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Developmental regulation of juvenile hormone synthesis: ovarian synchronization of volumetric changes of corpus allatum cells in cockroaches

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Summary

The corpus allatum (CA) cells of adult *Blattella germanica* females undergo cyclic volumetric changes in relation to juvenile hormone (JH) synthesis. In intact females the size of CA cells changes synchronously during the gonotrophic cycle, resulting in cyclic JH synthesis. In ovariectomized females volumetric changes among CA cells become asynchronous, resulting in highly variable but high rates of JH synthesis. Injection of the steroid hormone 20-hydroxyecdysone into ovariectomized females with active CA resulted in a transient decline followed by an increase in both CA volume and JH biosynthesis. This response was due to a change in the size distribution of CA cells and not in the total number of CA cells. In ovariectomized females, CA cells can be re-synchronized into a uniform population of small inactive cells with injection of 20-hydroxyecdysone and implantation of an artificial egg-case, mimicking the successive events of ovulation, oviposition and pregnancy.

Introduction

Juvenile hormone (JH), synthesized by the corpora allata (CA), is essential for the expression of sexual behavior, synthesis of yolk proteins, and for the successful development of the ovaries and various ovarian tissues in many insects (Koeppe et al., 1985; Schal and Smith, 1990). The regulation of CA activity in insects during the gonotrophic cycle involves modulation by ovarian signals and by brain allatotropins and/or allatostatins which are released in response to environmental (external) and physiological (internal) stimuli. Also, rates of JH synthesis by the CA have been shown to be stimulated by the presence of young ovaries and inhibited by the presence of mature ovaries or egg case in many insects (reviewed by Engelmann, 1970; Cassier, 1979; de Kort and Granger, 1981; Feyereisen, 1985; Tobe and Stay, 1985; Khan, 1988; Raabe, 1989; Rankin, 1990). However, it is not clear how the CA respond to these regulatory factors that result in the cyclic release of JH during the gonotrophic cycle.

It has been suggested that ecdysteroids, which are found in mature ovaries and in the hemolymph of adult females, are involved in the negative feedback (Lanzrein et al., 1981; Stay et al., 1984; Weaver et al., 1984; Rankin et al., 1985). Removal of the ovaries abolishes the hemolymph

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ecdysteroid peak that is associated with presence of mature oocytes in *Periplaneta americana* (Weaver et al., 1984) but not in *Diploptera punctata* (Stay et al., 1984). Injections of the steroid hormone 20-hydroxyecdysone into adult cockroaches result in declines in CA activity (Engelmann, 1959; Friedel et al., 1980; Stay et al., 1980) and in CA cell number in *D. punctata* (Szibbo and Tobe, 1981).

The CA in the adult female German cockroach, *Blattella germanica*, exhibit a discrete cycle of structural plasticity in relation to JH synthesis during the gonotrophic cycle (Belles et al., 1987; Chiang et al., 1989; Gadot et al., 1989b). During oocyte maturation, active CA are significantly larger than inactive glands and they contain larger cells with well developed cellular organelles (Chiang et al., 1989; Piulachs et al., 1989). In contrast to other cockroach species, in which ovariectomy suppresses the activity and cyclicity of the CA (Stay and Tobe, 1978; Lanzrein et al., 1981; Weaver, 1981), the CA in ovariectomized *B. germanica* exhibit delayed but high rates of JH synthesis and a clear cycle of activity (Gadot et al., 1990). CA cell numbers in both intact and ovariectomized females remain relatively constant despite cyclic changes in CA volume in the former and a gradual and continuous enlargement of the CA in the latter (Chiang et al., 1990b).

We now report on the roles of the ovaries, 20-hydroxyecdysone and the egg case in inducing cellular changes in the CA in relation to JH biosynthesis. We demonstrate that JH biosynthesis is regulated mainly through changes in CA cell size rather than in cell number or biochemical rate-limitation in the JH synthetic pathway. Our results indicate that the absolute rate of JH synthesis by each CA mainly depends upon the availability of cellular machinary while the cyclicity depends upon the degree of synchronization among CA cells.

Materials and methods

Insects

German cockroaches, *B. germanica*, were reared at 27 ± 1°C under a 12 h light/12 h dark photoperiodic regimen and supplied with pelleted dog food and water ad libitum. Newly-ecdysed adult females (day 0) were collected daily and maintained in groups (Gadot et al., 1989a). Intact females were allowed to mate on day 6 and they oviposited on day 9. Mated females carry the egg case externally for approximately 20 days before the onset of the second ovarian cycle. Insects were always immobilized by chilling on ice before performing various treatments.

