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A Novel Neuropeptide with Molt-Inhibiting Activity from the Sinus Gland of the Crayfish, *Procambarus clarkii*

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ABSTRACT—Molt-inhibiting hormone (MIH) activity was tested in terms of the inhibitory activity of ecdysteroid secretion by cultured Y-organs of the crayfish, *Procambarus clarkii*. Several neuropeptides were separated from a crude extract of sinus glands of *P. clarkii* by means of reverse-phase high-performance liquid chromatography (HPLC) and tested for MIH activity. An extremely high level of activity was found in one fraction in reverse-phase HPLC, suggesting that the novel neuropeptide in this fraction may be a *P. clarkii* MIH.

INTRODUCTION

At present, it is commonly accepted that the process of molting in crustaceans is regulated by at least two types of hormones, ecdysteroids (molting hormone) and molt-inhibiting hormone (MIH) (Chang, 1985; Skinner, 1985; Smith and Sedlmeier, 1990). It is likely that during the intermolt stage, ecdysteroid secretion by Y-organs is inhibited by the MIH produced by the eyestalk neurosecretory X-organ-sinus gland (SG) system; on the other hand, in the premolt stage, a decrease in MIH levels results in the release of the Y-organs from inhibitory control. Our previous studies in the crayfish, *Procambarus clarkii*, have also shown that eyestalk ablation at the intermolt stage leads to a rapid increase in the levels of circulating ecdysteroids, mainly 20-hydroxyecdysone, which is due to an increase in the rate of ecdysteroid secretion (3-dehydroecdysone as the major product, together with small amounts of ecdysone) by the Y-organs (Sonobe *et al.*, 1991).

MIHs were isolated from SGs of the shore crab, *Carcinus maenas* (Webster and Keller, 1986; Webster, 1991), the lobster, *Homarus americanus* (Chang *et al.*, 1990), and recently the kuruma prawn, *Penaeus japonicus* (Yang *et al.*, 1996), and their primary structures were determined (Chang *et al.*, 1990; Webster, 1991; Yang *et al.*, 1996). The alignment of the amino acid sequences suggests that these MIHs are members of a neuropeptide family called the CHH family or the CHH/MIH/VIH family (Keller, 1992), which includes crustacean hyperglycemic hormone (CHH), MIH and vitellogenesis-inhibiting hormone (VIH).

In addition to similarities in structural homology, overlapping physiological effects have been also observed in this family; a peptide with both molt-inhibiting and hyperglycemic activities has been isolated from *H. americanus* (Chang *et al.*, 1990). On the other hand, another peptide which exhibits more potent MIH activity than the MIH activity exhibited by CHH has been isolated from *C. maenas* and *P. japonicus* (Webster, 1991; Yang *et al.*, 1996). Recently, in the course of characterization of *P. clarkii* CHH (Prc-CHH), it has also been demonstrated that Prc-CHH-II, one of two variants of Prc-CHH, exhibited more potent MIH activity than did Prc-CHH-I (Yasuda *et al.*, 1994). However, by analogy with the diversity of SG neuropeptides in *C. maenas* (Keger *et al.*, 1989; Webster, 1991) and *P. japonicus* (Yang *et al.*, 1995, 1996), a possibility that an authentic MIH (Prc-MIH) may exist in the SGs of *P. clarkii* cannot be ruled out. Therefore, more information on SG neuropeptides in *P. clarkii*, especially Prc-MIH, is clearly required at present.

We report here a novel neuropeptide, likely an authentic Prc-MIH, which exhibits a much higher MIH activity than that exhibited by Prc-CHH-II.

MATERIALS AND METHODS

Animals

Freshwater crayfish *Procambarus clarkii*, carapace width 21–23 mm, were obtained from a local dealer, and used for a bioassay of MIH activity.

Extraction and purification of neurosecretory peptides from the SGs

Five hundred SGs were dissected out from eyestalks of *P. clarkii*, and stored at –80°C until extraction. Sinus gland neuropeptides were extracted and isolated by procedures described previously (Nagasawa *et al.*, 1996). Briefly, SGs were homogenized in

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30% acetonitrile containing 0.9% NaCl on ice. The homogenate was centrifuged at 15,000 rpm for 5 min at 4°C. The pellet was re-extracted twice in the same manner, and the combined extracts were concentrated under reduced pressure and subjected to analytical reverse-phase high-performance liquid chromatography (HPLC) using an Asahi Pak ODS-50 column (4.6×250 mm, Showa Denko, Tokyo). Peptides were eluted with a 65-min linear gradient of 0–65% acetonitrile in 0.05% trifluoroacetic acid (TFA) at a flow rate of 1 ml/min. The absorbance of the eluate was monitored at 225 nm. Fractions were collected manually and alphabetized in the order of elution. For further separation, a TSK gel ODS-120T column (4.6×250 mm, Tosoh, Tokyo) was used. The peptides were eluted with an 88-min linear gradient of 0-65% acetonitrile in 0.05% TFA at a flow rate of 1 ml/min.

