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Dissociated cell suspensions of *Carcinus maenas* Y-organs as a tool to study ecdysteroid production and its regulation

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Abstract. In vitro ecdysteroid production by dissociated Y-organ cells of the shore crab *Carcinus maenas* was characterized during short-term incubations. Under optimized conditions (M199 adjusted to crab osmolality and with the addition of 10% foetal calf serum), ecdysteroid production by dispersed cells increased linearly during 4-hour incubations, with little intra-assay variation. 25-deoxyecdysone was mainly produced. Purified *Carcinus* molt inhibiting hormone (CamMIH) produced a dose-dependent inhibition of ecdysteroid production by dispersed cells. The cells were about 50 times more sensitive than whole glands to MIH. Other structurally-related peptides were tested.
Key words. Ecdysteroid biosynthesis; neuropeptide regulation; Y-organs; crustacea.

In crustaceans as well as in insects, steroid hormones (ecdysteroids) are involved in the control of molting (reviews in refs 1, 2). In crustaceans, ecdysteroids are secreted by glands of epidermal origin, termed Y-organs (review in ref. 3). Most authors agree that there is only one type of glandular cell present in Y-organs (reviews in refs 2–4). The ecdysteroid synthesis by the Y-organs is repressed by a molt-inhibiting hormone (MIH) produced by neurosecretory cells in the X-organ sinus gland complex. MIH action prevents the increase in ecdysteroid concentration necessary to initiate and sustain premolt processes. Steroidogenesis and its control have been studied in several marine and fresh-water species but many aspects remain unclear (reviews in refs 1, 4, 5).

To our knowledge, only a few studies have dealt with ecdysteroid production by cell suspensions of molting glands. Using prothoracic glands of *Manduca sexta*[6], dissociated cells appeared a promising tool to study the effect of hormones and pharmacological agents. However, in the crab *Cancer antennarius*[7], the results obtained with dispersed Y-organ cells did not convince the authors of the usefulness of such a model. One limitation of in vitro studies using whole Y-organs is the high interindividual variations of steroid production observed even between individuals at the same physiological stage. In the crab *Carcinus maenas*, a reduction in variation was observed only between both glands of the same animal[8]. It was then possible to characterize and purify MIH[9], by treating one organ with the regulatory factor and using the other one as control. But the use of numerous equivalent cell aliquots would be more convenient in multifactorial studies which require several complementary experimental groups. In addition, the differential penetration of various secretagogues into whole glands may affect the biological response. At present, results concerning the second messengers involved in MIH action appear somewhat contradictory. According to the species, cAMP in crabs[10,11] or cGMP in crayfishes[12,13] appear to be the primary second messengers in MIH action and the effect of calcium is still controversial (review in ref. 5). The use of cell suspensions might be a more reliable method to study the intracellular regulation of ecdysteroidogenesis. Concerning the study of steroidogenesis itself, the last steps of ecdysteroid biosynthesis have been elucidated in several species[14–16], including the crab *Carcinus maenas*. Short-term kinetic studies or studies of the first steps of biosynthesis are restricted by the poor incorporation of presumed precursors into whole organs[17] and might also be improved by the use of dispersed cells.

In order to extend the possibilities of in vitro experiments with *Carcinus maenas*, the use of dispersed Y-organ cells was investigated. Suspensions of Y-organ cells from a group of crabs at the same physiological stage were prepared. After optimization of the incubation medium, the characteristics of ecdysteroid production were compared to ecdysteroid synthesis by whole Y-organs. In a first attempt to develop a convenient screening method for identifying activatory or inhibitory factors, the effect of the major regulatory peptide (MIH) was analyzed and compared to other structurally-related peptides.

Materials and methods

Animals. Experimental animals (carapace width 3–4 cm) were supplied by the biological station of Roscoff (Brittany, France) and kept in aquaria containing well-aerated and filtered reconstituted sea-water at a temperature of 16 °C (salinity of 35%). They were fed fresh

mussels twice a week. Molting stages were determined according to Drach and Tchernigovtzeff[18].

