Dynamics of in Vivo Release of Molt-Inhibiting Hormone and Crustacean Hyperglycemic Hormone in the Shore Crab, Carcinus maenas

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Very little is known regarding the release patterns or circulating titers of neuropeptides in crustaceans, in particular those concerned with regulation of molting hormone (ecdysteroid) synthesis, molt-inhibiting hormone (MIH), and crustacean hyperglycemic hormone (CHH), which is also an adaptive hormone, centrally important in carbohydrate metabolism. Furthermore, the currently accepted model of molt control is founded on an untested hypothesis suggesting that molting can proceed only after decline in MIH titer. Accordingly, we measured simultaneous circulating neuropeptide profiles for both MIH and CHH by RIA of purified hemolymph during the molt cycle at fine temporal scale during day/night cycles and seasonally. For CHH we additionally determined release patterns after physiologically relevant stress. Results show that both hormones are released exclusively and episodically, rather than continuously, with notably short half-lives in circulation, suggesting dynamic and short-lived variations in levels of both hormones. During the molt cycle, there are no overt changes in MIH titer, except a massive and unprecedented increase in MIH during late premolt, just before ecdysis. The function of this hormone surge is unknown. Treatment with various stressors (hypoxia, temperature shock) showed that CHH release occurs extremely rapidly, within minutes of stress. Release of CHH after stressful episodes during premolt (when gut endocrine cells synthesize large quantities of CHH) is exclusively from the sinus gland: CHH from the gut is never involved in the stress response. The results show a hitherto unsuspected dynamism in release of MIH and CHH and suggest that currently accepted models of molt control must be reconsidered. (Endocrinology 146: 5545–5551, 2005)

IN THE PAST FEW years, a large number of structurally related neuropeptides, generically known as members of the crustacean hyperglycemic hormone (CHH) peptide family have been identified from the X-organ sinus gland neurosecretory system of crustaceans using microsequencing and cDNA cloning strategies (1–3), and this list is being expanded continually. The CHH family peptides can be classified into two groups, depending on the presence, in the unprocessed peptide of a precursor-related peptide (type I, or CHH type) or lack of this precursor and a glycine residue at position 12 of the mature peptide, the insertion of which aligns peptide sequences in all members in this group (and if a gap is inserted at a corresponding position for CHH peptides, aligns all members), and which immediately highlights all members of this group as type II or molt-inhibiting hormone (MIH)/gonad-inhibiting hormone type (1).

Whereas the names of these hormones obviously reflect their first discovered biological activity (or their identity to peptides with well-established roles), it has become ever more apparent that many of them have multiple roles or probably that the physiologically relevant ones have yet to be established. For the best-known prototype hormone, CHH, apart from its defining role in regulation of carbohydrate metabolism (4), related activity as a secretagogue (5) and in lipid mobilization (6) has been shown. Furthermore, roles in molt control (7, 8) and ionic-osmoregulation (9–11) have been described. Involvement in reproduction (inhibition of protein and vitellogenin synthesis) has also been suggested (12). Further complexity is added when other members of the CHH family are considered. For example, whereas a distinctive (MIH type) mandibular organ-inhibiting hormone, which represses in vitro juvenile (methyl farnesoate) synthesis by the mandibular organs, exists in Cancer pagurus and other cancrids (13 and own unpublished observations) for other decapod crustaceans, CHH appears to fulfill this role (14, 15). Similarly (as alluded to earlier) in several crustaceans in which distinctive MIH-type peptides appear to be absent, CHH-type peptides are the functional MIHs (8, 16, 17).

Given this background, it is clear that we know very little regarding the physiologically relevant roles of most of the CHH group peptides. Whereas appropriate bioassays are useful in pointing to possible functions, we reasoned that these could be more credibly defined by in vivo measurement of circulating hormone titers. For example, the current model of molt control in crustaceans is completely based on the supposition that MIH is released only during intermolt, in which its action is to repress ecdysteroid synthesis by the Y-organs. Reduction in MIH release could thus permit increased ecdysteroid synthesis, leading to permissive entry to premolt and subsequent molting. Similarly, whereas it is known that CHH release occurs after stressful episodes in some crustaceans (18–20) and from gut endocrine cells immediately before ecdysis in Carcinus maenas (10), we know

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Abbreviations: CHH, Crustacean hyperglycemic hormone; DA, dopamine; MIH, molt-inhibiting hormone; SG, sinus gland.

