PONASTERONE A BINDING SITES IN HYPODERMIS DURING THE MOLTING CYCLE OF CRAYFISH *PROCAMBARUS CLARKII*

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The distribution of ecdysteroid binding sites in the hypodermal tissue of crayfish (*Procambarus clarkii*) was examined autoradiographically in correlation with the molting stage. The radiolabeled hormone analogue ponasterone A (25-deoxy-20-hydroxyecdysone) and thaw-mount autoradiographic techniques were used. These techniques enabled us to prevent the dislocation of soluble ponasterone A. Ecdysteroid binding sites were demonstrated only at certain molting stages, the small gastrolith period (Proecdysis D₀ to D₂ early). Ponasterone A binding sites appeared in nuclei and cytoplasm of epidermis, Leydig cells and pillar like cells through the stages. The notable appearance of nuclear ecdysteroid binding sites at proecdysis stage D₀ coincided with the induction of calcium reabsorption from the exoskeleton, suggesting a strong correlation of the two events. The findings also suggest that the cells involved in calcium transport are targets for ecdysteroids.

The hypodermis of crayfish consists of a monolayer of thin epithelial cells and some layers of Leydig cells. On the outside of the hypodermis, there are firmly attached cuticles that are composed of three strata, the epicuticle, exocuticle and endocuticle. Each of these cuticles is calcified, except the inner part of the endocuticle which is described as membranous layer (20). To maintain their calcium during the molting cycle, fresh water crayfish dissolve the cuticular Ca²⁺ and reabsorb it into the body before ec dysis via the hypodermal cells. The reabsorbed calcium is transported by the hemolymph to a specialized part of the stomach, the gastrolith-disc tissue. Gastrolith-disc tissue cells incorporate Ca²⁺ from the hemolymph and secrete it on the luminal side of the stomach, forming a solid pair of gastroliths which are stored until ec dysis occurs. After ec dysis the gastroliths are redissolved in the stomach, absorbed by the gut, and the calcium is reutilized for the calcification of the new cuticles of the exoskeleton (22). In accordance with the morphological changes of the hypodermis, Drach (8) divided the crustacean molting stages into A to E. Skinner (16) further described the relation of the molting cycle to the histological changes of the hypodermis and detailed the various histological and functional changes in correlation with the molting stages (17).

Hypodermal cells secrete first exuvial-fluid into the space between hypodermis and old cuticle, followed by the secretion of proteins which form the basic structure of the new outerskeleton at proecdysis stage D. At stage D also reabsorption of the calcium from the old exoskeleton through the hypodermis takes place. At the time of ec dysis (stage E), hypodermal cells change their transport direction for calcium and secrete calcium and organic matrices back into the new exoskeleton. Consequently, the hypodermis performs bi-directional calcium transport/absorption from the old exoskeleton, and calcium secretion to the new exoskeleton in a very short time (13, 22, 23).

The functional changes of the hypodermis are supposed to be controlled by molting hormones (ecdysteroids), and ecdysteroid receptors are demonstrated biochemically in the hypodermis of various crustacean species at intermolt stages (18). In other arthropods, the appearance of ecdysteroids is
related to defined endocrinologically controlled events which occur during postembryonic and adult development in connection with the molting cycles (19). The stage-specific expression of ecdysteroid receptors in an organ or cell determines whether or not cells will be targets for the hormone.

To get a better understanding which hypodermal functions are related to the actual presence and action of ecdysteroids, we investigated the appearance of specific high-affinity binding sites (receptors) for ecdysteroids by thaw-mount autoradiography.

MATERIALS AND METHODS

Animals
Adult crayfish (*Procambarus clarkii*) were purchased in September and maintained for 2 or 3 weeks in a shallow aquaria. They were fed by cat food (American Pet Food Co., Ala. USA) and kept under artificial light for 13 hr at room temperature. For the induction of molting, 20-hydroxyecdysone (Rhoto Pharmaceutical Ltd., Osaka, Japan) was injected with an insulin syringe through the arthrodial membrane between the merus and carpus. The hormone was dissolved in a van Harrevelt saline containing 2% (v/v) ethanol at a concentration of 20 μg/ml. A dose of 0.05 μg/g body weight was injected into the animal for 3 consecutive days.

