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Regulation of steroidogenesis in crayfish molting glands: involvement of protein synthesis

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Abstract

The involvement of continuous protein synthesis in the mechanisms of crustacean steroidogenesis was investigated using crayfish molting glands (Y-organs). During intermolt, Y-organ steroidogenic activity is low. Eyestalk ablation initiates premolt which is characterized by a rapid increase in the production of ecdysteroids. In vitro incorporation of [^{14}C]leucine into TCA-precipitable proteins was measured in Y-organs. A significant increase of de novo protein synthesis was observed in the premolt stage compared to the intermolt stage. On the other hand, cycloheximide totally suppressed protein synthesis within 2 h and simultaneously led to a strong inhibition of the ecdysteroid synthesis. Sinus gland extracts (containing molt inhibiting hormone) also induced both a limited but reproducible inhibition of Y-organ protein synthesis and a pronounced inhibition of ecdysteroid production within 2 h. The results suggest a functional link between protein synthesis in the Y-organ and sustained ecdysteroid production. The analysis of autoradiographs from one-dimensional gel electrophoreses revealed an overall increase in de novo synthesis of glandular proteins in early premolt but also a more specific effect on distinct proteins (increase of 150, 140, 50–60, 22 and 15–18 kDa proteins) which may be more directly involved in the regulation of ecdysteroidogenesis.

Keywords: Steroidogenesis; Regulation; Protein synthesis; Ecdysteroids; Crustaceans; Molting glands

1. Introduction

In arthropods, steroids (ecdysteroids) are involved in a major developmental event: the molt. Ecdysteroids are synthesized by well-individualized paired glands called prothoracic glands in insects and Y-organs in crustaceans (Spaziani, 1990; Lachaise et al., 1993). There are important variations of hemolymphatic ecdysteroid levels according to the molting stage: very low titers are measured during intermolt followed by a pronounced peak during premolt. These hemolymphatic fluctuations roughly parallel the evolution of the steroidogenic capacity of the molting glands (e.g. for *Orconectes limosus* in Keller and Schmid, 1979) which are under neuroendocrine control.

In insects, the major regulatory factor is a brain neuropeptide (prothoracicotropic hormone or PTTH) stimulating ecdysteroid production (Smith, 1993) but in crustaceans, a major factor of regulation is the molt inhibiting hormone (MIH) (Lachaise et al., 1993), an eyestalk derived peptide stored in a neurohemal organ (the sinus gland) which negatively controls ecdysteroid production. MIH would inhibit the ecdysteroid production tonically during intermolt but the relative importance of both acute (within minutes) and chronic (within days) effects of MIH are not precisely known.

In vertebrates, regulation of steroidogenesis by neuropeptides presents both acute (Liscum and Dahl, 1992) and chronic (Waterman and Simpson, 1989) aspects. Tropic peptides (ACTH, LH) exert a chronic stimulation on biosynthetic enzymes levels (at the transcriptional level) via rapid enhancement of transcription factors termed steroid hydroxylase-inducing proteins (SHIPs). The acute stimulation of steroid production depends primarily on cholesterol supply to the first steroidogenic enzyme (cytochrome P450scc system) located in the inner mem-

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brane of mitochondria. Short-lived proteins involved in cholesterol trafficking are rapidly stimulated by regulatory peptides. Thus, both acute and chronic stimulation of vertebrate steroidogenesis involve protein synthesis.

In arthropods, the ecdysteroid biosynthetic pathway has not yet been fully elucidated (Grieneisen, 1994). However, the importance of translation in regulation of ecdysteroid production has been pointed out for several years in insects (Keightley et al., 1990). Recent studies on *Manduca sexta* (Kulesza et al., 1994; Rybczynski and Gilbert, 1994) have evidenced the importance of increased protein synthesis for sustained ecdysteroid production. In crab, it was shown that MIH might act via an inhibition of protein synthesis (Mattson and Spaziani, 1986).

