. Hyaline cells are ovoid or fusiform in shape with a mean nuclear to cytoplasmic ratio (N/C ratio) of 0.26 ± 0.07 and characterized by the absence of granules in the cytoplasm; SGCs are fusiform in shape with a mean N/C ratio (0.22 ± 0.06) similar to that of HCs and small cytoplasmic granules of approximately 1 μm in diameter, whereas GCs are ovoid in shape with a relatively small (0.15 ± 0.04) mean N/C ratio and large cytoplasmic granules of approximately 2–3 μm in diameter (Fig. 3; Table 2).

CHH levels in the extracts derived from each type of hemocytes were determined by the ELISA. The results show that CHH was not detectable in HCs, but were present in SGCs and GCs, the levels of which are 497.8 ± 49.4 and 202.8 ± 86.7 fmol/mg protein, respectively (Table 2).

To determine the effect of CHH on the membrane-bound guanylyl cyclase activity of hemocyte, membrane preparations were incubated in the absence or presence of different doses of purified CHH. Cyclic guanosine 3′,5′-monophosphate (cGMP) levels were then quantified after incubation. Data presented in Fig. 4 show that CHH within a physiologically relevant range dose-dependently increased the guanylyl cyclase activity in the membrane fractions of hemocytes.

4. Discussion

Data reported in the present study for the first time provide evidence that *CHH* gene is expressed, in additional to neuroendocrine tissues where CHH has been originally identified and extensively studied (Fanjul-Moles, 2006; Chung et al., 2010), in circulating hemocytes, which are known to assume important immune functions in crustaceans (Cerenius et al., 2011).

Deduced amino acid sequence of the putative mature peptide encoded by the cloned *Prc-CHH* has a C-terminal amidation signal sequence (-GK), processing of which would result in the C-terminally amidated CHH, which has been purified from the sinus gland (Yasuda et al., 1994). Presently, it is not known if transcripts encoding other CHH or CHH-L are expressed in hemocytes. More cloning efforts are required to reveal a complete repertoire of the transcripts expressed in hemocytes.

Table 2
Morphometric measurements and CHH levels of the different types of hemocyte in the crayfish *Procambarus clarkii*.

Cell type	Cell length[a] (μm)	Cell width[a] (μm)	N/C ratio[a,b]	CHH levels[c] (fmol/mg protein)
Hyaline cell	11.3 ± 0.4	8.0 ± 0.2	0.26 ± 0.07	ND
Semi-granular cell	12.9 ± 0.3	8.5 ± 0.5	0.22 ± 0.06	497.8 ± 49.4
Granular cell	14.2 ± 0.3	11.0 ± 0.2	0.15 ± 0.04	202.8 ± 86.7

ND: not detectable.
[a] Data are presented as a mean ± S.D.; $n=30$ for each cell type.
[b] Nuclear to cytoplasmic ratio.
[c] Data are presented as a mean ± S.D.; $n=5$ for each cell type.

Presence of hemocyte-derived CHH at the protein level was confirmed by a quantitative CHH-specific ELISA that revealed modest levels of CHH in crayfish hemocytes. Although CHH levels in hemocytes are far much lower than those in the sinus glands, they are approximately 2 to 10-fold higher than those in the cerebral ganglia and thoracic ganglia, where comparable CHH levels had been previously reported (Chang et al., 1999). Assuming the hemolymph volume (mL) is 25% of its body mass (g) (Prosser and Weinstein, 1950), the total hemocyte content of CHH estimated for an adult animal of 25 g, the average mass of the animals used in this study, is 1090 fmol/animal-approximately 2.5% of the sinus gland CHH content. It should be mentioned that hematopoiesis is an active process that promptly replenishes the circulating hemocyte levels, which dramatically decrease during an infection (Söderhäll et al., 2003; Wu et al., 2008). Thus, hemocytes represent a modest but steady source of CHH.

Three types of hemocyte were resolved using a density gradient centrifugation. Morphological and cellular characteristics of each cell type are in general consistent with those previously reported for the same species (Lanz et al., 1993). Quantification of CHH levels in each cell type by ELISA revealed a cell type-specific distribution of CHH, with CHH being detected in the granulated SGCs and GCs, but not in the agranulated HCs.