Ovariectomy and synchronization of insects

Ovariectomy was performed in early last instar nymphs by pressing the ovaries out of the abdomen through longitudinal lateral slits in the 7th tergite. Ovariectomized females were grouped with males for at least 8 h daily from day 3 until mating was observed; thereafter, each mated female was kept with two males. Only females that mated before day 8 were used. Before its CA were used in biochemical and developmental assays, each ovariectomized female was dissected and checked to ensure the absence of ovaries.

Injection of 20-hydroxyecdysone

Injections were made through the base of the coxae of the right metathoracic leg. 20-Hydroxyecdysone (Sigma, St. Louis, MO, U.S.A.) was injected in 2 μl Ringer solution containing 10% ethanol for the dose of 100 μg and 1% ethanol for other doses. Sham controls were injected 10% or 1% ethanol Ringer solution only.

Egg case implantation

Before implantation egg cases were infiltrated with molten bees-wax overnight to prevent dehydration. The waxed egg case was implanted into the oothecal chamber and attached with a small amount of bees-wax. Sham controls were operated the same way except without attaching the egg case.

Radiochemical assay

Pairs of CA were removed from adult females at various times after adult emergence or after experimental treatments. The rate of JH synthesis from each pair of CA was determined according to the methods of Pratt and Tobe (1974) as modified by Gadot et al. (1989b) by measuring the incorporation of radioactivity from L-[*methyl*-^{3}H]methionine into JH released in the culture

medium. Farnesoic acid (about 70% pure) was a generous gift from Dr. F. Baker (Zoecon Corp., Palo Alto, CA, U.S.A.). Oocyte length of experimental insects was measured whenever possible.

Morphometric measurements

CA volume was determined by the formula $v = 4/3 \cdot \pi \cdot abc$, where a, b and c are the radii of the three principal axes measured with an ocular filar micrometer under a dissecting microscope. The total number of CA cells was counted from a monolayer of CA cells. Individual CA were partially digested with 0.1% collagenase for 15 min at 28°C, stained with 0.01% crystal violet in 0.1 M citric acid for 5 min, and then spread into a cell monolayer under a coverslip on a gelatin subbed slide (Chiang et al., 1989, 1990a).

The maximum diameter of each CA cell was measured under a compound microscope at ×400 and served as an indicator of cell size since dissociated CA cells of *B. germanica* are largely globular (Chiang et al., 1990a). To prepare cell suspensions, individual CA were digested with 0.1% trypsin in isotonic cockroach saline and then dispersed by gentle vortexing (Chiang et al., 1989). CA cells were randomly sampled in a hemocytometric grid. Variations are represented by SEM.

Results

Synchronous volumetric changes of CA cells in intact females

In intact females CA cells exhibit synchronous changes in size in relation to the cyclic changes in JH synthesis during the first two ovarian cycles (Fig. 1). CA cells are mostly small in the inactive glands of newly-ecdysed adult females. As the CA become active in the first (days 0–7) and second (days 29–34) gonotrophic cycles, CA cells enlarge synchronously and JH synthesis increases. Both parameters peak in insects with terminal oocytes 1.6–2.0 mm in length (days 6–7 and 33–34) and decline dramatically just before and during ovulation (days 8–9 and 37–38). In gravid females, CA cells remain small and JH synthesis is undetectable until the egg-case hatches. More than 95% of the cells in highly active CA (days 7 and 34) had maximun diameters greater than 9 μm, while the diameters of only 25–55% of the cells in relatively