Conventional morphology
The integumentary tissues which are part of carapace and cover gills were used. The tissues were excised and cut in small pieces (2 mm × 2 mm). If a mineralized exoskeleton was formed, the integument was separated from the calcified cuticles. All tissues were fixed with 2.5% glutaraldehyde in phosphate buffer, pH 7.4 for 2 hr at 4°C. After dehydration in graded concentrations of ethanol, the tissue blocks were embedded in LR White (London Resin Co. Hampshire UK). Sections, 2.5 μm thick were cut on a microtome, and stained with 0.1% Toluidine Blue N.

Autoradiography
The same integumentary tissues which were used in conventional morphology, were excised and cut in strips (0.5 mm × 3 mm). They were preincubated in van Harrevelt saline for 1.5 hr, followed by a 1 hr incubation in 4 nM tritium labeled ponasterone A (PNA, sp. act. 178 Ci/mmol; a gift from Dr. P. Maroy, Dept. of Genetic, University of Szeged, Hungary) or 125iodine labeled PNA (sp. act. 1575 Ci/mmol; a gift from Dr. P. Cherbas, Dept. of Biology, University of Indiana at Bloomington, USA). The tissues were rinsed two times for 30 min and mounted in O.C.T. compound (Miles Laboratories, Naperville, IL), frozen in liquified propane. The thaw-mount autoradiographic procedure was done as described by Stumpf (21). Four μm frozen sections were cut at −34°C, thaw-mounted on nuclear emulsion (NTB3, Eastman Kodak, Rochester, NY) coated slides, and exposed in light-proof desiccator boxes at −15°C. After exposure times from 2 weeks (125I) to five months (3H), slides were developed in Kodak D-19 developer diluted 1:1 with tap water, fixed with Kodak-fixer and stained with methyl green-pyronin.

RESULTS

The histological changes of the hypodermis are divided into stages ranging from A to E by Drach (8), further detailed by Skinner (16). Ueno (22) described five molting stages which correlate to characteristic changes in gastrolith disc tissue. In this section we refer to both classifications for molting stages. The alphabetical number in parentheses shows the stages described by Drach (8) and Skinner (16).

Intermediate (A) period
At this stage, the hypodermis consisted of a thin layer of epidermis, and contained some Leydig cells. Especially in the carapace portion, two layers of epithelium faced each other with some Leydig cells in between. Between the epithelium, an open vascular space was formed with some pillar like cells maintaining the vascular space. Both epithelia had a flat shape and little cytoplasm (Fig. 1). The distribution of radioactivity, as indicated by the presence of silver grains, was diffuse and without any concentration in the epithelium, Leydig cells and open vascular system (labeling intensity per unit compared to competition) (Fig. 2).

Small gastrolith period (Proecdysis D0-D2 early)
At this stage, the hypodermis consisted of a thin layer of epidermis, and contained some Leydig cells. Especially in the carapace portion, two layers of epithelium faced each other with some Leydig cells in between. Between the epithelium, an open vascular space was formed with some pillar like cells maintaining the vascular space. Both epithelia had a flat shape and little cytoplasm (Fig. 1). The distribution of radioactivity, as indicated by the presence of silver grains, was diffuse and without any concentration in the epithelium, Leydig cells and open vascular system (labeling intensity per unit compared to competition) (Fig. 2).
Aftermolt (Metecdysis A, B)

FIG. 5. Large gastrolith period (Proecdysis D2 late). The hypodermal cells (ue) show remarkable increase in size. c newly formed cuticles. L: Leidig cell. p: pillar like cell. le: epithelium which face to gill. × 840

FIG. 6. Large gastrolith period (Proecdysis D2 late). No distinctive deposition of radioactivity is notably observed over the new exocuticle and exocuticle. The gastroliths become large in size above the gastrolith epithelium (22). The hypodermal cells showed remarkable fourfold increase in size compared to the intermolt stages (Fig. 5). The presence of silver grains was diffuse over nuclei and cytoplasm of the hypodermal cells and without any notable concentrations over cell compartments (Fig. 6).