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little regarding day-to-day variation in and release patterns of CHH family peptides, which are clearly necessary to understand the physiological significance of these hormones. To address these questions, we used highly sensitive RIAs for both CHH and MIH to investigate diel patterns of release, the effects of stressors on release of CHH, and influence of the molt cycle on MIH titers.

Materials and Methods

Animals

Specimens of Carcinus maenas were collected by baited traps around low water level in the Menai Strait (Wales, UK). After capture they were immediately selected (males, 45–55 mm carapace width, intermolt stage C2) and placed in a recirculating seawater system (10–12 per 50 liters) under conditions of ambient temperature and photoperiod. Great care was taken to ensure crabs were unstressed during this period. Tubes were added for refuge so that each crab could avoid interaction with others. Crabs were left to acclimate for 72 h before experimentation and fed once (fish and mussel) during this period. For some experiments premolt crabs were used (molt staging according to Ref. 21).

RIA

RIAs for CHH and MIH were performed as described and validated previously (MIH: Ref. 22; CHH: Ref. 23). Standards were triplicated and unknowns duplicated. The MIH assay was made much more sensitive previously (MIH: Ref. 22; CHH: Ref. 23). Standards were triplicated and values for 3 h. Crabs that had been eyestalk ablated 48 h before experimentation served as controls and also to determine whether known extra eyestalk sources of CHH, such as from gut endocrine cells, could be involved in CHH release after hypoxic stress. Hemolymph samples were assayed for CHH and glucose, as described above. Assays for circulating glucose and lactate were performed as previously described (19).

To estimate half-life of CHH and MIH, small quantities (2.5 kBq) of iodinated peptides were injected, followed by estimation of hemolymph radioactivity 2 min after injection (to allow the bolus to perfuse), followed by sampling at T = 5, 10, 15, 30 min. These assays gave identical results to those previously described, in which native peptides were injected, followed by estimation of undegraded peptide by RIA (19, 23, 25) and thus gave reasonably accurate estimations of half-life.

Results

Normal crabs

Hormone levels of crabs that were presumably unstressed (after adaptation to laboratory conditions, ambient temperature, and photoperiodic conditions) were taken at monthly intervals over 24-h periods, sampling at least 10 animals at each time point (2 h). For each blood sample from subsequently molt-staged animals, levels of CHH and MIH were determined. In total, more than 1000 hemolymph samples were processed. Initial analysis of individual experiments revealed that there was, as expected, a high level of stochasticity. However, when all results (intermolt crabs) were collated (Fig. 1), elevated levels of CHH or MIH were rather infrequently observed. Nevertheless, few instances of simultaneously high levels of both CHH and MIH were observed. Comparison of elevated levels of hormones (MIH > 5 fmol/ml, CHH > 5 fmol per 100 μl) between night and daytime showed that during daytime, only 28 (5.1%) samples showed elevated MIH levels, compared with 55 (11.9%) during nighttime. For comparison, elevated CHH levels were similar during the day (13.7%) and night (14.7%).

Experiments designed to estimate the half-lives of MIH and CHH showed that both are cleared from the hemolymph quite dynamically. For both peptides, half-lives are in the order of 5–10 min (Fig. 1, inset). Thus, if these hormones are released in an episodic manner, the possibility of obtaining hemolymph samples close to a release event is small. However, assuming that the elevated hormone levels (defined earlier) arise from episodic release, all CHH or MIH hormone levels constrained within these parameters may be considered to be those arising from release events that have occurred within two half-lives (10–20 min) of release.

Analysis of changes in seasonal levels of CHH and MIH for intermolt animals (Fig. 2) showed that during winter, CHH levels were significantly lower (ca. 25 fmol/ml) than those of animals sampled in the summer (50–55 fmol/ml) and that there were no significant differences between samples collected during day or nighttime. For MIH, during the winter, levels were significantly higher during nighttime (2.8 fmol/ml) than during daytime (2.1 fmol/ml).