Chemicals. Purified MIH and CHH (Crustacean Hyperglycemic Hormone) from *Carcinus maenas* were kindly provided by Dr. S. Webster (Bangor, UK). MIH/CHH and VIH (vitellogenic inhibiting hormone) from *Homarus americanus* were purified by Dr. D. Soyez in our laboratory. The antiserum (DUL2) was a generous gift of Pr. J. Koolman (Marburg, RFA). Ecdysone was supplied by Simes (Milan, Italy). 25-Deoxyecdysone was chemically prepared by Prof. R. Lafont. Tritiated ecdysone (specific activity 30 Ci/mmol) was routinely prepared as previously described[19] from [23, 24 – $^3H_4$]2-deoxyecdysone (a generous gift from Dr. Hetru, Strasbourg, France). Collagenase/dispase was from Boehringer. Medium 199 (M199), antibiotics and Trypan blue were purchased from Sigma. Double glass distilled water was used to prepare the medium and buffers. All other chemicals were of analytical grade.

In vitro incubations. Culture medium (pH = 7.4) was based on medium 199 with Hank's salts. The medium was Hepes-buffered (10 mM) and the osmolality (1080 mOsm) was adapted for *Carcinus maenas*. The salt concentrations were adjusted according to the ionic haemolymph composition established by Webb[20]: NaCl, 21.0 g/l; KCl, 0.49 g/l; $CaCl_2$, 1.30 g/l; $MgCl_2$, 1.70 g/l; Na_2SO_4, 2.13 g/l; $NaHCO_3$, 0.35 g/l. Penicillin (0.16 g/l) and streptomycin (0.10 g/l) were also added. 10% foetal calf serum (FCS) was added in designated experiments.

After dissection, the Y-organs used for the preparation of cell suspensions were kept at 4 °C in serum-free medium and minced with scissors. Y-organ cells were dissociated by incubation for about 1 h at 20°C in serum-free medium containing 0.25% of collagenase/dispase, with mild stirring. After dissociation the cells were washed 3 times with 2 ml of fresh culture medium with 10% FCS. Cell viabilities were determined using the Trypan blue (0.4%) dye exclusion test. The cells were pelleted by centrifugation at 300 *g* (4 °C) for 10 min and diluted with incubation medium to give a final concentration of at least 1.5×10^6 cells.ml^{-1} (about 3 Y-organ equivalent/200 µl).

Aliquots of cells (200 µl) or whole Y-organs (in 200 µl medium) were incubated for designated times in 5 ml glass tubes (at 20 °C, in darkness, under constant agitation). In designated experiments, neuropeptides dissolved in incubation medium were added to give final concentrations of 10^{-11} M to 10^{-8} M.

Assay of ecdysteroid secretion. After incubation, the tissue was pelleted (12,000 *g* for 10 minutes) and kept for total protein assay according to the method of Bradford (Biorad reagent) with catalase as standard. Ecdysteroids secreted into the medium were generally measured in duplicated 5–50 µl aliquots by radioimmunoassay (RIA) according to Chang and O'Connor[21] without prior extraction. [^{3}H]-ecdysone (6000 cpm/tube) was used as a tracer and DUL2 as antiserum (final dilution 1/10,000). DUL2 recognizes both 25-deoxyecdysone and ecdysone which are secreted by *Carcinus* Y-organs[15,16] (300 fmol/tube ecdysone or 25-deoxyecdysone are needed for a 50% displacement of radioactive ecdysone). The usable range of the ecdysone standard curve is from 30 to 4,000 fmol/tube. Results were expressed in ecdysone equivalents. Blank values were below the sensitivity of the standard curve. In one series of experiments, these two ecdysteroids were rapidly separated using simple chromatography on reversed phase C18-minicolumns (Sep-pak cartridges from Waters; elution of ecdysteroids by 5 ml of acetonitrile/water (30:70) followed by 2.5 ml of pure acetonitrile). Biological samples were eluted in 1 ml fractions. Fractions were evaporated to dryness, resuspended in 250 µl RIA buffer and 50 µl aliquots were measured by RIA.

Statistical analysis. Results are expressed as mean ± SE and compared using Student's test.