Ecdysteroid production and its control have been extensively studied in a freshwater crustacean species, *Orconectes limosus* (Keller and Schmid, 1979; Jegla et al., 1983; Von Gliscynski and Sedlmeier, 1993; Sedlmeier and Fenrich, 1993; Böcking et al., 1993; Böcking and Sedlmeier, 1994). The present study was undertaken in order to determine whether in crayfish Y-organs, steroidogenesis and protein synthesis were linked. The effect of cycloheximide on ecdysteroid production was analyzed. In addition, de novo protein synthesis by crayfish Y-organs was examined according to the molting stage of the animals and to the effect of a crude MIH-preparation .

2. Materials and methods

2.1. Animals

Crayfish, *Orconectes limosus,* were obtained from the Havel river in Berlin. They were maintained in tanks under running tap water at 10–12°C and under a 14 h/10 h light/dark photoperiod. Animals were fed once a week with cat food pellets. Only males were used in our experiments. Premolt was induced by eyestalk ablation. Eyestalkless animals were transferred to compartmentalized aquaria at 18°C. Molting stages were determined according to Willig and Keller (1973).

2.2. Chemicals

All chemicals were of analytical grade. Non-radioactive ecdysone was supplied by Simes (Milan, Italy). α-[23,24-^{3}H(N)]Ecdysone (specific activity approximately 2.5 TBq/mmol) was purchased from NEN DuPont (Dreieich, Germany); L-[U-^{14}C]Leucine (11 Gbq/mmol) and L-[^{35}S]methionine (43.5 TBq/mmol) were from Amersham (Braunschweig, Germany).

2.3. Tissue preparation and incubation

Y-Organs were located according to Burghause (1975) and dissected under ice-cold Van Harreveld saline (Van Harreveld, 1936). Subsequently, glands were transferred into 200 μl control Medium 199 with Hank's salts modified according to Keller and Schmid (1979), or medium containing experimental agents. Incubations were performed in sterile 96-well microtiter plates (NUNC, Denmark) at room temperature and under constant agitation. To determine the effect of cycloheximide or sinus gland extracts, the two glands from the same animal were individually incubated one as a control and the contralateral one receiving treatment. Cycloheximide was added at a concentration of 4×10^{-5} M (2.5 μg/incubation), a concentration similar to those generally used both in invertebrate (Mattson and Spaziani, 1986; Keightley et al., 1990) and vertebrate (Waterman and Simpson, 1989; Payne and Sha, 1991) studies. Sinus gland extracts (SGE) were prepared according to Webster and Keller (1986). Briefly, after dissection of the sinus glands, neuropeptides were extracted twice using ice-cold 2 M acetic acid. The supernatant was dried under reduced pressure and the material was redissolved in culture medium just prior to incubations. One sinus gland equivalent was used per incubation, since this concentration was previously shown to induce maximal inhibition of ecdysteroid synthesis by crayfish Y-organs (Sedlmeier and Fenrich, 1993).

2.4. In vitro assay of ecdysteroid secretion

After incubation for designated times, total ecdysteroid concentrations were measured in duplicate 20–100 μl aliquots of incubation medium without previous extraction by radioimmunoassay (RIA) according to Chang and O'Connor (1979). The ecdysteroid content of the Y-organs themselves was very low compared to the ecdysteroids found in the medium (less than 10% of a 4-h production) and was therefore neglected (data not shown). [^{3}H]Ecdysone was used as a tracer; DUL-2 antiserum was kindly provided by Professor J. Koolman (Marburg, Germany). This antibody recognizes nearly equally ecdysone and 3-dehydroecdysone which are both secreted by *Orconectes* Y-organs (Böcking et al., 1993). Results were expressed as ecdysone equivalents if not specified. The usable range of the ecdysone standard curve was 15–2000 pg ecdysone/tube.