Aftermolt (Metecdysis A, B)

FIG. 2. Intermolt stage (Anecdysis C4). Silver grains diffuse over the epithelium and open vascular system (by). n nucleus. b blood cell. Exposure time 61 days. × 735

FIG. 3. Small gastrolith period (Proecdysis C4). Hypodermal cells (ue) which face to carapace increase in size and become rich for the cytoplasm. Blood vessel (bv) is stained 0.1% Toluidine Blue N. Bar = 10 μm.

Figs. 1, 3, 5, 7. Conventional morphology of the carapace portion. There are two layers of epithelium and some Leydig cells in the middle. Between the epithelium, an open vascular space is formed with some pillar like cells. The sections are 2.5 μm thick, and stained 0.1% Toluidine Blue N. Bar=10 μm.

Figs. 2, 4, 6, 8, 9. Autoradiograms of hypodermal tissue. The tissues were preincubated 1.5 hr in van Harrevelt saline, followed by 1 hr incubation in 3H labeled PNA (Fig. 8) or 125I labeled PNA (Figs. 2, 4, 6, 9). Bar = 10 μm.

FIG. 1. Intermolt stage (Anecdysis C4). Both epithelia (ue, le) have a flat shape, and poor cytoplasm. A wide blood vessel (bv) and pillar-like cells (p) are notable between the epithelium. ue: epithelium which face to carapace. le: epithelium which face to gill. c: endocuticle. b: blood cell. × 840

FIG. 2. Intermolt stage (Anecdysis C4). Silver grains diffuse over the epithelium and open vascular system (bv). n nucleus. b blood cell. Exposure time 61 days. × 735

FIG. 3. Small gastrolith period (Proecdysis D4). Hypodermal cells (ue) which face to carapace increase in size and become rich for the cytoplasm. Blood vessel (bv) is stained stronger than intermolt stage. b: blood cell. c: endocuticle. × 1,050

FIG. 4. Small gastrolith period (Proecdysis D4). The radio-activity is observed notably in the nucleus (n) and cytoplasm of both epidermis, Leydig cells (L) and pillar like cells. Exposure time 15 days. × 1,104

FIG. 5. Large gastrolith period (Proecdysis D2 late). The hypodermal cells (ue) show remarkable increase in size. c newly formed cuticles. L: Leidig cell. p: pillar like cell. le: epithelium which face to gill. × 350

FIG. 6. Large gastrolith period (Proecdysys D2 late). No distinctive deposition of radioactivity is notably observed over the nucleus (n). Exposure time 15 days. × 424

DISCUSSION

1. 20-Hydroxyecdysone analogue ponasterone A

Ponasterone A (PNA) is an agonist of 20-hydroxyecdysone (20E) (5, 12). In insects, PNA shows an increased affinity for the ecdysteroid receptors KD = 0.2-3.0 nM compared to 20E KD = 30 nM (2). Similar differences in the affinity of the ecdysteroid receptors for 20E and PNA are known of several crustaceans (18). Different from insects, crustaceans synthesize small amounts of PNA as well as 20E. Nothing is known about the function of the endogenous ponasterone A. Although we cannot deny the possibility of different functions for PNA and 20E in crayfish. Injection of PNA at a concentration ten times less than 20E initiated in 70% of the animals (Procambarus clarkii) the premolt stage within 6 days after treatment (Ueno, unpublished data). These results confirm a 20E agonistic action of ponasterone A in vivo in Procambarus clarkii as it is known and reviewed for various insects (2).