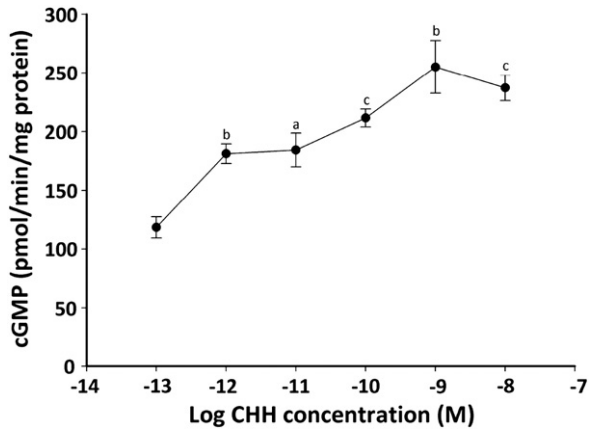

Fig. 4. Effect of CHH on hemocyte membrane-bound guanylyl cyclase activity in the crayfish *Procambarus clarkii*. Membrane fractions were prepared from hemocyte preparation and incubated in the absence (as the basal reaction) or presence of various concentrations of CHH. After incubation for 30 min at 37 °C, the reaction was stopped and centrifugation, and the supernatant dried and quantified for cGMP levels using a competitive cGMP assay kit. Data are presented as a mean ± S.D. ($n=4$). [a],[b],[c] represent statistically different from the basal level at 5%, 1%, and 0.1% levels, respectively.

Presently, the regulatory functions of the hemocyte-derived CHH can only be speculated. One scenario would be that it, when released into hemolymph, functions like the sinus gland-derived CHH as a hyperglycemic regulator by acting on the glycogen depots, mainly the muscle and hepatopancreas (see Fanjul-Moles, 2006). However, considering the relative levels of CHH present in hemocytes, it argues against the possibility that the hemocyte-derived CHH would significantly contribute to its hemolymph levels. Indeed, it had been shown that eyestalk-ablated animals failed to exhibit a hyperglycemic response when subject to stressful conditions, which are known to stimulate the release of CHH from the sinus glands of the eyestalk ganglia, indicating that CHH of extra-eyestalk sources is of little significance in evoking hyperglycemia (Fingerman et al., 1981; Reddy et al., 1994; Webster, 1996; Lorenzon et al., 1997).

Alternatively, it is suggested that the hemocyte-derived CHH may act in an autocrine/paracrine manner on hemocytes. Data concurrent with the suggestion are those showing that CHH increased in a dose-dependent manner the membrane-bound guanylyl cyclase activity of hemocytes. Previous results have revealed that cGMP plays the role of a second messenger for the actions of CHH on the target tissues and that CHH activates the cGMP pathway through activation of membrane-bound guanylyl cyclase activity (Sedlmeier and Keller, 1981; Sedlmeier, 1982; Goy et al., 1987; Goy, 1990; Nagai et al., 2009; see Mykles et al., 2010 and Covi et al., 2009 for reviews).

In this respect, hemocyte-derived CHH may regulate carbohydrate metabolism in hemocyte, as crustacean hemocyte has been found to be an important site for carbohydrate storage and metabolism (Johnston and Davies, 1972; Johnston et al., 1970, 1973). Another possibility is that the hemocyte-derived CHH may have direct immunoregulatory functions. Accumulated data gathered from vertebrate studies have demonstrated endogenous production of neuropeptides in immune tissues and firmly established that these immune tissue-derived peptides play pivotal roles in immune regulation (see Weigent and Blalock, 1997; Blalock and Smith, 2007). The distinct pattern of CHH expression observed in different types of hemocytes, which are functionally distinct (Johansson et al., 2000; Cerenius et al., 2011), implies specific functional roles for the hemocyte-derived CHH. Interestingly, a recent study reported that injection of CHH into shrimps (*Litopenaeus vannemei*) significantly increased total hemocyte count, serum protein levels, and phenoloxidase activity, and that, in pathogen-challenged shrimps, CHH increased the pathogen clearance rate and the survival rate of the animals, suggesting an immune stimulatory role for CHH (Wanlem et al., 2011).

In conclusion, this is the first report for any crustacean species that a neuropeptide-encoding gene (*i.e.*, *CHH*) is expressed in hemocytes; its expression in hemocytes is cell type-specific, restricting to the semi-granular and granular cells. Despite the regulatory functions are presently unknown for the hemocyte-derived CHH, it is suggested that hemocyte-derived CHH may be acting as an autocrine/paracrine agent on hemocytes. It is plausible to assume that, although CHH levels in hemocytes are modest, release of CHH by tissue-infiltrating hemocytes would result in physiologically relevant levels of CHH in tissues, into which hemocytes are actively recruited during an infection (Vincent and Lotz, 2005; Soonthornchai et al., 2010). Though presently not supported by direct experimental data, the proposition that the hemocyte-derived CHH assumes immunoregulatory functions is worth examining.

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