Bioassay

To assay MIH activity, the *P. clarkii in vitro* Y-organ culture system was used (Sonobe *et al.*, 1991). Briefly, Y-organs were dissected out 3 days after eyestalk removal, and rinsed in saline. They were then placed in glass dishes containing 0.5 ml culture medium in such a way that each dish contained one of a bilateral pair of Y-organs: one organ served as a control, and the other as the experimental organ. After incubation for 6 hr at 25°C, the culture medium was removed from each dish and stored at –20°C until radioimmunoasay (RIA) for ecdysteroids. Percent inhibition of ecdysteroid secretion was calculated as described by Soumoff and O'Connor (1982).

To prepare samples of peptides for MIH assay, a peak or pooled zone material in HPLC was dried and re-dissolved in culture medium. Peptide concentrations were expressed as SG equivalents per 0.5 ml culture medium.

Radioimmunoassay

To determine the amounts of ecdysteroids secreted by the Y-organs, an aliquot (usually 50 µl) of the culture medium was directly subjected to RIA. The RIA was performed according to procedures described previously (Sonobe *et al.*, 1991). An antiserum used was prepared against 20-hydroxyecdysone 6-carboxymethyloxime. The cross-reactivity factor for 3-dehydroecdysone, the main ecdysteroid secreted by the Y-organs of *P. clarkii* (Sonobe *et al.*, 1991), using ecdysone as the competing standard with the antiserum was 2.6 in our laboratory (unpublished data).

RESULTS AND DISCUSSION

To isolate Prc-MIH, the following three series of experiments were carried out.

Firstly, the supernatant of the SG homogenate was subjected to the bioassay for MIH activity. In order to examine the effect of MIH on *in vitro* secretion of ecdysteroids by the Y-organs, the Y-organs were cultured in medium that contained a crude extract of 0.5 SG equivalents. An aliquot of the culture medium was withdrawn every 3 hr as described in our previous paper (Sonobe *et al.*, 1991), and subjected to RIA for ecdysteroids. As shown in Fig. 1, the control Y-organs secreted ecdysteroids rapidly for about 6 hr before reaching a plateau. In contrast, the Y-organs incubated with the SG extract maintained a low level of secretory activity throughout the whole incubation periods. The maximal inhibition was about 50% of the control Y-organs. On the basis of these results, a 6-hr incubation was adopted for further experiments. Subsequently, the inhibitory effect induced by the crude extract was examined at doses between 0.002 and 5 SG equivalents. As shown in Fig. 2, the inhibitory effect was increased in a log-linear manner with increasing crude extract concentration from 0.002 to 0.125 SG equivalents, but decreased with increasing the concentration of crude extract from 0.5 to 5 SG equivalents. Maximum level of inhibition, approximately 50%, was observed at doses be-

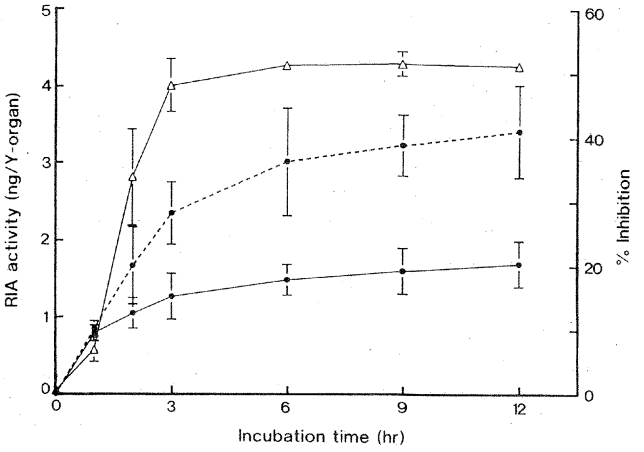

Fig. 1. Time-course profile of ecdysteroid secretion by the Y-organs *in vitro*. The Y-organs 3 days after eyestalk removal of the donors were incubated in the medium in the presence (•—•) or absence (•---•) of SG crude extract at a concentration of 0.5 SG equivalents. An aliquot of the medium was withdrawn every 3 hr and subjected to RIA for ecdysteroids. Results are expressed as mean±SE of three independent bioassays. Percent inhibition (△—△) of ecdysteroid secretion by the Y-organs was caluculated by comparing the RIA activity in the experimental medium with that in the control one (see Materials and Methods).