Analysis of MIH and CHH titers according to molt stage showed that during intermolt (C4) and early premolt (D1–D2), despite reasonable sample sizes, there were no significant changes in hormone titer (Table 1). However, during late premolt (despite the difficulties in taking adequate hemolymph samples at this time), there were unprecedented
changes in levels of MIH. In animals sampled during late premolt (D3–4) MIH levels were enormously elevated, to the extent that sinus gland levels of stored MIH were significantly depleted (Fig. 3).

Stressed crabs

For crabs exposed to thermal stress, CHH release was immediately apparent, within 5 min of exposure (Fig. 4). An interesting feature was that both hyper- and hypothermic stress elicited somewhat similar hormone release profiles. For hyperthermic stress, crabs were also exposed to brief periods (5 min) of repeated thermal shock and showed that each exposure to high temperature resulted in hormone release and accumulated hyperglycemia (Fig. 5).

Hypoxia was a potent elicitor of CHH release. In both intermolt and premolt animals, 2-h periods of extreme hypoxia (<5% O₂ saturation) elicited quite dramatic increases in CHH levels (Fig. 6).

Field crabs

Because one of the accepted roles of CHH concerns its action as an adaptive hormone, we were interested in determining relevant changes in hormone levels in the intertidal environment. In situ measurement of instantaneous hormone titers and correlative glucose and lactate levels were measured from crabs at different times during the tidal cycle from a site around midtide level (Table 2). The results indicated that for crabs exposed to long periods of emersion, CHH levels were somewhat elevated, but paradoxically, glucose levels were somewhat lower in these animals, compared with others in which emersion times were shorter. During emersion, blood lactate levels remained unchanged.
Discussion

In the present study, we measured instantaneous circulating levels of both CHH and MIH from large numbers of crabs during intermolt and early and late premolt to define temporal release patterns of these peptides and defined release patterns in normal and stressed animals to gain further information on mechanisms of molt control and regulation of energy mobilization. Measurement of both hormones from more than 1000 crabs during 24-h cycles initially suggested stochastic release patterns. Bearing in mind the very short half-lives that we measured, high K+ evoked release of CHH from Cardi- soma carnifex X-organ-SG in vitro of about 0.02% min⁻¹ of the total CHH content of a single SG has been observed (26). Because Carcinus SG contain 30–50 pmol MIH (27), assuming that similar rates of MIH release could be sustained over 1 h, then up to 1.2% of the total content of the MIH in both SG could be released per hour (0.7–1.2 pmol). Because levels of CHH in the hemolymph are about 10 times higher than MIH, which correlate well with ratios in the SG, the same type of calculation can be applied for CHH, with similar results. However, sustained patterns of release would undoubtedly be untenable; rapid depletion of SG peptide would occur. Turnover rates of SG peptides are probably in the order of months (28), and there is firm evidence that in crustaceans (29, 30), as in other organisms (31–33), that immediate release pools of neuropeptides preferentially contain freshly synthesized rather than aged hormone. Thus, episodic rather than sustained release of CHH and MIH seems likely.

Comparison of all results from intermolt crabs from night or day revealed a pattern suggesting that either MIH or CHH was released; high titers of both peptides were very rare. Only two to three hemolymph samples above thresholds of 5 fmol/ml MIH and 5 fmol per 100 μl CHH were observed in more than 1000 samples analyzed. There was also some evidence to suggest that MIH release was greatest during nighttime. It is interesting to note that short (5 min) exposure of the Y-organ to MIH (which is likely to be analogous to single release events of hormone) results in measurable in-

**TABLE 1.** Comparison of hemolymph titers of CHH and MIH during intermolt (C₄) and early premolt (D₀₋₁)

<table>
<thead>
<tr>
<th></th>
<th>CHH (fmol/ml) C₄</th>
<th>CHH (fmol/ml) D₀₋₁</th>
<th>MIH (fmol/ml) C₄</th>
<th>MIH (fmol/ml) D₀₋₁</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>21.5</td>
<td>2.9</td>
<td>24.8</td>
<td>3.1</td>
</tr>
<tr>
<td>SEM</td>
<td>1.0</td>
<td>0.1</td>
<td>1.3</td>
<td>0.17</td>
</tr>
<tr>
<td>n</td>
<td>313</td>
<td>324</td>
<td>147</td>
<td>166</td>
</tr>
<tr>
<td>Significance</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values were taken from 24-h experiments during spring (February through April). NS, No significant differences (Kruskal-Wallis ANOVA and Dunn’s multiple comparison).