Results

Characteristics of the dissociated cells. Several enzymes (trypsin or enzyme cocktails) and treatments (mechanical dispersion, sifting the cells) were initially tested but did not lead either to healthy cells or to any ecdysteroid production in vitro. The enzymatic dissociation of the Y-organ cells by collagenase/dispase under mild stirring was monitored by microscopic observation and was generally achieved within 1–2 h. About 10^5 cells were recovered per Y-organ. There was no significant difference in the cell number recovered per Y-organ from premolt or intermolt animals, but a higher protein content of both whole Y-organs and equivalent as dispersed cells was found in premolt (table). The protein content per Y-organ equivalent after dissociation was only about 30% of that of whole Y-organ (table) indicating that there was an important loss of tissue during the cell dissociation. However, the viabilities of the dispersed cells were in the 80–90% range at the end of incubations (4 h).

Several attempts were made to improve in vitro ecdysteroid production (2 h incubations) from dispersed cells which, in medium 199 alone was low. Incubation in Grace's medium adapted to crab osmolality led to a significantly lower ecdysteroid production (56 ± 9% of control value in M199 alone; n = 7 replicates). Addition of foetal calf serum (FCS) induced a significant stimulation of ecdysteroid production (216 ± 22% of control value in M199 alone; n = 7 replicates). A 10% concentration of FCS was optimal for the dispersed cells, 5% leading to a weaker effect and 20% giving no further amelioration (data not shown). As a comparison, a reproducible but weaker effect of FCS on steroid pro-

Table. Characteristics of dissociated cells as compared to whole Y-organs

		intermolt	premolt
Number of cells/Y-organ equivalent		91,000 ± 13,000 (n = 12)[a]	99,000 ± 16,000 (n = 8)[a]
Protein content (μg/Y-organ or Y-organ equiv.)	*dispersed cells*	11.2 ± 3.2 (n = 7)[a]	20.6 ± 3.1 (n = 6)[a]
	whole Y-organs	41.2 ± 3.1 (n = 36)[b]	67.1 ± 5.6 (n = 30)[b]
	cell/Y-organ ratio	0.27	0.31

[a]Number of experiments for the cell number and protein content.
[b]Number of Y-organs for the Y-organ protein content.
Results are expressed as mean ± SE.

duction by whole Y-organs was observed only after 18 h (50% stimulation). The FCS effect could not be mimicked by BSA alone at a concentration of 0.4% (ecdysteroid production was 113 ± 14% of control value in M199 alone). M199 + 10% FCS was routinely used in further experiments.

Secretory patterns of in vitro ecdysteroid production. All experiments were performed in spring when both intermolt and premolt animals can be found. In several experiments, ecdysone and 25-deoxyecdysone were separated using sep-pak C18 cartridges. Tritiated standard ecdysone was eluted in the first 5 ml acetonitrile/water (30:70) solution and 25-deoxyecdysone in the following 2.5 ml of pure acetonitrile. 25-deoxyecdysone was the major steroid produced in vitro both in premolt (fig. 1A) and in intermolt (fig. 1B). The ratios between ecdysone and 25-deoxyecdysone were about the same for dispersed cells and whole organs (fig. 1A and 1B). The total ecdysteroid production by dispersed Y-organ cells was much higher in premolt stage (1600 ± 149 fmoles/μg protein in 2 h incubations: n = 12 replicates) than in intermolt stage (98 ± 6 fmoles/μg protein in 2 h incubations; n = 8 replicates). The ecdysteroid production by dispersed cells was significantly lower than that by whole organs but the ratio varied according to the molting stage (from 1/2 in premolt to 1/16 in intermolt). This difference could not be explained and cells from premolt animals were preferentially used.

The time-course of ecdysteroid production by dispersed cells was established in optimal premolt conditions (fig. 2). Medium was replaced after 15, 30, 60, 120, 180 and 240 min and ecdysteroid levels in the media were assessed at each time interval. Even if lower than by whole glands, ecdysteroid production by dissociated cells increased linearly during the 4 h incubation (fig. 2). Moreover, in that experiment, it was clearly verified that the production of ecdysteroid was much more variable between Y-organs than between cell aliquots.