2.5. Measurement of protein synthesis

To determine the time course of [^{14}C]leucine incorporation into proteins, pairs of glands were incubated for designated times in 200 μl of medium containing 0.5 μCi of [^{14}C]leucine, tissue was then homogenized in 250 μl of ice-cold 0.01 N NaOH. The protein content of the Y-organs was evaluated using the total protein assay (Pierce BCA assay) on a 50-μl aliquot of the homogenate. Bovine serum albumin (BSA) was used as standard. Proteins were precipitated in the remaining tissue homogenate and in the medium with trichloroacetic acid (TCA) using a method modified from Mattson and Spaziani (1986). Two hundred micrograms of BSA and 1 ml of 10% TCA were added. The samples were vortexed and centrifuged for 10 min at 2000 $\times$ g. The pellets were washed twice by sequential additions of 250 μl NaOH (0.01 N) and 1 ml of

10% TCA. The pellets were resuspended in 200 μl of water, dissolved in 1 ml of scintillation cocktail (Aqualuma plus, Baker Chemicals) and counted using an Isocap 300 β-counter (Searle, Chicago).

The same protocol was used to study the variation of de novo protein synthesis according to the molting stage and to the effect of cycloheximide, and in a first series of experiments to analyze the effect of SGE.

To study the effect of SGE, another protocol was also used: groups of 4–5 glands were incubated for 3 h in 200 μl of medium 199 free of unlabelled leucine or methionine to which [^{14}C]leucine (1 μCi) or [^{35}S]methionine (10 μCi) was added, respectively. At the end of incubation, the glands were rinsed during 1 h in 200 μl of normal medium 199. The tissue was further processed (homogenization and TCA precipitation of the proteins) as described for the first series of experiments except that BSA was omitted.

2.6. Electrophoresis of labeled proteins

In a further series of experiments, [^{35}S]methionine labeled proteins were analyzed by electrophoresis followed by autoradiography. [^{35}S]Methionine was generally used instead of [^{14}C]leucine to take advantage of its higher specific activity. Following 3-h incubations with radiolabelled methionine (10 μCi in methionine-free medium 199) and one additional hour in normal medium 199, pools of glands were homogenized and proteins were precipitated with TCA. The pellets were boiled for 5 min in a denaturing solubilizing buffer (30% glycerol, 6% SDS, 10% β-mercapto-ethanol, 0.002% bromophenol blue, 1 mM DTT, 0.12 M Tris–HCl buffer, pH 6.8). After liquid scintillation counting of an aliquot, similar amounts of radioactivity were loaded on gels in order to facilitate the detection of specific proteins. The analysis by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) was performed on continuous gradient gels 9–18% acrylamide. Gels were fixed and stained in Coomassie blue R250/methanol/acetic acid/water (0.125 g/40 ml/10 ml/50 ml) and destained in methanol/acetic acid/water (25 ml/8 ml/67 ml). Autoradiographs were obtained from dried gels using Kodak X-Omat films, exposed at −80°C for 2–4 weeks.

2.7. Statistical analysis

Results are expressed as mean ± SE and compared using Student's t-test.

3. Results

3.1. Time-course incorporation of radiolabeled leucine into proteins and production of ecdysteroids by Y-organs in vitro

Incorporation of leucine into Y-organ proteins increased linearly during at least a 24-h period (Fig. 1A; results obtained for early premolt animals). Under our in vitro conditions (Fig. 1B), the ecdysteroid production by the Y-organs regularly increased throughout 21 h of incubation but the highest production was observed during the first 2 h of incubation. Thus, the first 20 h of in vitro incubation appeared suitable for studies concerning protein or ecdysteroid synthesis.

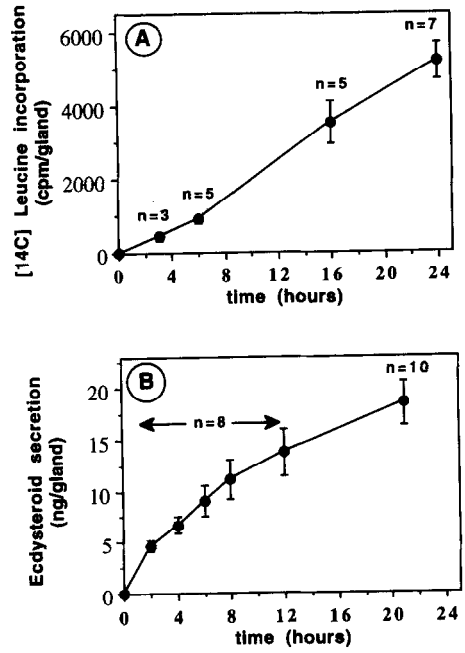

Fig. 1. Time-course of (A) [14C]leucine incorporation into TCA precipitable proteins and (B) ecdysteroid production by *Orconectes limosus* Y-organs. Glands from premolt animals were incubated in vitro during the indicated times. Each point represents the mean ± SE of n incubations from pairs of glands.