**FIG. 2.** Seasonal differences in CHH (gray bars) and MIH (black bars) hemolymph levels. Samples were taken during summer (June to August) and winter (December to February) and divided into daytime (L) or nighttime (D) samples (n = 122–209 for each sample). Bars, ± 1 SEM. Winter CHH levels were significantly lower than summer levels (*, P < 0.001). Nighttime MIH levels were elevated during winter, compared with daytime (*, P < 0.05). Kruskal-Wallis ANOVA and Dunn’s multiple comparisons used for statistical analysis.

**FIG. 3.** Changes in circulating levels of MIH in the hemolymph (solid bars) and MIH levels in the SG (gray bars) during intermolt and late premolt (n = 4–15 animals at each stage, bars, ± 1 SEM. Large asterisk indicates highly significant increases (P < 0.001, Kruskal-Wallis ANOVA and Dunn’s multiple comparisons) in circulating MIH, small asterisk, significant decrease (P < 0.05, Student’s t test) in SG MIH content during late premolt, compared with intermolt.

**FIG. 4.** Effect of temperature change on CHH release. Crabs (n = 5–8) were acclimated at 15 C and then thermally stressed (5 min) at 5–35 C. Bars, ± 1 SEM. Hemolymph samples were taken before and immediately after thermal stress. Asterisks indicate significant increases (P < 0.05, Student’s matched-pair t test) in CHH levels.
hormone levels (offset for clarity). Given an estimated hormone half-time of 5 min (see Fig. 1, approximations of presumptive decline in hemolymph CHH levels, an estimated hormone half-time of 5 min (see Fig. 1, inset).

Inhibition of ecdysteroid synthesis (34) and that after MIH exposure the Y-organ remains repressed for long periods of time (35, 36). Because CHH potentiates the action of MIH in a greater than additive manner (37), it is possible that seasonal changes in day length might alter MIH or CHH release patterns, in ways that could subsequently affect ecdysteroidogenesis by the Y-organ.

Because release of both hormones is episodic, it should be stressed that any analysis of hormone titers in which results are pooled will reduce apparent changes because in the majority of animals, hormone levels were basal. Thus, any responses will be difficult to detect. However, in view of the unprecedented numbers of samples taken in this study, some analysis seemed worthwhile. For seasonal changes (intermolt crabs) in CHH and MIH levels, CHH levels during winter were about half that in summer, which likely reflects inhibition of ecdysteroid synthesis necessary for premolt (37–41). This long-held hypothesis was tested by measurement of MIH levels during intermolt and early and late premolt. Mean MIH and CHH levels in intermolt (C4) and early premolt (D0–1) were very similar (MIH: 3 fmol/ml; CHH: 20–25 fmol/ml hemolymph), and it was noteworthy that ratios of both peptides were in accord with their concentrations in the sinus gland (1.7 MIH to CHH) (27). For the only other study (the crayfish Procambarus clarkii) in which MIH levels have been measured (using a very sensitive time-resolved fluoroimmunoassay), intermolt MIH levels were about 6 fmol/ml but dropped during early premolt to 1.3 fmol/ml. Intriguingly, subsequent pre- and postmolt MIH levels were very similar to those of intermolt (42). When we investigated MIH levels at a fine temporal scale during late premolt and ec dysis (Fig. 3), a remarkable, and unprecedented release of MIH was observed about 1 h before ec dysis (D3.4), when MIH reached high levels (40 fmol/ml), declining rapidly before ec dysis. This was reminiscent of the CHH surge in Carc. maenas, in which high levels of CHH (up to 2 pmol/ml), which arise by release of CHH from gut endocrine cells, precede ec dysis (10).