Sensitivity of the dispersed Y-organ cells to regulatory factors. Purified *Carcinus* molt inhibiting hormone (CamMIH) was used to assess the effect of this major regulatory factor on ecdysteroid production by dispersed Y-organ cells. A nanomolar concentration of MIH significantly inhibited in vitro ecdysteroid production by premolt Y-organ cells within 30 min of addition. The maximal effect was reached after 1 h (fig. 3A). The dose-dependent inhibition of ecdysteroid production by MIH was determined over a $10^{-12} - 10^{-8}$ M range

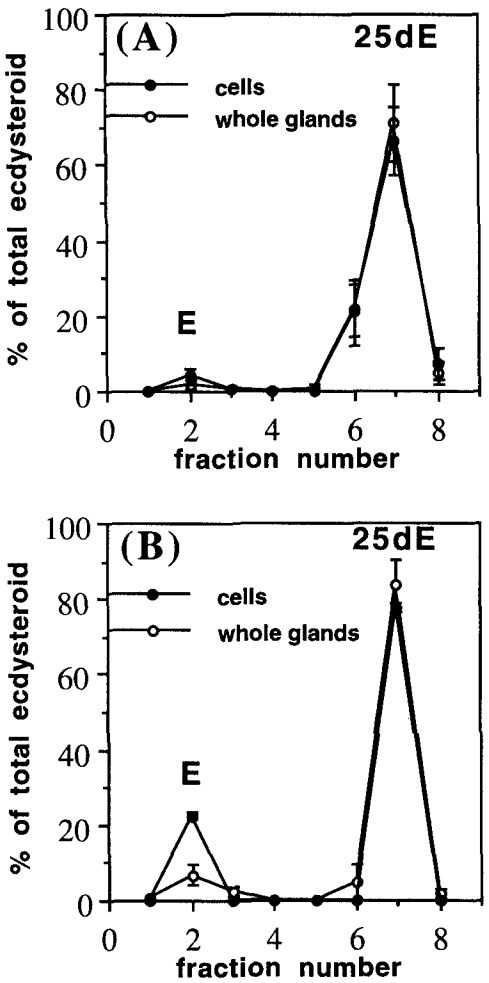

Figure 1. Separation of ecdysone and 25-deoxyecdysone produced in vitro by dispersed cells and whole Y-organs during 2 h incubations. Steroids were eluted by 5 ml acetonitrile/water (30:70) followed by 2.5 ml pure acetonitrile. Fractions were 0.5 ml for fraction 1 and 1 ml thereafter. *A* Separation of ecdysteroids produced by premolt Y-organs (n = 4 replicates for the whole glands and cell aliquots). *B* Separation of ecdysteroids produced by intermolt Y-organs (n = 3 replicates for the whole glands and pooled aliquots from n = 2 different experiments for the cells).

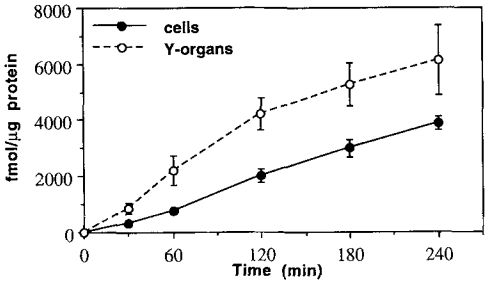

Figure 2. Time-course of in vitro ecdysteroid production by dispersed Y-organ cells and whole Y-organs from premolt animals. Dispersed cells (3-4 replicates) and whole glands (3 replicates) were incubated in M199 + 10% FCS, at 20 °C. Medium was replaced at each period interval. Results are expressed as mean ± SE of the cumulative ecdysteroid production in fmol/µg protein.

