Changes in the Amounts of the Molt-Inhibiting Hormone in Sinus Glands during the Molt Cycle of the American Crayfish, Procambarus clarkii

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ABSTRACT—The purposes of this study are to determine the molt cycle of the American crayfish, *Procambarus clarkii*, and to quantify the amounts of the molt-inhibiting hormone (Prc-MIH) in the hemolymph and neurohemal sinus glands during the molt cycle of the American crayfish. The molt cycle was classified into six stages based on the changes in volumes of gastroliths in the stomach and ecdysteroid titers in the hemolymph. A sandwich-type enzyme immunoassay using specific antibodies raised against N-terminal and C-terminal segments of Prc-MIH was developed for the Prc-MIH assay. It is sensitive to as little as 0.5 fmol of Prc-MIH (3.3×10^{-12} M). In the hemolymph, no Prc-MIH could be detected at any of the molt stages tested. However, in the sinus gland, it was demonstrated that the amount of Prc-MIH changes in a molt-stage-specific manner during the molt cycle. It was particularly noteworthy that the initiation of a molting sequence (i.e., entering the early premolt stage) corresponded to the increase in Prc-MIH content in the sinus gland, because the finding is consistent with the hypothesis that crustaceans enter the premolt stage when the MIH secretion from the sinus gland is reduced or ceases.

INTRODUCTION

Molting in crustaceans is regulated by ecdysteroids (molting hormone) secreted from the Y-organ. Regarding the regulation of ecdysteroid secretion from the Y-organ, it has been demonstrated that (1) the eyestalk ablation during the intermolt stage activates ecdysteroid secretion from the Y-organ to induce the precocious molting (Keller and Schmid, 1979; Jegla *et al.*, 1983; Sonobe *et al.*, 1991), (2) a neuropeptide, the molt-inhibiting hormone (MIH) that is secreted from the X-organ sinus gland system in the eyestalk, inhibits ecdysteroid secretion from the Y-organ cultured *in vitro* (Soumoff and O'Connor, 1982; Mattoson and Spaziani, 1985; Webster, 1986), and (3) the MIH activity can be assayed by inhibition of ecdysteroid secretion from the Y-organ cultured *in vitro* (Webster and Keller, 1986; Huberman and Aguilar, 1989; Sefiani *et al.*, 1996; Terauchi *et al.*, 1996). Based on these results, MIHs have been isolated from sinus glands and their primary structures were determined in various crustaceans

(Chang *et al.*, 1990; Webster, 1991; Aguilar *et al.*, 1996; Chung *et al.*, 1996; Nagasawa *et al.*, 1996; Yang *et al.*, 1996). Furthermore, cDNAs encoding these peptides have been also isolated in a number of species (Klein *et al.*, 1993; Lee *et al.*, 1995; Ohira *et al.*, 1997). Thus, at present, it is presumed that MIHs inhibit molting by suppressing ecdysteroid secretion from the Y-organ during the intermolt stage; on the other hand, molting is induced by being freed from the inhibitory regulation by MIH during the premolt stage (for reviews, Skinner, 1985; Jegla, 1989; Webster, 1998). To prove the hypothesis, therefore, it is important to examine whether MIH titer in the hemolymph changes in correlation with changes in ecdysteroid titers in the hemolymph during the molt cycle, and whether MIH content in the neurohemal sinus glands changes during the molt cycle.

In this study, we first determined the molt cycle of the crayfish, *Procambarus clarkii*, based on the changes in volumes of gastroliths in the stomach and ecdysteroid titers in the hemolymph, and subsequently attempted to quantify Prc-MIH levels in the hemolymph and sinus glands by a sandwich-type enzyme immunoassay (EIA).

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MATERIALS AND METHODS

Animals

Freshwater crayfish, *Procambarus clarkii*, were obtained from local dealers. They were maintained in tanks at a constant temperature of 19°C in the laboratory and fed a pellet diet 3 times per week. Female crayfish, carapace width 20±1 mm, were used for our experiments.

Molt staging

Molt stages were determined based on quantitative changes in the volumes of gastroliths in the stomach and ecdysteroid titers in the hemolymph. The gastrolith growth was monitored using an X-ray-apparatus E-3 (Softex, Japan). The volume of the gastrolith, which is a semidisk-shaped structure, was calculated according to the formula for calculating one-half of the volume of an ellipsoidal body revolving on its short axis: $1/2[4/3\pi X(1/2Y)^2]$ (X and Y indicate short and long axes of the gastrolith on the radiograph).