The MIH surge seems to be particularly dramatic in that there is a significant reduction in sinus gland MIH content at this time, which indicates a massive exocytotic event. Whereas this correlates well with the low levels of ecdysteroids characteristic of late premolt and might explain the rapid termination of ecdysteroid biosynthesis characteristic of this stage of the molt cycle (43), our studies on in vitro ecdysteroid biosynthesis show that the Y-organ becomes refractive to the inhibitory influence of MIH (5 nm) both in terms of inhibition of ecdysteroid synthesis and second messenger (cGMP) signaling during late premolt and early postmolt (D2-3, A-B) (27). Thus, if the in vitro situation is physiologically relevant to that in vivo, in which peak MIH levels are much lower (40 pm), it seems unlikely that the MIH surge is directly important in terminating ecdysteroid synthesis. Furthermore, the precipitous premolt decline in circulating ecdysteroid levels also occurs in eyestalkless animals (44–46). Unless there is an eyestalk source of MIH, which seems very unlikely, it is difficult to reconcile a role of MIH in termination of ecdysteroid synthesis before molting. Whereas our previous studies have shown that the MIH receptor is only expressed by the Y-organ (intermolt), using

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**TABLE 2. In situ measurements of CHH, glucose, and lactate levels**

<table>
<thead>
<tr>
<th>Time after midwater (h)</th>
<th>CHH (fmol/100 μl hemolymph)</th>
<th>Glucose (μg/100 μl)</th>
<th>Lactate (μg/100 μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–1.5</td>
<td>3.9 ± 0.9</td>
<td>2.1 ± 0.3</td>
<td>4.7 ± 1.2</td>
</tr>
<tr>
<td>2–3</td>
<td>4.8 ± 1.6</td>
<td>2.8 ± 0.7</td>
<td>4.4 ± 1.3</td>
</tr>
<tr>
<td>4.5–5</td>
<td>7.3 ± 1.3</td>
<td>1.5 ± 0.2</td>
<td>4.2 ± 0.5</td>
</tr>
</tbody>
</table>

Hemolymph samples were taken from approximately 30 crabs from a site around midtide level, from midwater until inundation on the next advancing tide. Samples were immediately frozen in liquid nitrogen.

* Significant differences (P < 0.05, Student’s t test) from samples taken 1–1.5 h after midwater. Means ± 1 SEM are shown.

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**Fig. 5.** Effects of repeated thermal stress on CHH levels. Crabs (n = 10) acclimated at 15 C were given hourly thermal stress for 5 min, 30 C. Gray bars show hemolymph glucose levels, filled circles, hemolymph CHH levels (offset for clarity). Bars, ± 1 SEM. Dotted lines show approximations of presumptive decline in hemolymph CHH levels, given an estimated hormone half-time of 5 min (see Fig. 1, inset).

**Fig. 6.** Effect of hypoxia (2 h) on hemolymph CHH levels in intact and eyestalk-ablated intermolt and premolt crabs (n = 6–8 at each stage). Gray bars, t = 0; black bars, t = 2 h. Bars, ± 1 SEM. For intact intermolt crabs, hypoxia resulted in greatly elevated CHH levels (P > 0.001); this was also seen in premolt animals (P < 0.05, Student’s matched-pair t test).
classical radioligand binding studies (22), it is possible that other tissues might express MIH receptors during late premolt, which would uncover novel roles for MIH. Identification of the MIH receptor is now timely.

Our studies have shown that not only MIH release is episodic and that basal, low levels of this hormone do not change during the molt cycle but also that levels do not decline during premolt. Thus, these results contradict the long-established hypothesis of molt control, whereby high levels of MIH are proposed to inhibit ecdysteroidogenesis during intermolt, and falling titers of MIH in premolt subsequently directly lead to a freeing of the Y-organ from the inhibitory influence of MIH, resulting in increased ecdysteroidogenesis and circulating ecdysteroid titers. Furthermore, because we have shown that during late premolt, there is a massive and unprecedented release of MIH, which has no known function, it is clear that the current model of molt control via MIH needs a complete reappraisal.