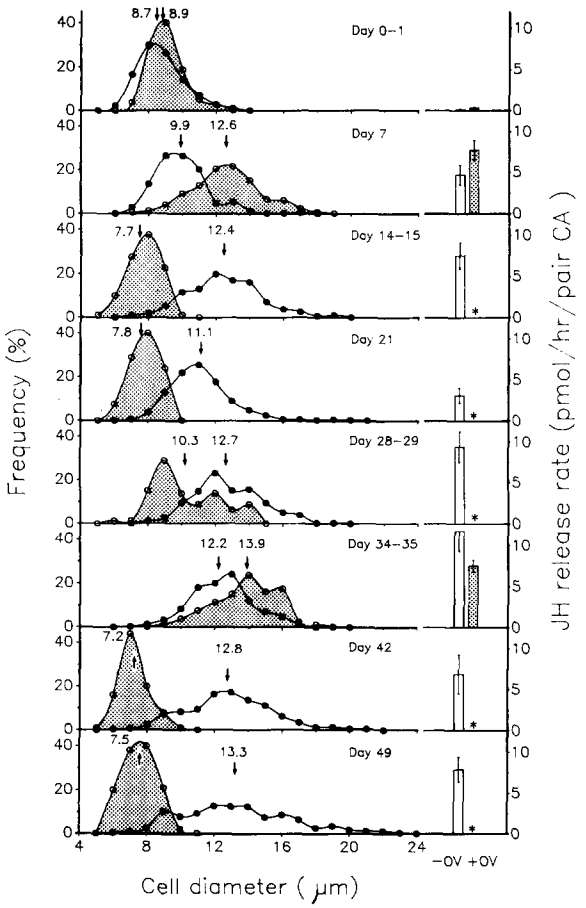

Fig. 1. Frequency distributions of maximum cell diameters and JH synthesis rates (±SEM) of CA from intact (stippled, +OV) and from ovariectomized (not-stippled, −OV) females. Only mated females were used as described previously (Gadot et al., 1990). Each frequency distribution consists of 300–400 cells from eight different CA taken from four insects. Arrows and adjacent numbers indicate the mean (μm) of the distribution. SEM is less than 1.5% of the respective mean for all distributions. An asterisk (*) indicates undetectable rates of JH synthesis in gravid females.

low-activity CA (days 0 and 29) exceeded 9 μm. Fewer than 1% of the cells in inactive CA of gravid females (days 15 and 21) were larger than 9 μm in diameter (Fig. 1).

CA activation in ovariectomized females

We selected only females that mated by day 8 (53%, $n = 172$) in order to obtain a relatively synchronous group of females. The CA of ovariectomized females that refused to mate by

day 28 exhibited low rates of JH biosynthesis (0.9 ± 0.2 pmol/h/pair CA, $n = 6$) and relatively small mean cell size (10.3 ± 0.1 μm). Both parameters were significantly lower than the corresponding values in 28-day-old mated females (Fig. 1) (t-test, $p < 0.05$).

Juvenile hormone synthesis and the size of CA cells attained similar peak values respectively in ovariectomized and intact females (t-test, $p > 0.05$ for both parameters) but were significantly delayed in ovariectomized females (7 days later). Also, the frequency distributions of CA cell sizes during the first peak of JH synthesis are similar in intact and in ovariectomized females (Kolmogorov-Smirnov test, $p > 0.05$). These results indicate that in *B. germanica*, the ovary is not required either for the synchronous enlargement of CA cells or for the activation of the CA to normal high rates of JH synthesis; however, the presence of the young ovary accelerates both processes.

Asynchrony of CA cells in ovariectomized females

Both JH synthesis and the mean size of CA cells declined moderately by day 21 and then increased again and remained high for at least 3 more weeks in the absence of the ovaries and the egg case (Fig. 1). During this period, volumetric changes among CA cells became asynchronous as indicated by the gradual increase in the range of cell sizes and the flattening of their frequency distribution: on day 49 CA cells ranged from very small (5 μm diameter) to hypertrophic (24 μm diameter). These results suggest that inhibitory factors, possibly from mature ovaries and/or the egg-case, may be responsible for the synchronous cyclic changes in the size of CA cells in intact females.

Dose–response of 20-hydroxyecdysone inhibition

We used 28- to 42-day-old mated ovariectomized *B. germanica* to study the effects of ecdysteroids because their CA exhibited a plateau of high JH synthesis and correspondingly large mean cell size (Fig. 1). Three days after injection of 20-hydroxyecdysone into 28- to 30-day-old ovariectomized females dose-dependent inhibitions of both the spontaneous and the farnesoic acid (FA)-stimulated rates of JH synthesis were evident, as well as a decline in CA volume (Fig. 2).

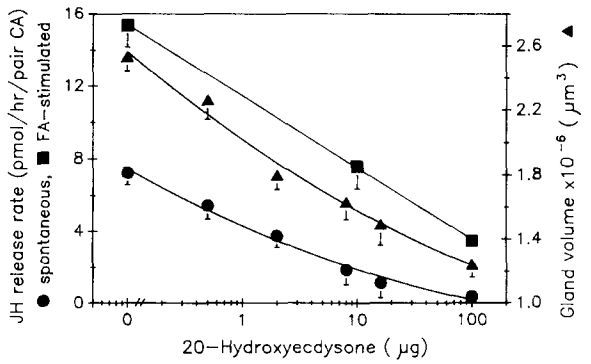

Fig. 2. Spontaneous and farnesoic acid-stimulated rates of JH synthesis and CA volume (±SEM) 3 days after injection of 20-hydroxyecdysone into 28- to 31-day-old ovariectomized females. 20-Hydroxyecdysone was injected in 2 μl Ringer solution containing 10% ethanol for the dose of 100 μg and 1% ethanol for other doses. Sham controls were injected 10% or 1% ethanol Ringer solution. Each mean was determined from at least eight CA from four insects for gland volume or five pairs of CA for rate of JH synthesis.