3.2. Incorporation of leucine into Y-organ proteins according to the molting stage of the animals

Like in intact premolt animals, in vitro ecdysteroid production increased rapidly in eyestalkless animals: from 317 ± 26 pg/2 h per Y-organ in intermolt to 4153 ± 317 pg/2 h per Y-organ 4 days after eyestalk ablation (n = 18 incubations). Incorporation of leucine into proteins was measured during 20 h in in vitro incubations of Y-organ pairs from animals at different molting stages. The early premolt (early D0 stage) was characterized by a significant and rapid increase of the rate of de novo protein synthesis: a nearly 2-fold increase of leucine incorporation per μg of protein was measured 2 days after eyestalk ablation (Fig. 2A). A more general trophic effect further developed as premolt proceeded (late D0 to D3 premolt stages) and was evidenced both by a prolonged increase in leucine incorporation into proteins as expressed in cpm/Y-organ (Fig. 2B) and by an increased total protein content of the glands (Fig. 2C).

Newly synthesized Y-organ proteins secreted into the incubation medium were not analyzed since they generally represented less than 10% of the total TCA precipi-

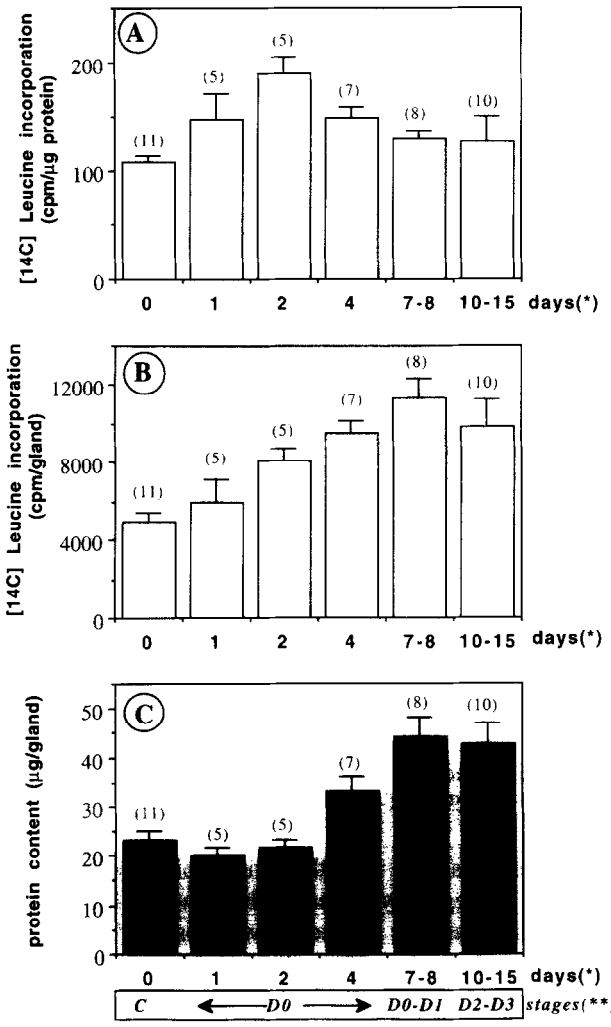

Fig. 2. Variations of Y-organ proteins according to the molting stage of the animals: (A) Incorporation of [^{14}C]leucine into TCA precipitable proteins per Y-organ; (B) Incorporation of [^{14}C]leucine into TCA precipitable proteins per μg protein and (C) protein content of the Y-organs. Results are expressed as mean ± SE of (n) separate pairs of glands. (*) Number of days after eyestalk ablation. (**) Molting stages were determined according to Willig and Keller (1973).

table proteins and were not separated as specific bands in autoradiography (data not shown).