Studies on the response of Carc. maenas to stress were exclusively concerned with CHH because in every stressful scenario used (hypoxia, temperature shock, salinity change), increases in MIH levels were never observed. The first reports regarding hypoxia and its influence on CHH were from crayfish (Orconectes limosus), in which hypoxic episodes are associated with remarkable increases in CHH titer, consistent with this hormone’s presumed adaptive role (18). Emersion stress (which is associated with hypoxia as evidenced by hyperlactemia) also induces CHH release in subtidal crustaceans such as Can. pagurus (19) and Homarus americanus (20). In the present study, we confirmed this response in Carc. maenas, and show that this is entirely due to release of CHH from the eyestalk because eyestalk ablation completely removed hypoxia-induced CHH release in premolt animals. Thus, hypoxia induces release of CHH only from eyestalk neuroendocrine cells. Despite the significant accumulation of CHH in gut endocrine cells during late premolt (10, 47), this material is not released and can be discounted as being of relevance to the CHH-mediated stress response in Carc. maenas. With regard to neurotransmitter-mediated signaling mechanisms involved in this CHH release, very little is known. However, it may be pertinent to mention that dopamine (DA) seems to be important. In Carc. maenas, DA has been suggested to be involved in CHH release (48), and this has recently been conclusively demonstrated in Procambus clarkii (49). Because prolonged hypoxia increases γ-amino butyric acid levels in the brain of Carc. maenas (50), it is possible that it might be involved in the hypoxia-induced CHH release observed in this study. It would thus be interesting to measure correlative profiles of DA, γ-amino butyric acid, and CHH during hypoxic episodes.

Thermal stress is a second and possibly highly important environmental variable, which should lead to CHH release. Elevated temperatures have been shown to cause CHH release in O. limosus (18), H. americanus (20), and Can. pagurus (19), and a similar increase in CHH release after hyperthermic episodes was seen in the current study: We have shown that repeated episodes of thermal shock lead to repeated episodes of CHH release, which, considering the half-life of CHH in the blood, are episodic rather than prolonged. These are correlated with accumulating hyperglycemia (Fig. 5). Because it has been elegantly shown that CHH neurones in the X-organ of Can. borealis hyperpolarize in the presence of physiologically relevant levels of glucose (51), it seems very likely that CHH release involves feedback inhibition and might thus be intrinsically episodic or pulsatile. However, the most environmentally pertinent question might be: are these manipulations environmentally relevant? For an intertidal organism, such as Carc. maenas, which exhibits refuge behavior to minimize thermal stress and evaporative loss, hyperthermic stress is probably uncommon. However, hypothermic stress, on inundation with the rising tide, is undoubtedly important in an environmental context: temperate, boreal intertidal crustaceans regularly face twice-daily inundation in which seawater temperature is considerably different, and frequently lower, than that of the exposed intertidal environment. When hypothermic stress was applied, significant increases in CHH release were observed after 5 min exposure to subambient (5–10 C) temperatures. This response was asymmetrical: ca.15 C increases in temperature were needed to elicit the same magnitude of response after hyperthermic stress. This response was not observed in H. americanus (20) or Can. pagurus (19), which is understandable because these species are cold water stenothermal crustaceans.

In this context, it is interesting to note that hypothermic shock in the tropical palammonid prawn Macrobrachium rosenbergii also leads to hyperglycemia (52). Thus, it is tempting to suggest that thermally elicited CHH release is entirely related to life history strategy. Relating to this, hyper/hyposaline stress results in release of CHH in H. americanus (20), as befits a stenohaline organism; in Carc. maenas, which is a model euryhaline organism, we failed to measure episodic CHH release, even at salinities approaching its maximal environmental limits (3 parts per thousand seawater). Given the evidence from laboratory-based experiments, suggesting that CHH is an adaptive hormone, it was of interest to measure CHH titers in the field. After prolonged air exposure (low water), CHH levels increased. These changes did not occur as a result of increased lactate levels, which were invariant (suggesting that the crabs did not undergo hypoxia during low water). Whereas it was impracticable to measure hormone titers or glucose levels during inundation, it is tempting to suggest that increasing CHH levels during the advancing tide will result in high water hyperglycemia. Thus, in an adaptive context, increasing levels of CHH might increase glucose before extensive foraging and feeding behavior over high water.

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