In the presence of exogenous FA, which bypasses the earlier rate-limiting steps in JH synthesis (Tobe and Pratt, 1976; Feyereisen, 1985), JH biosynthesis did not recover from the inhibition by injected 20-hydroxyecdysone suggesting that 20-hydroxyecdysone exerts its effects on the CA mainly through mechanisms other than biochemical rate-limitation prior to the last two steps in JH biosynthesis.

The decline in JH synthesis induced by 20-hydroxyecdysone was not accompanied by a decrease in the number of CA cells in *B. germanica*. The number of cells per CA, determined by total counts from CA cell monolayers 5 days after injection of 10 μg 20-hydroxyecdysone, was not significantly different from that in non-injected 28-day-old ovariectomized females (1981 ± 68, $n = 7$ and 1916 ± 79, $n = 7$, respectively; t-test, $p > 0.05$).

Time course of 20-hydroxyecdysone inhibition

To elucidate the long-term effects of 20-hydroxyecdysone and its possible role in maintaining pregnancy, we examined changes in CA volume, rates of JH synthesis and CA cell size 3, 5 and 12 days after injection of 10 μg 20-hydroxyecdysone into 28- to 31-day-old ovariectomized females (Fig. 3). Following similar response patterns, the values

of all three parameters declined 3 and 5 days after injection, but 12 days later they increased to the levels obtained from sham-injected insects.

Re-synchronization of CA cells in ovariectomized females

To mimic the successive events that occur during ovulation and pregnancy in intact females, we injected 10 μg 20-hydroxyecdysone and implanted a wax-infiltrated egg case into 17-day-old ovariectomized females, in which the CA undergo a moderate but incomplete decline in activity (Fig. 1). Four days later, the mean size of CA cells decreased significantly and their size distribution was uniformly synchronized resulting in barely detectable rates of JH synthesis (Fig. 4) that continued for at least one more week (data not shown)

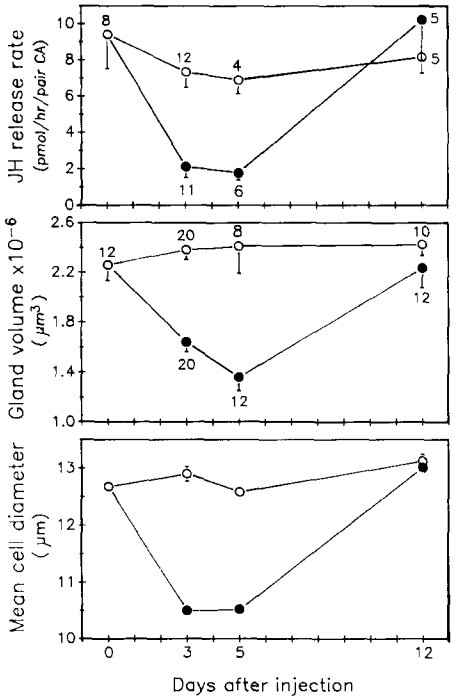

Fig. 3. The effects of 10 μg 20-hydroxyecdysone injection on JH synthesis, CA volume and the mean size of CA cells (±SEM). All three parameters are significantly lower 3 and 5 days after injection into 28- to 31-day-old ovariectomized mated females (solid circles) than in sham-injected females (open circles) (*t*-test, $p < 0.05$). Twelve days after the injection, the values of the three parameters are similar in treated and sham-injected females. The number of measurements is adjacent to the respective mean. Mean cell diameter is derived as in Fig. 1.

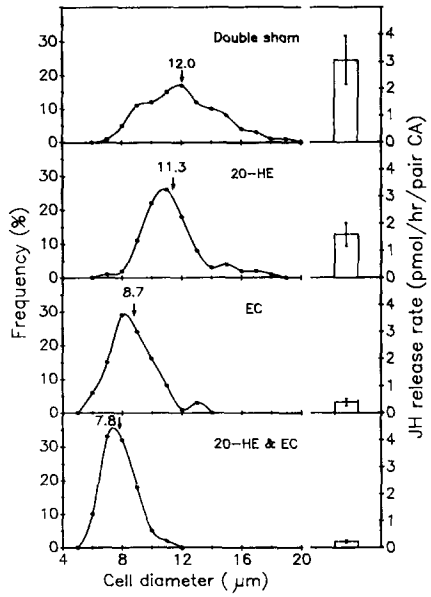

Fig. 4. Effects of 20-hydroxyecdysone (20-HE) injection and egg case (EC) implants on the distribution of CA cell diameters and JH synthesis (±SEM). Ovariectomized mated females were injected with 10 μg 20-HE in 1% ethanol Ringer solution and/or implanted with egg cases on day 17 and examined on day 21. Egg cases infiltrated with bees-wax were implanted into the genital vestibulum. Controls were sham-injected with 1% ethanol Ringer solution and sham-implanted without attaching the egg case. Figures were generated as described in Fig. 1.