3.3. Effects of the translation inhibitor, cycloheximide, on steroidogenesis

In order to determine whether the presence of newly synthesized proteins was required to enable the strong increase of ecdysteroid production in early premolt, the effect of the translation inhibitor cycloheximide was studied at this molting stage (Fig. 3A). The incorporation of leucine into proteins was totally inhibited within 2 h (211.8 ± 46.3 and 3.3 ± 0.2 cpm × h^{-1} per gland in controls and treated, respectively; $n = 4$ incubations). Ecdysteroid production was rapidly inhibited (50% inhibition within 2 h) in the presence of cycloheximide. The inhibition was more than 70% after 4 h. It was verified in an additional experiment that the inhibition of ecdysteroid production was not due to an inhibition of ecdysteroid secretion into the incubation medium (data not shown). After 4 h, cycloheximide was removed from the culture medium but the effect lasted and was even stronger after two additional hours (nearly 90%). The effect of 4 h cycloheximide treatment on ecdysteroid synthesis was reversible as ecdysteroid production by control and treated Y-organs was similar 16 h after removal of cycloheximide (Fig. 3A), excluding a cytotoxic effect.

To determine whether short-lived proteins were also necessary for basal ecdysteroid production, a similar experiment was performed using Y-organs from intermolt animals (Fig. 3B). Due to high interindividual variations between controls, the inhibition of ecdysteroid production by cycloheximide was not statistically significant during the first 2 h but thereafter it became very similar to that observed in premolt: 65% inhibition was observed within 4 h, a nearly 90% inhibition during the 2 h following the removal of cycloheximide and reversibility of the inhibition was observed during the next 16 h.

3.4. Effects of sinus gland extracts (SGE) on ecdysteroid and protein synthesis

In order to investigate whether the inhibitory effect of MIH on ecdysteroid synthesis acted via an inhibition of protein synthesis, Y-organs from early premolt animals were incubated in vitro with sinus gland extracts (one

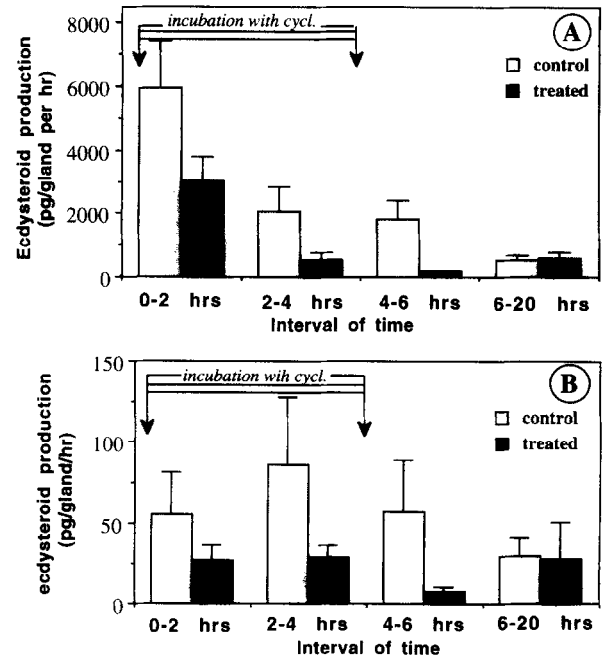

Fig. 3. Effect of cycloheximide on ecdysteroid in vitro production by Y-organs from (A) early premolt and (B) intermolt animals. Results are expressed as mean ± SE of five separate incubations of individual Y-organ glands. Glands were incubated with or without 2.5 μg cycloheximide during the first 4 h. Thereafter fresh control medium was used. Medium was changed at each interval of time.

Table 1

Effect of sinus gland extracts (1SG equ./inc.) on ecdysteroid production and amino acid incorporation into proteins by crayfish Y-organs

	Ratio of treated to control early premolt glands	
Ecdysteroid production (2 h)	0.65 ± 0.05	(n = 9)*
[^{14}C]Leucine incorporation		
16 h	0.83 ± 0.10	(n = 9)*
3 h + 1 h	0.64 ± 0.14	(n = 3)**
[^{35}S]Methionine incorporation		
3 h + 1 h	0.70 ± 0.09	(n = 3)**

Values are mean ± SEM of the calculated ratios; n = number of separate experiments.