To examine the changes in ecdysteroid titers in the hemolymph by radioimmunoassay (RIA), the hemolymph (30 μl) was collected from the same animal during the molt cycle. The RIA procedure was performed according to the method of Sonobe *et al*. (1991). The antiserum was prepared against 20-hydroxyecdysone-6-carboxy-methyloxime hapten. This antiserum exhibited about 2.5-fold stronger reactivity against 20-hydroxyecdysone than that against ecdysone. Ecdysteroid titers were expressed in nanograms ecdysone equivalent, since small quantities of other ecdysteroids in addition to 20-hydroxyecdysone, which was the main ecdysteroid in the hemolymph of the American crayfish, were detected (Sonobe *et al*., 1991).

Production of antibodies

Two kinds of synthetic fragments of Prc-MIH, Prc-MIH(55−75)-NH$_2$ that had been extended on its N-terminus with a cysteine residue and Prc-MIH(1−7) that had a cysteine residue on its C-terminus, were coupled to bovine serum albumin (BSA) with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester. The amino acid sequences of Prc-MIH(1−7) and Prc-MIH(55−75)-NH$_2$ show no similarity with any segment of the hyperglycemic hormone (Yasuda *et al*., 1994) which is another hormone in the sinus gland of the American crayfish. Each conjugate was mixed with complete Freund's adjuvant by sonication and subcutaneously injected into two rabbits. Animals were boosted three times at 2-week intervals with a mixture of the conjugate and incomplete Freund's adjuvant. Three days after each injection, the rabbits were bled and antibody activity was tested by enzyme-linked immunosorbent assay (ELISA) using the synthetic Prc-MIH(55−75)-NH$_2$ and Prc-MIH(1−7) fragments. The serum obtained after the final bleeding (10 days after the last injection) was positive at 5000-fold dilution of the antiserum. Immunoglobulin (IgG) was prepared using a protein A-linked affinity column (Hitrap rProtein A, Pharmacia Biotech, Sweden), and then purified using affinity columns conjugated with Prc-MIH(1-7) or Prc-MIH(55−75)-NH$_2$ fragment. The anti-Prc-MIH(55−75)-NH$_2$ IgG was biotinylated according to the method of Bayer and Wilchek (1990).

Immunoblotting

The sinus gland extract, which was prepared using 30% acetonitrile containing 0.9% NaCl (Terauchi *et al*., 1996; Nagasawa *et al*., 1996), and synthetic Prc-MIH (Kawakami *et al*., 1999) were analyzed by immunoblotting. These samples were dissolved in 125 mM Tris-HCl buffer, pH 6.8, containing 4% (w/v) SDS, 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.002% bromophenol blue, and boiled for 2 min. They were electrophoresed in 15% polyacrylamide-slab gel (1 mm thick) containing 0.1% SDS at 20°C for 90 min (SDS-PAGE), and electrophoretically transferred onto polyvinyliden difluoride membranes (Atto, Japan) for 30 min at a current of 2 mA/cm^2. The membranes were preincubated for 30 min in 10 mM Tris-HCl buffer saline (TBS) containing 0.1% casein, and then incubated with anti-Prc-MIH(1−7) or anti-Prc-MIH(55−75)-NH$_2$ IgG (5 μg/ml) for 2 hr at 25°C. Bound antibodies were detected with goat anti-rabbit peroxidase conjugated IgG (1:1000 dilution, Wako Chemical, Japan), and the peroxidase activity was visualized using 0.5% of 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma, USA) in TBS containing 0.02% H$_2$O$_2$. To investigate the specificity of IgG, anti-Prc-MIH(1−7) or anti-Prc-MIH(55−75)-NH$_2$ IgG (5 μg/ml) was preincubated separately with Prc-MIH(1−7) or Prc-MIH(55−75)-NH$_2$ (100 μg/ml) overnight at 4°C. These IgGs were used in the immunoblotting analyses as described above.

Immunohistochemistry

Eyestalk ganglia were dissected from fresh crayfish in crustacean saline (Van Harreveld, 1936), fixed in Bouin's solution for 24 hr, dehydrated through an ethanol and xylene series, embedded in Paraplast and sectioned at 10 μm. Subsequently, the Paraplast was dissolved away from the sections with xylene, the sections were soaked in TBS, pH 7.4, containing 6% H$_2$O$_2$ for 30 min to eliminate the effect of endogenous peroxidase activity in the tissue, and then washed with TBS. After blocking with 0.1% casein, sections were incubated with the anti-MIH(55−75) antiserum (1:1000 dilution) overnight at 4°C. Subsequently, the extra antibody solution was removed by washing with TBS containing 0.1% Tween-20. The tissue sections were then incubated in goat anti-rabbit IgG antibody conjugated to peroxidase for 1 hr at 4°C. After that they were washed for 45 min in TBS, and the peroxidase activity was developed with a 0.03% DAB/H$_2$O$_2$ solution. Excess substrate was removed by washing with distilled water, followed by dehydrating and mounting on slides.