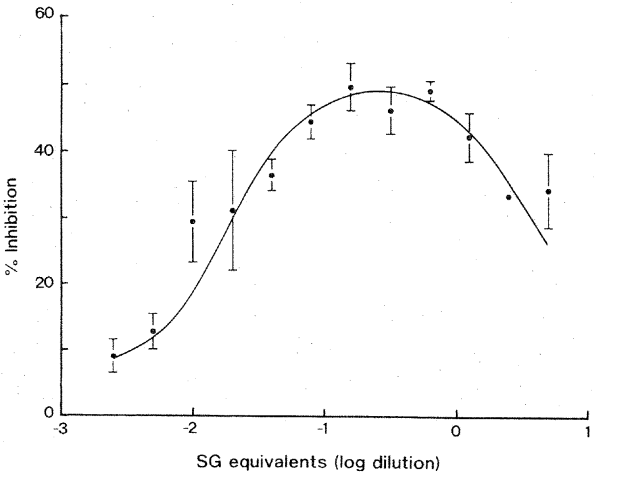

Fig. 2. Dose-response effects of the SG crude extract on ecdysteroid secretion by *P. clarkii* Y-organs. The Y-organs were incubated in 0.5 ml of culture medium at the indicated dose (SG equivalents) for 6 hr. Ecdysteriods in the medium were quantified by RIA. Points represent percent inhibition of ecdysteroid secretion determined by comparison with the RIA activity in untreated control cultures (see Materials and Methods). Results are expressed as mean±SE of three independent bioassays.

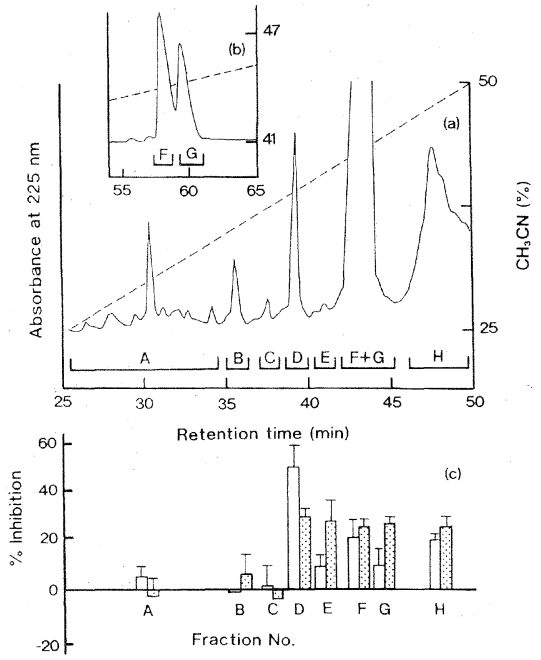

Fig. 3. HPLC profile of neuropeptides extracted from the SGs of *P. clarkii*, and MIH activity in each fraction. (a) HPLC elution profile of the extract of 200 SGs chromatographed on the Asahi Pak ODS-50 column. (b) Further separation of peptides having a retention time of 43 min [as shown in (a)] on the TSK gel ODS-120T column. Chromatographic conditions in (a) and (b) are detailed in the text. (c) Effects of neuropeptides purified by reverse-phase HPLC on Y-organ ecdysteroid secretion. Aliquots of each fraction were examined at a dose of 5 SG (▨) or 0.5 SG (□) equivalents. The bioassay system is the same as for Fig. 2. Results are expressed as mean±SE of three or four independent bioassays.

tween 0.125 and 0.5 SG equivalents. The ability of the Y-organs to respond to a concentration as low as 0.002 SG equivalents indicates that the Y-organ culture system provides an extremely sensitive tool for the detection of the MIH activity.

Secondly, the crude extract was subjected to reverse-phase HPLC using the Asahi Pak ODS-50 column (Fig. 3a), and subsequently a large peak having a retention time of 43 min was further separated using the TSK gel ODS-120 column (Fig. 3b). The eight fractions obtained by the above, temporarily termed A-H, were each subjected to the bioassay at doses of 5 and 0.5 SG equivalents. As shown in Fig. 3c, when 5 SG equivalents of peptide were added to the culture medium, approximately 20–30% inhibition was observed for fractions D-H. At a dose of 0.5 SG equivalents, fraction D showed the highest inhibitory activity of about 50%; this value was higher than that obtained at the dose of 5 SG equivalents for fraction D. However, fractions E-H showed lower inhibitory activities (about 10–20% inhibition) at a dose of 0.5 SG equivalents than at that of 5 SG equivalents. At either concentration, little inhibitory activity was detected in fractions A-C (data not shown). These results suggest that peptide concentrations which exhibit the maximum levels of inhibitory activity may be different in each fraction.