[a] Individual contralateral glands or [b]pools of glands were compared.

sinus gland equivalent per Y-organ). Within 2 h incubations, the ecdysteroid synthesis by Y-organs treated with SGE was significantly depressed (nearly 35% inhibition; Table 1) compared to the contralateral untreated ones.

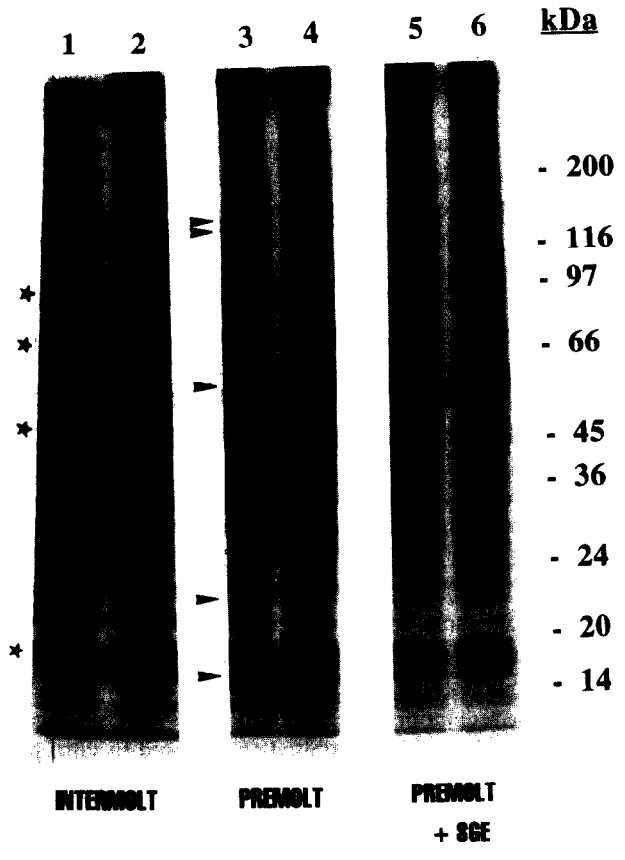

Fig. 4. Pattern of Y-organ proteins analyzed by 1D-electrophoresis followed by autoradiography. Tissues were incubated during 3 h in medium containing [^{35}S]methionine (10 μCi). Lanes 1 and 2, Y-organs from intermolt animals; lanes 3 and 4, Y-organs from early premolt animals; lanes 5 and 6, Y-organs from early premolt animals with MIH. Arrows indicate proteins whose rates of synthesis increase in premolt. Asterisks indicate proteins whose rates of synthesis decrease in premolt. Similar results were seen in replicate experiments (n = 3).

Y-Organs were further incubated during 16 h with radiolabelled leucine in the presence or absence of SGE. The protein synthesis by treated Y-organs was only slightly inhibited (17% inhibition, Table 1) compared to the contralateral untreated ones. Due to high interindividual variations, this difference was not statistically significant (paired Student's t-test). When pools of Y-organs were incubated for shorter periods (3 h) in leucine- or methionine-free Medium 199 with either radioactive leucine or methionine, a significant inhibition of protein synthesis (30–36% inhibition depending on the radiolabelled amino acid; Table 1) was noticed.

3.5. Electrophoresis of newly synthesized proteins

Additional experiments focussed on the characterization of synthesized proteins in Y-organs from intermolt and early premolt (4 days after eyestalk ablation) animals by the means of SDS-PAGE electrophoresis. Fig. 4 gives a representative picture of the variations of Y-organ protein patterns. The general pattern of Y-organ proteins (Fig. 4) was roughly similar in intermolt (lanes 1 and 2) and premolt (lanes 3 and 4) stages indicating a general enhancement of (overall) protein synthesis in premolt. However, the incorporation of [^{35}S]methionine into some proteins was more markedly increased in early premolt (approximate molecular weights 150, 140, 50–60, 22 and 15–18 kDa, see arrows in Fig. 4). A few protein bands appeared to be more actively synthesized in intermolt (approximate molecular weights 100, 78–75, 50, 20 kDa; see asterisks in Fig. 4).