Preparation of samples for the sandwich-type EIA

The hemolymph (250 μl) was collected in series during the molt cycle and mixed immediately with equal volume of buffer solution (0.1 M phosphate buffer, pH 7.0, containing 0.01 M sodium citrate and 0.01 M EDTA) to prevent clotting (Kallen *et al*., 1990). The mixture was centrifuged, and the supernatant was used for the sandwich-type EIA.

The Prc-MIH in the sinus gland was extracted as described previously (Terauchi *et al*., 1996). Briefly, sinus glands were homogenized with micro tissue grinder in 30% acetonitrile containing 0.9% NaCl on ice. The homogenate was centrifuged at 10,000 rpm for 5 min at 4°C. The pellet was re-extracted two times in the same manner. The combined extract was evaporated under reduced pressure and dissolved in blocking buffer (see below) for the sandwich-type EIA.

The sandwich-type EIA

To determine Prc-MIH titer in the hemolymph and Prc-MIH content in the sinus gland, the sandwich-type EIA was carried out with anti-Prc-MIH(1−7) and anti-Prc-MIH(55−75)-NH$_2$ IgGs. Polystyrene beads (3.2 mm in diameter, Immuno Chemical, Japan) were incubated with anti-Prc-MIH(1−7) IgG (10 μg/ml of 0.1 M phosphate buffer, pH 7.5) for 16 hr at 4°C to coat the beads with primary antibody. Subsequently, the beads were washed five times with blocking buffer (0.01 M phosphate buffer, pH 7.0, containing 0.1% casein, 0.4 M NaCl, 0.01 M MgCl$_2$ and 0.1% NaN$_3$). The primary antibody-coated beads were incubated with 150 μl of standard synthetic Prc-MIH (Kawakami *et al*., 1999, 2000; Sonobe *et al*., 2001) solution or sample solution for 14 hr at 4°C. The beads were incubated with biotinylated secondary antibody, anti-Prc-MIH(55−75)-NH$_2$ IgG solution (100 ng/ml of blocking buffer), for 1.5 hr at 25°C with shaking (180 strokes/min), and then reacted with streptavidin-β-D-galactosidase conjugate (0.25 U/ml, Roche Diagnostics, Germany) for 1 hr at 25°C with shaking. After that, bound β-D-galactosidase was reacted with 4-methylumbelliferyl-β-D-galactoside, and then the product, 4-methyl-umbelliferone, was measured using a spectrofluorophotometer RF-5300PC (Shimadzu, Japan) according to the method of Ishikawa and Kato (1978).

RESULTS

Stages of the molt cycle

To determine molt stages of the American crayfish, fluctuations in the volumes of gastroliths and ecdysteroid levels in the hemolymph were monitored during the molt cycle.

Figure 1 shows morphological changes of gastroliths. No gastrolith was observed 21 days before molting, suggesting that the crayfish were in the intermolt stage. The first traces of gastroliths, thin plate-like structure of paired gastroliths, could be detected about 18 days before molting. The gastroliths grew gradually into hard semidisk-shaped structures until molting. At this time, the gastroliths reached the maximum size. Following molting, the gastroliths rapidly degenerated and were scarcely observed 5 days after molting. Figure 2 expresses quantitatively the changes in gastrolith volumes during the molt cycle.

Figure 2 shows quantitative changes of ecdysteroids in the hemolymph during the molt cycle as well. Ecdysteroids in the hemolymph were at low basal level (0.74 ng/ml) approximately 21 days before molting, indicating that they are in the intermolt stage. The ecdysteroid levels increased slightly from the intermolt levels to 1.56 ng/ml during 20-14 days before molting. The levels subsequently increased sharply, reaching a maximum level of 120 ng/ml 6-5 days before molting. During the last 5-4 days before molting, ecdysteroid titers decreased rapidly and reached a low basal level (0.72 ng/ml) just before molting. The low levels were maintained after molting.