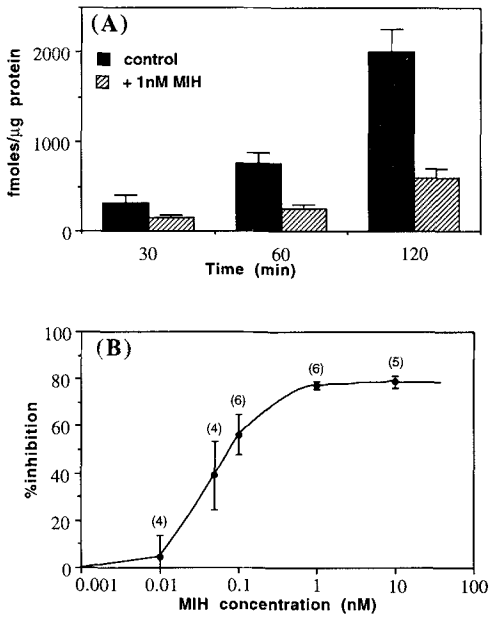

Figure 3. *A* Time-course effect of purified CamMIH (1nM) on in vitro ecdysteroid production by dispersed Y-organ cells from premolt animals. Medium (M199 + 10% FCS with or without dissolved MIH) was replaced at each period interval. Results are expressed as mean ± SE (n = 4 replicates) of the cumulative ecdysteroid production in fmol/µg protein. *B* Dose response curve showing the ability of increasing concentrations of purified CamMIH to repress in vitro ecdysteroid synthesis by Y-organ cells from premolt animals. Percentages of inhibition are calculated as ecdysteroid production in treated aliquot/mean ecdysteroid production of 4 control aliquots. Results are expressed as mean percentage of inhibition ± SE. (n): number of replicates.

(fig. 3B). The dose-response curve displayed features observed in earlier studies from Webster and Keller[9], namely a log-linear dose response relationship tending towards an asymptote. Maximal inhibition was reached for about 10^{-9} M MIH. The concentration required to repress half-maximally ecdysteroid synthesis was much lower for the dispersed cells (about 5×10^{-11} M) than for whole glands (about 1.5×10^{-9} M as determined by Webster and Keller[9]). The inhibitory effect of MIH was observed in intermolt and premolt stages for both whole glands and cell suspensions (fig. 4), but maximal inhibition was higher for the cells (nearly 80% in premolt) than for the glands (nearly 60% in premolt).

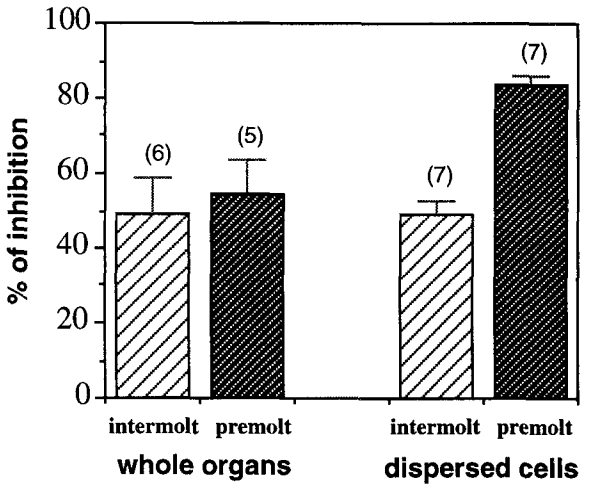

Figure 4. Maximal effect of purified *Carcinus* MIH on ecdysteroid in vitro production by whole Y-organs (10^{-8} M MIH) and dispersed Y-organ cells (10^{-9} M MIH) from intermolt and premolt animals. Dispersed cells and whole glands were incubated for 2 h in M199 + 10% FCS, at 20 °C. Percentages of inhibition are calculated as ecdysteroid production in treated aliquot/mean ecdysteroid production of 4 control aliquots for the dispersed cells and production by one gland in medium + MIH/production by the contralateral gland in control medium for the whole glands. Results are expressed as mean percentage of inhibition ± SE. (n): number of replicates.

In order to define the specificity of the MIH inhibitory effect, other related peptides were tested. A 10^{-8} M concentration of *Carcinus* hyperglycemic hormone (CamCHH) as compared to 10^{-10} M of CamMIH produced a 50% inhibition of ecdysteroid production (fig. 5). In addition, the effect of two structurally-related heterologous peptides was tested at the same concentrations. MIH/CHH and VIH (vitellogenesis inhibiting