2. The correlation between the changes of ecdysteroid titer and ecdysteroid binding sites

The ecdysteroid titers in hemolymph in crayfish were examined by Keller and Schmid (10) and Jegla et al. (9), in Orconectes limosus. Two peaks in the ecdysteroid titer were observed; the first peak starts at the beginning of proecdysis (D0), and the second peak appears at the proecdysis stage D2 (9). The injection of 20E stimulates the endogenous ecdysteroid synthesis of crayfish. The present histological data show that high affinity binding sites for ecdysteroids appears at the small gastrolith period (Proecdysis D0 to D2).
early), which coincides with the first peak in the ecdysteroid titer in the hemolymph (9). So far it is not certain if the increase of binding sites occurs strictly synchronous or with some delay to the increase in ecdysteroid titer. In Drosophila, ecdysteroid receptor synthesis is induced by 20E and the highest amount of receptors are found shortly after the peak in the ecdysteroid titer (6). Similar receptor dynamics may occur in crustaceans as well. A more direct response could also take place. Compared to insects, crustaceans have no hemolymph-binding proteins for ecdysteroids (14), which may lead to a faster rise in the active free ecdysteroid concentration resulting in a more rapid induction of receptors.

3. The role of ecdysteroid binding sites

Experimental data in insects, summarized by Riddiford (15), Yund and Osterbur (24) and Bidmon and Sliter (2) show that the function of molting hormone is the coordination and orchestration of embryonic- and postembryonic development, as well as metamorphosis and reproduction. Ecdysteroid receptors are differentially expressed in Calliphora, Sarcophaga and Manduca showing that only a few stage-specific target cells exist in insects at certain times during development (1, 3, 4). These stage-specific ecdysteroid targets are noticeably involved in the induction of stage-specific events, as changes in behavior or ecdysis (7). Crustaceans differ from insects by the presence of a calcified cuticle. Since calcium is rare for fresh water crayfish they have developed ways to save and reutilize their cuticular calcium by dissolving and reabsorbing it prior to ecdysis. The appearance of nuclear ecdysteroid binding sites at proecdysis stage Do coincides with the induction of calcium reabsorption from the exoskeleton. This indicates ecdysteroid dependency of the hypodermal cells during calcium mobilization. The radioligand in pillar-like cells and another epithelium which fences to gills, may be involved in the induction of protein synthesis for the new exoskeleton. The diffuse distribution of binding sites in cytoplasm and the absence of nuclear binding in aftermolt (Metecdysis A,B) indicates that nuclear ec-

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**FIG. 7.** Aftermolt (Metecdysis A). The size of epidermal cells (ue) decrease. Blood vessel (bv) is stained stronger than the large gastrolith period. c: endocuticle. ×840

**FIG. 8.** Aftermolt (Metecdysis A). The silver grains show diffuse distribution without any differences in concentration among special types of cells or cell compartments of the hypodermis. n: nucleus. Exposure time 111 days. ×960

**FIG. 9.** Small gastrolith period (Proecdysis D0, competition experiment). There are no notable activities in the epithelium and adjacent tissues. n: nucleus. Exposure time 15 days. ×864
ecdysteroid binding is not essential for the calcium secreting system at that period. These observations indicate further that ecdysteroids are more related to the induction of processes such as calcium mobilization, protein synthesis or sclerotization, than to their maintenance.

The obtained data reveals a strong relation between molting changes and the distribution of ecdysteroid hormone binding sites. The correlation between calcium absorption by hypodermis and the existence of increased numbers of predominantly nuclear ecdysteroid binding sites clearly show that cells involved in calcium transport are targets for ecdysteroids. Nuclear ecdysteroid binding is indicative for a genomic action of ecdysteroids, which may be related to the induction of protein synthesis such as calcium transport proteins.

REFERENCES