On the bases of patterns of changes in gastrolith growth and ecdysteroid titers in the hemolymph, the molt cycle of the American crayfish was classified into six overall stages (Fig. 2). The individual stages are characterized as follows: (1) The intermolt stage is characterized by no gastroliths in addition to hard cuticle. The ecdysteroid titer is maintained at a very low level. (2) The early premolt stage (approximately 20-14 days before molting) is characterized by the presence of small gastroliths, achieving about 5% of the maximum volume of gastroliths. The ecdysteroid titer is elevated slightly but still remains rather low. (3) The middle premolt stage (12-5 days before molting) is characterized by the gradual increase in the deposition of gastroliths, achieving about 50% of the maximum volume of gastroliths. Ecdysteroid titer exhibits a steep increase to its maximum level. (4) The late premolt stage (during the last 4-2 days before molting) is characterized by the rapid increase in the volume of gastroliths, and the sharp decline in the ecdysteroid titer. (5) The very late premolt stage (during 1 day before molting) is characterized by the culmina-
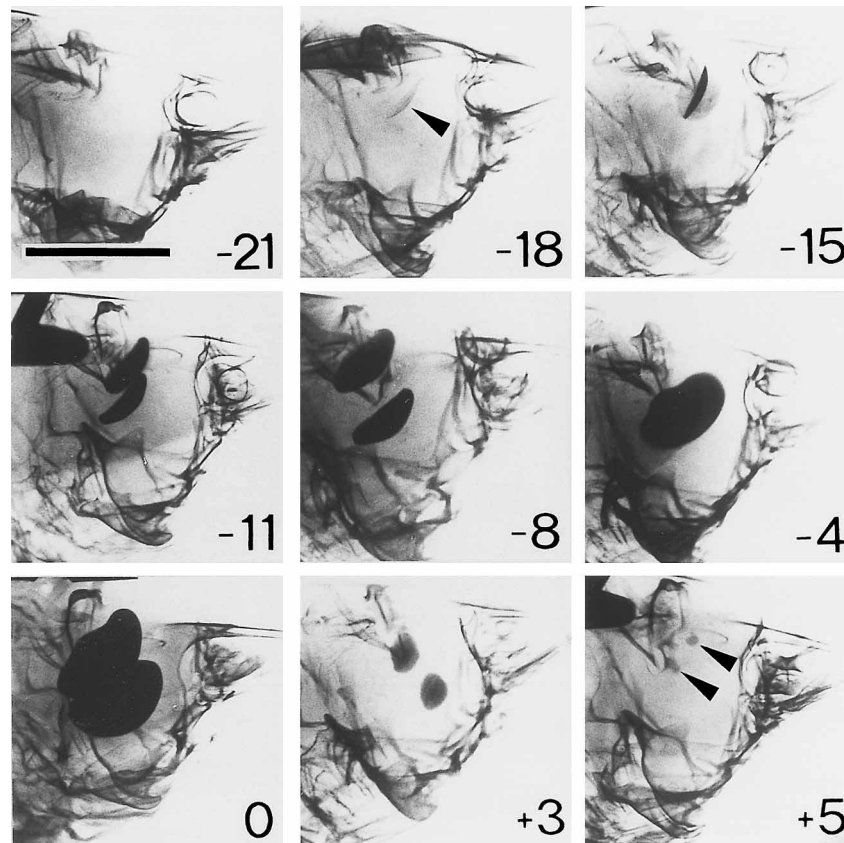

Fig. 1. Morphological changes in gastrolith growth during the molt cycle in the American crayfish, *Procambarus clarkii*. The morphological changes of gastroliths were monitored in the same animals by X-ray radiography. Minus and plus numbers on the photographs represent days before and after molting, respectively. The arrows indicate the thin plate-like gastroliths. The scale bar represents 10 mm. The photographs are of the same magnification.
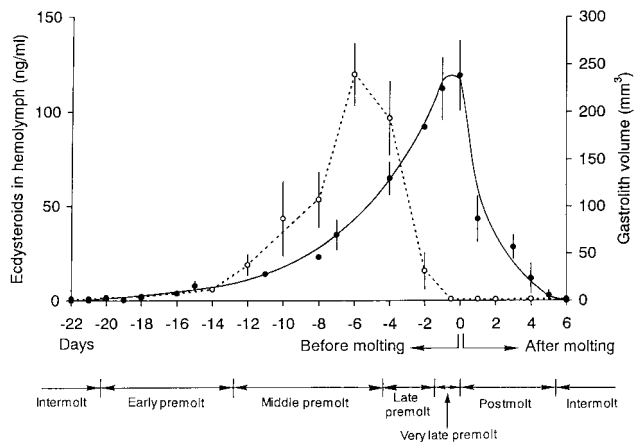
Fig. 2. Changes in gastrolith volumes and ecdysteroid titers in the hemolymph during the molt cycle of the American crayfish. Gastrolith volumes (●—●) were calculated from X-ray radiograph (see Materials and Methods). Ecdysteroids titers (○---○) in the hemolymph were determined by RIA. Results are expressed as mean±SE of nine animals. Stages of the molt cycle were classified into six overall stages according to the changes in the volumes of gastroliths and ecdysteroid titers in the hemolymph as designated in the text.

tion of the gastrolith volume, and the ecdysteroid titer reaching a very low level. Additionally, this stage was recognized by the appearance of soft cutaneous zones on the ventral rim of the branchiostegites. (6) The postmolt stage (during about 5 days after molting) is characterized by degradation of the gastroliths in addition to progressive hardening of the new cuticle.