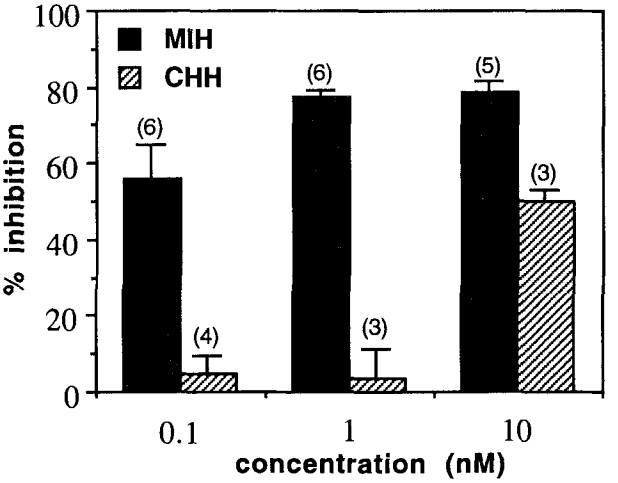

Figure 5. Effect of graded concentrations (0.1, 1 and 10 nM) purified CamMIH and CHH on ecdysteroid in vitro production by Y-organ dispersed cells from premolt animals. Results are expressed as mean percentage of inhibition ± SE (see fig. 4 for details of the calculation). (n): number of replicates.

hormone) from *Homarus americanus* (HoaMIH/CHH and HoaVIH) did not present any inhibitory effect on ecdysteroid in vitro production by *Carcinus* Y-organ cells.

Discussion

In the present study, suspensions of dispersed Y-organ cells were incubated in vitro. The aim was to get a homogeneous material which would permit the study of steroidogenesis and its control in a crustacean at a cellular level. The enzymatic dispersions yielded a single type of cells with large nuclei as expected from histological descriptions (reviews in refs 2–4). The lower protein content of dispersed cells as compared to whole glands might be partly explained by the loss of loose connective tissue, blood vessels and basal sheath which surround the compact glandular tissue of the Y-organ in *Carcinus maenas*[2]. Increased protein content of the premolt Y-organs reflected an increased abundance of cytoplasmic material described during premolt[2], indicative of higher steroidogenic activity at this stage.

To our knowledge, most in vitro studies regarding steroidogenesis and its control in crustaceans used incubations of whole Y-organs (in marine crabs[15,16,22–29], prawns[30] and fresh-water crayfishes[14,31–34]). The steroidogenic potential of dispersed cells was only tested in *Cancer antennarius*[7]. Optimal in vitro conditions for *Carcinus maenas* were based on enriched culture medium M199 (a widely-used medium for crustaceans, review in ref. 35) and addition of heterologous serum. Mattson and Spaziani[7] observed that the combination of these two elements was necessary to improve ecdysteroid production. It is demonstrated in the present work that the stimulatory effect of FCS could not be mimicked by BSA. Further studies will be needed to identify the stimulatory factor present in the serum. In our experiments, as in similar studies in crustaceans[7] and insects[6], ecdysteroid production by dispersed molting gland cells was significantly lower than by whole organs. On the other hand, the ecdysteroid output by dispersed cells increased linearly during several hours and the improved reproducibility as compared to whole Y-organs should allow easier in vitro studies of small responses to regulatory factors.

The characteristics of in vitro ecdysteroid production were analyzed for dispersed cells and whole organs according to the molting stage. Using an antiserum which recognizes ecdysone and 25-deoxyecdysone (DUL2) with almost equal affinity and simple separation procedure, it was demonstrated that both dispersed cells and whole glands from premolt crabs produce predominantly 25-deoxyecdysone, as already mentioned by Lachaise et al.[25] for whole glands. Only a slight increase in ecdysone proportion was noticed during intermolt. The physiological significance of limited ecdysone production besides the major 25-deoxyecdysone production remains unclear. At present, it is assumed that ecdysone and 25-deoxyecdysone are converted by peripheral tissues to their 20-hydroxylated metabolites, 20-hydroxyecdysone and ponasterone A[16] (the major circulating ecdysteroid in premolt) but no specific physiological effect for any of these steroids has been described. The higher E/25dE ratio observed when using a radioactive exogenous putative precursor[15,16] (tritiated 2,22,25 trideoxyecdysone or ketodiol) might be explained by a different pathway of intracellular transport of this steroid as compared to endogenously synthesized intermediates, and consequently easier access to the microsomal[4] 25-hydroxylase.