Lastly, the dose-response characteristics of the inhibitory activities were quantitatively examined in each of fractions D-H. As shown in Fig. 4, although each of fractions E-H showed some degree of inhibitory activity, the maximum levels of inibitory activity detected in these fractions were much lower than the maximum level of inhibitory activity detected in fraction D at all concentrations examined. The dose-response curve for fraction D was almost similar to that for the crude extract: the maximum level of inhibition was approximately 52%, and half-maximum inhibition was attained at a concentration of about 0.01 SG equivalents. These

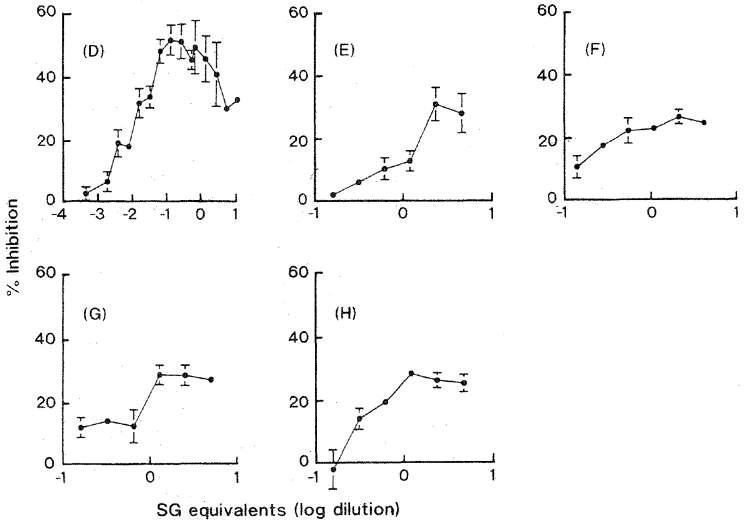

Fig. 4. Dose-response effects of neuropeptides purified by reverse-phase HPLC on Y-organ ecdysteroid secretion. Each neuropeptide in fractions D-H obtained by the HPLC (Fig. 3), shown as (D), (E), (F), (G) and (H) in the figure, was assayed at doses of the indicated SG equivalents. The assay system is the same as for Fig. 2. Results are expressed as mean±SE of three or four independent bioassays.

values were almost the same as those obtained in *C. maenas* MIH (Cam-MIH) (Webster and Keller, 1986; Hurberman and Aquilar, 1989). As shown in Fig. 4D as well as in Fig. 2, the inhibitory effect decreased at the doses over 1 SG equivalent. There is no plausible explanation for the decrease in inhibitory effect at present, but a similar pattern has also been observed in the crab *Pachygrapsus crassipes* (Soumoff and O'Connor, 1982).

We believe that a novel neuropeptide in fraction D is authentic Prc-MIH for the following three reasons. (1) As discussed above, the dose-response curve for the novel neuropeptide was comparable to that for the crude extract (compare Fig. 2 with Fig. 4D). This suggests that almost all of the MIH activity in the crude extract was recovered in fraction D. (2) According to our preliminary experiments using a mass spectrometer and a protein sequencer, two neuropeptides in fractions F and G proved to be identical to the peptides for Prc-CHH-I and Prc-CHH-II, respectively, which were characterized by Yasuda *et al.*(1994). Although both Prc-CHH-I and Prc-CHH-II showed MIH activity (Yasuda *et al.*, 1994), our present experiments demonstrated that the levels of their activities were much lower than the level of the activity in fraction D (Fig. 3D, F and G). (3) The yield of the novel neuropeptide from 500 SGs was approximately 17 μg. In preliminary experiments using a matrix assisted laser desorption ionization time-of-flight mass spectrometer (MALDI TOF-MS), it was estimated that the isolated novel neuropeptide has a molecular weight of 8629 Da. Based on the dose-response characteristics, it is considered that the neuropeptide is active in the picomolar range (Fig. 4D). This implies that the neuropeptide is sufficiently effective at the physiological concentration in the same range as Cam-MIH (Webster, 1991).

Recently, we determined the amino acid sequence of the novel neuropeptide, which will be reported elsewhere (Nagasawa *et al.*, 1996).

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