Specificity of antisera

First, the specificity of antisera raised against Prc-MIH(1–7) and Prc-MIH(55-75)-NH$_2$ was examined by Western blotting. Western blotting analyses with anti-Prc-MIH(1-7) (Fig. 3, A) and anti-Prc-MIH(55–75)-NH$_2$ IgG (Fig. 3, B) showed that the synthetic Prc-MIH was detectable as a single sharp band at a level of approximately 8,700 Da in SDS-PAGE (Fig. 3, lanes 1 and 3). The blotting profiles were similar to those of the sinus gland extract (Fig. 3, lanes 2 and 4) and also corresponded to the molecular weight (8,652) expected by the amino acid sequence of Prc-MIH (Nagasawa *et al.*, 1996). When the anti-Prc-MIH(1–7) IgG and anti-Prc-MIH(55–75)-NH$_2$ IgG were preabsorbed with Prc-MIH(1–7) and Prc-MIH(55–75)-NH$_2$, respectively, no bands of Prc-MIH were detectable in Western blotting analyses (data not shown).

Next, the specificity of anti-Prc-MIH(1–7) and anti-Prc-MIH(55–75)-NH$_2$ antisera was examined by immunohistochemical means. Figure 4 shows a reconstruction of an immunopositive neurosecretory system from consecutive sections using anti-Prc-MIH(55–75)-NH$_2$ antiserum. The immunoreactive neurosecretory cells of Prc-MIH were localized in the perikarya of the X-organ, which is adjacent to the medulla terminalis. By examining the immunopositive neurosecretory cells on every consecutive section, it was revealed that the number of Prc-MIH cells in the X-organ was 20–25. In addition to these cells, a few immunoreactive cells were observed
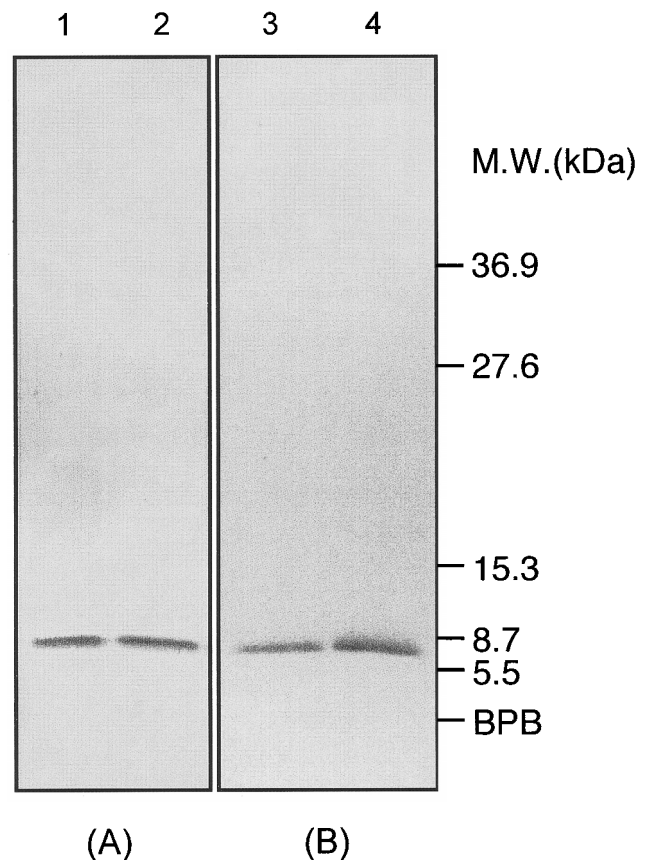

Fig. 3. Western blot analyses of sinus gland extract and synthetic Prc-MIH. Synthetic Prc-MIH (0.4 pmol, lanes 1 and 3) and sinus gland extract (0.1 sinus gland equivalent, lanes 2 and 4) were subjected to SDS-PAGE, and peptides were immunoblotted using IgGs raised against Prc-MIH(1–7) (A) and Prc-MIH(55–75)-NH$_2$ (B). The molecular weight scales were determined using the Kaleidoscope polypeptide standard (Bio-Rad, USA), and bromophenol blue (BPB) was used as a dye front.

in the medulla terminalis. The sinus gland, located on the opposite side of the X-organ and between the medulla externa and medulla interna, was strongly immunostained. Parts of the immunostained axonal tract originating from Prc-MIH cells penetrated the medulla terminalis and extended to the sinus gland through the medulla interna. To check the non-specific binding of the antisera, the primary antiserum was substituted by preimmune rabbit sera, anti-BSA rabbit antiserum or anti-rabbit IgG goat IgG conjugated horseradish peroxidase. No immunostaining was observed in the sections (results not shown). Comparable results to our immunohistochemical study have been described in the eyestalks of the kuruma prawn *Penaeus japonicus* (Shih *et al.*, 1998), the spiny lobsters *Jasus lalandii* and *Panulirus homarus*, and the shore crab *Carcinus maenas* (Marco and Gäde 1999).