The lower incorporation of [^{35}S]methionine into Y-organ proteins observed after SGE treatment of premolt glands (Table 1) seemed to be a general effect of MIH on overall protein synthesis. None of the proteins which were more intensely radiolabelled in intermolt recovered their intensity in the course of the 4 h in vitro incubations with SGE (Fig. 4; lanes 5 and 6).

4. Discussion

In the present study as in previous works (Keller and Schmid, 1979; Jegla et al., 1983), premolt was artificially induced by eyestalk ablation. It was characterized by normal premolt events like an increase in the steroidogenic capacities of the Y-organs, an increase in ecdysteroid titers in the hemolymph, formation of gastroliths and setae, and finally molting (after 15–18 days under our experimental conditions). As indicated by the effects of eyestalk ablation, the regulatory mechanisms underlying the important changes of ecdysteroid biosynthesis imply primarily the action of a molt inhibiting hormone (MIH) located in the eyestalks. It is generally assumed that steroidogenesis in intermolt lies mainly under the control of MIH. MIH exerts acute effects since in the present study, inhibition of ecdysteroid synthesis is observed within 2 h. Even more rapid effects have been demon-

strated in the crab *Carcinus maenas* (Saidi et al., 1994; Toullec and Dauphin-Villemant, 1994). In addition, chronic effects of MIH are evidenced since steroid in vitro production by Y-organs from intermolt animals remains far below production from premolt animals (as shown by the comparison of Figs. 3A,B). Moreover, a lack of reversibility of MIH action on ecdysteroid in vitro production by Y-organs from premolt animals was shown in several crab species (Soumoff and O'Connor, 1982; Saïdi et al., 1994).

Protein synthesis is involved in regulation of steroidogenesis in vertebrates and arthropods but our knowledge concerning crustaceans is very limited (Smith and Sedlmeier, 1990). In the present study, a significant increase in de novo protein synthesis was demonstrated in premolt as the ecdysteroid production increases. It started within the two first days after eyestalk ablation and thereafter reflected a general increase in the Y-organ protein content. The qualitative analysis of newly synthesized Y-organ proteins suggested that in premolt the overall protein synthesis is enhanced but that synthesis of specific proteins is also stimulated. These results agree with preliminary studies performed in the crab *Carcinus maenas* (Carpentier et al., 1992) and with recent reports in the insect *Manduca sexta* where PTTH stimulates both ecdysteroid production and general but also specific protein synthesis (Keightley et al., 1990; Kulesza et al., 1994; Rybczynski and Gilbert, 1994).

The general increase in protein synthesis in Y-organs of premolt crayfish may fit with the hypothesis proposed for vertebrates that for long-term stimulation, neuropeptides (e.g. ACTH on adrenals) exert general trophic effects on the steroidogenic tissues (Mazzochi et al., 1986; Arola et al., 1993).

We can only speculate about the identity and function of the specific proteins which are more actively synthesized in premolt or intermolt. In vertebrates, sustained steroid production requires optimal levels of biosynthetic enzymes. The regulation at the transcriptional level of cytochrome P450 enzymes which are proteins with molecular weights of 48–58 kDa has been widely documented (Hall, 1986; Waterman and Simpson, 1989; Hanukoglu, 1992; Parker and Schimmer, 1993). In arthropods, even if the biosynthetic pathway of ecdysteroids has not yet been fully elucidated (Rees, 1985; Grieneisen, 1994), it is generally assumed that the first step (7,8-dehydrogenation) and the final steps (hydroxylations at C2, C22 and C25) of biosynthesis are catalyzed by cytochrome P450 enzymes (Kappler et al., 1988; Grieneisen et al., 1993; Grieneisen, 1994). Thus, some variations of the protein pattern in the 50–60 kDa range observed in the present study may be related to an increased synthesis of cytochrome P450 enzymes in active Y-organs (premolt stage). On the other hand, an inhibition of the expression of some steroidogenic enzymes might represent a long-term effect of MIH.