It is generally assumed that ecdysteroid biosynthesis in crustaceans is chronically repressed during intermolt by the molt inhibiting hormone (MIH) originating from the X-organ-sinus gland, a neurohemal complex. The fact that Y-organ cells from intermolt animals continue to produce much less ecdysteroids in vitro than premolt Y-organ cells indicate profound modifications of the steroidogenic capacities (e.g. modification of the steroidogenic enzyme content) in response to this neuropeptide. These results are similar to those obtained in crayfish[31,32,34] but differ from the findings of Mattson and Spaziani[7] indicating that in *Cancer antennarius*, rinsing intermolt Y-organs was sufficient to recover the steroidogenic potential of premolt Y-organs. On the other hand, a rapid effect of MIH (within 30 min) was observed on ecdysteroid production by dispersed cells (the present study) and on whole glands (Saïdi and Lachaise, pers. commun.). Further studies on the kinetic effect of MIH will be of great interest to distinguish more precisely acute (e.g. action through cyclic nucleotides[11]) and chronic effects of this neuropeptide.

Cell suspensions of *Carcinus* Y-organs appear a promising tool to study the regulation of ecdysteroidogenesis at the cellular level. Preliminary experiments using too drastic dissociation conditions indicated damage of the membrane-bound receptors to MIH (as revealed by a comparison of MIH-binding to membrane preparations from whole glands and dispersed cells kindly performed by Dr. S. Webster). Using optimal conditions of dissociation, purified CamMIH[9] was a potent inhibitor of ecdysteroid production in vitro by Y-organ cell suspensions from both intermolt and premolt animals. The sensitivity of dispersed cells to MIH (5×10^{-11} M for 50% maximal inhibition) was more than 50 times higher than that of whole glands[9]. This low value fits well with the K_D for MIH obtained with membrane preparations from Y-organs (1×10^{-10} M according to Webster[36]). This higher sensitivity of dispersed cells, already pointed out by Mattson and Spaziani[7] in *Cancer*, might be explained by a better accessibility of MIH to cell membrane receptors. As in whole glands[9], a weaker inhibitory effect of hyperglycemic hormone (CamCHH) on ecdysteroid production was noted (50% inhibition

for 10^{-8} M as compared to 10^{-10} M for CamMIH). In a recent study, Webster[36] showed that the Y-organs possessed both MIH and CHH receptors with nearly equivalent binding capacities towards MIH and CHH respectively, but that there is also some cross-reactivity between the two peptides (there is a 25% sequence homology between CamMIH and CamCHH). The physiological effect of CHH on Y-organs through CHH receptors has not yet been elucidated[36] but the consistently different inhibitory effects of MIH and CHH on ecdysteroid biosynthesis suggest that in that respect both peptides act through MIH receptors.

Finally, in order to gain some information on the specificity of the MIH effect, some other structurally-related peptides were tested. MIH belongs to a family of peptides specific to crustaceans, the MIH (molt inhibiting hormone)/CHH (crustacean hyperglycemic hormone)/VIH (vitellogenesis inhibiting hormone) neuropeptides[4, 37, 38]. Chang et al.[39] have sequenced a peptide from *Homarus americanus* sinus glands, similar to HoaCHH identified by Tensen et al.[40] and which displays MIH and CHH activities in homologous in vivo assay. In our in vitro assay, a 10^{-8} M concentration of the HoaMIH/CHH had no inhibitory effect on ecdysteroid production. This means that the inhibitory effect if any is at least 100 times lower than that of CamMIH. HoaVIH has greater structural homology (48%) to CamMIH[41] than even CamCHH. However, this peptide (at a 10^{-8} M concentration) did not inhibit ecdysteroid production by dispersed *Carcinus* Y-organ cells. In contrast, MIH from two other crabs (*Necora* and *Cancer*) tested by Webster[36] presented no obvious differences in biological activity from CamMIH. These results may emphasize the specificity of MIH receptors and the evolutionary difference between astacurans and brachyurans as already mentioned in CHH studies (in *Orconectes*, CamCHH is almost ineffective while the HoaCHH is quite effective[37]). The Y-organ cell suspensions would provide a good standardized in vitro assay to test systematically the relative potencies of fully characterized MIH molecules. In conclusion, our study shows that dispersed cells may provide an improved method for the study of steroidogenesis and its control at a cellular level in crustaceans molting glands. This system can provide a convenient screening method for identifying activatory or inhibitory factors.

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