These results obtained by Western blotting analyses and immunohistochemical observation indicate that both IgGs, anti-Prc-MIH(1–7) IgG and anti-Prc-MIH(55–75)-NH$_2$ IgG, are specific for the Prc-MIH.
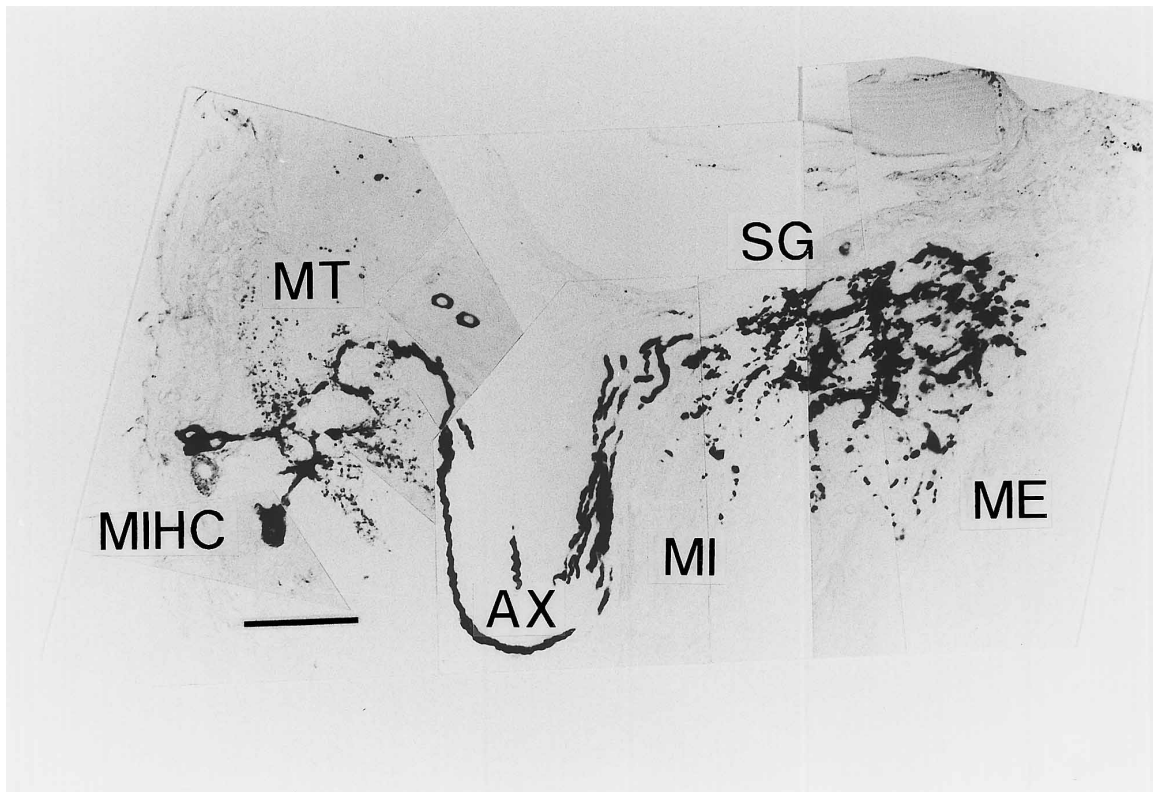
Fig. 4. Composite photograph of the immunopositive neurosecretory systems in the eyestalk. Consecutive eyestalk sections were immunostained and reconstructed to observe axonal profiles between the Prc-MIH cells in the X-organ and sinus gland. AX: axonal track; ME, MI, MT: medulla externa, medulla interna, medulla terminalis, respectively; MIHC: Prc-MIH cells; SG: sinus gland. Scale bar represents 100 μ m.
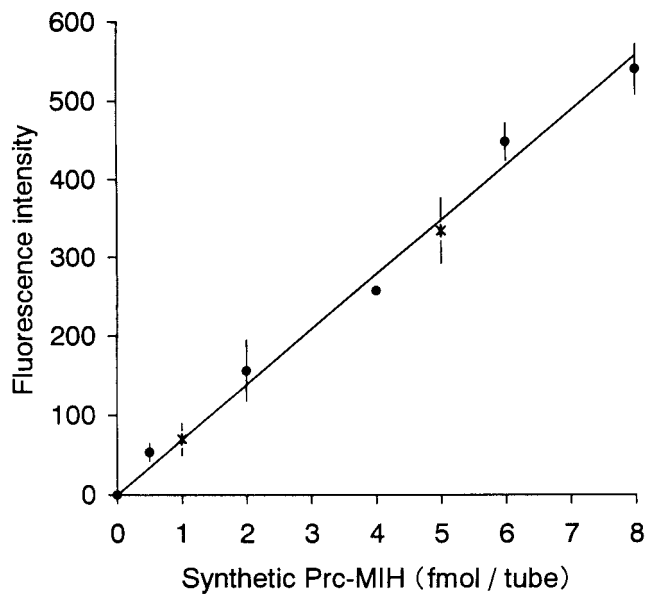