Several proteins appear to be more actively synthesized in intermolt. In *Orconectes limosus,* MIH was shown to act primarily through an increase in cGMP (Sedlmeier and Fenrich, 1993). Increased activity of cGMP dependent protein kinases (Von Gliscynski and Sedlmeier, 1993) has been observed in Y-organs from intermolt animals and the molting stage dependent phosphorylation of several endogenous Y-organ proteins in intermolt appeared to be dependent of cyclic nucleotides (Böcking and Sedlmeier, 1994). Thus, it can be hypothesized that some regulatory proteins (cyclases, kinases) involved in signal transduction of MIH action are more actively synthesized during intermolt and that these variations might be evidenced here.

Concerning the short-term regulation of ecdysteroid synthesis, our results show that the addition of the translation inhibitor cycloheximide to the incubation medium of crayfish premolt and also intermolt Y-organs induced a rapid (within 2 h) inhibition of ecdysteroid production. This suggests that short-lived proteins are required for the sustained as well as basal ecdysteroid production. A few studies performed in the crab *Cancer antennarius* support the hypothesis of a regulation of cholesterol supply according to the molting stage: an important increase in cholesterol uptake by the Y-organs in vitro was observed in de-eyestalked animals (artificially induced premolt) compared to intact intermolt animals (Watson and Spaziani, 1985a,b; Spaziani et al., 1989). In molting gland cells of arthropods, activities of steroidogenic enzymes have been evidenced in both mitochondrial and microsomal compartments (Kappler et al., 1988; Rudolph et al., 1992; Grieneisen et al., 1993). Consequently, an intracellular transport of biosynthetic intermediates is required for ecdysteroidogenesis and this transport may also be a regulated process. In vertebrates, it has been shown that intracellular cholesterol trafficking plays a key role in the short-term regulation of steroidogenesis and the importance of several sterol carrier proteins of relatively low molecular weight (below 30–35 kDa) has been proposed but not unequivocally established (Strott, 1990; Liscum and Dahl, 1992). Further studies are needed to determine whether some small proteins more actively synthesized in premolt might be related to any of these proteins.

The regulatory neuropeptide MIH is also involved in short-term regulation of ecdysteroid biosynthesis. Treatment with SGE rapidly inhibited ecdysteroid in vitro production and also induced within a few hours a limited but reproducible inhibition of methionine incorporation into proteins. Our results are in agreement with previous studies performed on crabs (Soumoff and O'Connor, 1982; Mattson and Spaziani, 1986; Carpentier et al., 1992). Under the present experimental conditions, a general reduction of protein synthesis was observed after a few hours applications of SGE but it was not possible to demonstrate inhibition of specific proteins. However, changes in the synthesis of minor proteins may have es-

caped the 1D-SDS-PAGE analysis and their detection would require techniques with higher resolution. On the contrary, a clear cut difference in protein pattern could be shown between Y-organs from premolt and those from intermolt animals (see above discussion). This suggests that short-term (applications of SGE during a few hours) and long-term (intermolt situation) effects of MIH are different. A similar scheme has been proposed in vertebrate regulation of steroidogenesis by neuropeptides. Only a few proteins are involved in the acute response to neuropeptides, both proteins involved in intracellular cholesterol trafficking (see above discussion) and transcription factors necessary for the further long-term effects of neuropeptides (the steroid hydroxylase inducing proteins as proposed by Waterman and Simpson, 1989) while the major effect on the level of steroidogenic enzymes may be delayed for more than 8 h (Waterman and Simpson, 1989; Hanukoglu, 1992).

In conclusion, there are many parallels between vertebrate and arthropod control of steroidogenesis even if cellular and molecular mechanisms are much better understood in vertebrates. Protein synthesis appears to be involved in both acute (possible effect on cholesterol or early ecdysteroid precursors supply to rate limiting enzymes) and chronic (possible effect on enzymes levels) regulation of ecdysteroid production in crustaceans. A number of potential regulatory proteins have been evidenced in the present study. Further studies are needed using more specific tools (detection of evolutionary conserved enzymes by immunoblots for example) in order to identify specific regulatory proteins since this cannot be directly deduced from analysis of the protein patterns obtained in electrophoresis.

Acknowledgments

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