Fig. 5. Standard curve of Prc-MIH by the sandwich-type EIA. Synthetic Prc-MIH, diluted in series (0.5, 2, 4, 6, 8 fmol/150 μ l of blocking buffer), was used as a standard. Asterisks indicate the amounts of Prc-MIH recovered from the hemolymph into which exogenous Prc-MIH (1 or 5 fmol) has been mixed. Bars represent mean±SE of triplicate assays.
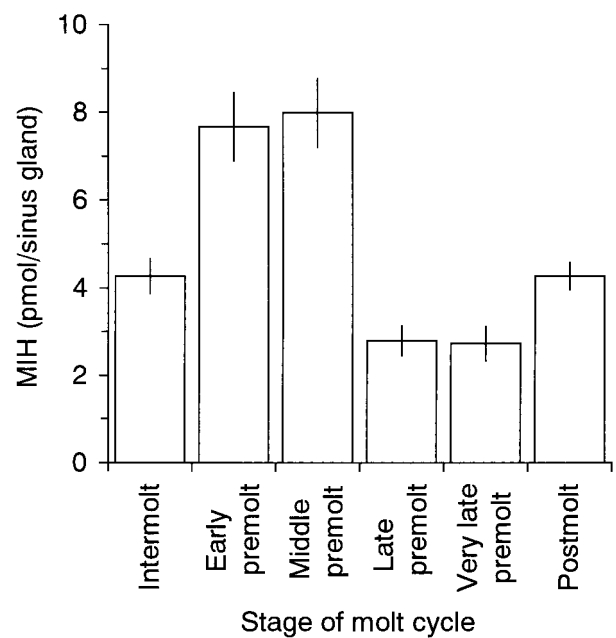

Fig. 6. Fluctuation of Prc-MIH content in the sinus gland during the molt cycle. Seven to twenty sinus glands were dissected by stage and analyzed individually. Results are expressed as mean±SE. The molt stages are as designated in the text.

Changes in the amounts of Prc-MIH in hemolymph and sinus glands

Figure 5 shows a standard curve using synthetic Prc-MIH. The lower limit of detection of this assay was 0.5 fmol of Prc-MIH in 150 μl of assay solution(3.3×10^{-12} M). Although the assay was tried at several different stages of the molt cycle, no MIH was detected in the hemolymph. The synthetic Prc-MIH was mixed with the fresh hemolymph (1 or 5 fmol of Prc-MIH/150 μl of hemolymph samples) *in vitro*, and then the amounts of Prc-MIH in the hemolymph were quantified by the sandwich-type EIA described in Materials and Methods. More than 90% of exogenous Prc-MIH were recovered from the hemolymph samples (Fig. 5).

On the other hand, in sinus glands (Fig. 6), sufficient levels (4.27 pmol) of Prc-MIH were measured during the intermolt stage. The Prc-MIH titer increased to 7.67 pmol at the early premolt stage, and the relatively high levels (7.99 pmol) were maintained during the middle premolt stage. However, the titers dropped rapidly to the lower basal level (2.80 pmol) at the late and very late premolt stages. The MIH level again increased during the postmolt stage and reached almost the same level as in the intermolt stage.

DISCUSSION

In crustaceans, changes in hemolymph ecdysteroid levels during the molt cycle exhibit the same general pattern and are closely related to morphological changes in the epidermis (Stevenson, 1979; Hopkins, 1983; Jegla *et al.*, 1983; Soumoff and Skinner, 1983; Skinner, 1985; Jegla, 1989). Thus, molt stages are in general determined on the bases of the morphological feature of the epidermis. However, in the American crayfish, *Procambarus clarkii*, the morphological changes in the epidermis have not been described with reference to classification of the molt cycle. In the freshwater crayfish, *Orconectes virilis* (McWhinnie, 1962) and *Orconectes limosus* (Willig and Keller, 1973) and the land crab, *Gecarcinus lateralis* (McCarthy and Skinner, 1977), it has been demonstrated that the growth of gastroliths correlates with ecdysteroid titer in the hemolymph and suggested that molt stages can be determined based on observations of the growth of gastroliths. Thus, in the first series of experiments, molt stages of the American crayfish were determined based on visualizing gastroliths using X-rays and monitoring ecdysteroid titer using small volumes of hemolymph (Fig. 2). The classification of the molt cycle in the American crayfish by our criteria was consistent with that in the freshwater crayfish and the land crab described above. This method was rather convenient for following same animals during the molt cycle by non-destructive means.

It has been widely accepted that a molting sequence is initiated (i.e., the animals enter premolt) when MIH secretion from the sinus gland reduces or ceases probably due to environmental cues (Skinner, 1985; Jegla, 1989; Webster, 1998). In keeping with this concept, MIHs were isolated, sequenced and cloned in a number of species. However, relatively little is known about the synthesis, secretion and precise physiological actions of MIH. Recently, Ohira *et al.* (1997) demonstrated that the MIH mRNA level in the eyestalk of the kuruma prawn does not change significantly during the molt cycle and predicted that the synthesis and/or secretion of MIH are regulated post-transcriptionally. On the other hand, Lee and co-workers (1998) demonstrated that the MIH mRNA level in the eyestalk of the blue crab, *Callinectes sapidus*, undergoes a stage-specific change which negatively correlates with the ecdysteroid titer in the hemolymph. From this result, they suggested that changes in the MIH mRNA level play a critical role in the regulation of molting. However, neither group has examined stage-specific changes in not only MIH titer in the hemolymph, but also MIH content in the neurohemal sinus gland, which afford important clues to understand the role of MIH in regulating cycles of molting.

In the second series of experiments, we attempted to quantify the hemolymph MIH titer by the sandwich-type EIA using specific antibodies against Prc-MIH (Figs. 3 and 4). Although the lower limit of detection of this EIA (0.5 fmol/assay: 3.3×10^{-12} M) was almost the same as that utilized to quantify the changes in crustacean hyperglycemic hormone (CHH) in the hemolymph (Webster, 1996; Chang *et al.*, 1998; Chung *et al.*, 1999), no MIH titers could be detected in the hemolymph at any of the molt stages tested. In addition, the observation that more than 90% of exogenous Prc-MIH mixed with the fresh hemolymph *in vitro* were recovered from the hemolymph samples (Fig. 5) indicates that there is no substance in the hemolymph that interferes with the measurement of Prc-MIH. These results suggest a possibility that in comparison with the CHH level, the MIH level is not so high. It has recently been demonstrated in the shore crab *Carcinus maenas* that MIH and CHH act synergistically in inhibiting ecdysteroid secretion from the Y-organ *in vitro* (Webster, 1998). It has also been demonstrated in the crayfish that CHH inhibits the ecdysteroid secretion from the Y-organ *in vitro*, although the activity is less effective than the authentic MIH (Yasuda *et al.*, 1994; Aguilar *et al.*, 1995, 1996; Terauchi *et al.*, 1996). These results do not exclude the possibility that not only MIH but also CHH functions as a molt regulator *in vivo*.

In the third series of experiments, the pattern of changes in the Prc-MIH content in the sinus glands was analyzed (Fig. 6). During the early premolt stage, a time when the hemolymph ecdysteroid titer began to rise gradually (Fig. 2) and the MIH mRNA level was high (Lee *et al.*, 1998), the Prc-MIH content was about two fold higher than that during the intermolt stage, a time when the hemolymph ecdysteroid titer remains at a low level (Fig. 2) and the MIH mRNA level was high (Lee *et al.*, 1998). The rise of Prc-MIH content in the sinus gland might result from a decline of Prc-MIH secretion at the premolt stage. In the middle premolt stage when the hemolymph ecdysteroid titer increased sharply (Fig. 2), although the MIH mRNA level declined (Lee *et al.*, 1998), the Prc-MIH content was maintained at a high level as in the early premolt stage. This fact suggests that other regulatory mechanisms besides *de novo* synthesis of MIH, such as feedback inhibition of MIH secretion by hemolymph ecdysteroids in the crab *Cancer*

antennarius (Mattson and Spaziani, 1986), might be involved in regulating the MIH secretion in the American crayfish as well. The decrease in the Prc-MIH content in the sinus gland during late and very late premolt prior to molting was accompanied by a steep decline in the hemolymph ecdysteroid titer (Fig. 2). However, the steep decline in the hemolymph ecdysteroid titer might not be attributed to the action of Prc-MIH, because eyestalk ablated crustaceans show similar ecdysteroid titer curves as natural late premolt animals (Hopkins, 1983). In addition, a feedback action of circulating ecdysteroids on Y-organ activity was also suggested to trigger the steep decline in hemolymph ecdysteroid titer (Dell *et al.*, 1999). Therefore, although Prc-MIH has been secreted from sinus glands at this stage, the physiological function of Prc-MIH remains to be solved. During the postmolt stage, a time when hemolymph ecdysteroids remained at a low level (Fig. 2), the Prc-MIH content in the sinus gland was recovered to the level of the intermolt stage (Fig. 2). The increase in the Prc-MIH content is causally linked to a steep increase in *de novo* synthesis of MIH (Lee *et al.*, 1998).

In summary, a novel result in this study was that we were able to detect stage-specific changes in the MIH content in the sinus glands during the molt cycle. The observed changes are generally consistent with the concept that MIH negatively regulates ecdysteroid secretion from the Y-organ. It seems justified to assume that the secretory activity of MIH from the sinus glands is subjected to the *de novo* synthesis of MIH and the feedback of circulating ecdysteroids.

ACKNOWLEDGEMENTS

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