as in gravid intact females. Females that were either injected with 20-hydroxyecdysone or implanted with an egg case exhibited intermediate values of both CA cell size and JH synthesis. However, the mechanical signals from egg case implantation resulted in a more potent inhibition of CA activity after 4 days than did 20-hydroxyecdysone injection.

Discussion

It has been hypothesized that JH synthesis may be regulated rapidly through biochemical rate-limiting steps or slowly through developmental changes in CA cellular components (Feyereisen, 1985; Tobe and Stay, 1985). In *B. germanica*, both the spontaneous and FA-stimulated rates of JH synthesis exhibit similar patterns in normal (Gadot et al., 1989b) and in treated (ovariectomized or 20-hydroxyecdysone injected) females, suggesting

that the regulation of cyclic JH synthesis is mainly through mechanisms other than biochemical rate limitation prior to the last two steps in JH synthesis.

Developmental responses of the CA to ovarian factors were studied by monitoring changes in the size of CA cells. Corpus allatum cell size appears to reflect cell mass rather than the plasticity of the cell membrane. This is supported by ultrastructural evidence showing that the quantity of cellular components of CA cells exhibit cyclic changes that correspond to the ovarian cycle in several cockroaches, including *B. germanica* (Scharrer and von Harnack, 1958; Johnson et al., 1985; Piulachs et al., 1989). Also, CA cells in suspension have been shown to synthesize JH at normal rates suggesting that the dissociation procedures did not interfere with their normal functions (Chiang et al., 1989).

Our present results suggest that in *B. germanica* the absolute rate of JH synthesis by each CA mainly depends upon the availability of cellular machinary, a condition reflected in the total cell volume (i.e., mean cell size × number of cells per CA), while the cyclicity depends upon the degree of synchronization among CA cells. In intact females the cycle of JH synthesis is imposed by synchronous changes in the size distribution of CA cells and by factors that sustain this synchrony during the protracted pregnancy. In old ovariectomized females the cycle of JH synthesis is not apparent due to loss of synchrony among CA cells, as reflected by a wide range of cell sizes. It is important to note that while the total cellular machinary and JH biosynthesis remain relatively constant in old ovariectomized females, each individual CA cell may still exhibit cyclic changes in size and activity. However, a cycle of gland activity is not apparent because the cells are relatively asynchronous.

The CA of intact and ovariectomized adult *D. punctata* females have been reported to undergo significant cell proliferation (Szibbo and Tobe, 1981; Tobe et al., 1984), yet in ovariectomized females the CA remain relatively inactive (Stay and Tobe, 1978). Injection of 20-hydroxyecdysone into ovariectomized *D. punctata* has been shown to either reduce or prevent the increase in the number of CA cells (Tobe et al., 1984). However, these results with *D. punctata* were based on counts of nuclei in representative sections of fixed CA which produce inconsistent results even within the same species (Engelmann, 1957; Scharrer and von Harnack, 1958). Using two independent methods to determine total CA cell number (sampling from cell suspensions and parametric counts from cell monolayers) we recently showed that during CA activation cell number remains relatively constant in intact adult females of *B. germanica* and *D. punctata* (Chiang et al., 1989) and in ovariectomized *B. germanica* (Chiang et al., 1990b). The present results show that 20-hydroxyecdysone exerts a relatively long-term inhibition of the CA in vivo (for at least 5 days) by inducing declines in CA volume, the size of CA cells and in JH synthesis without significant changes in total cell number. Furthermore, these effects are reversible, suggesting that alone, an ecdysteroid peak from the mature ovaries is insufficient to sustain pregnancy (small CA cells, low CA activity) in intact females.

We hypothesize that in intact *B. germanica* females, factors from the young ovary cause a synchronous increase in the size of CA cells, resulting in a rapid activation of the CA. Factors from mature ovaries, probably including ecdysteroids, cause the initial decline in both the size of CA cells and the rate of JH synthesis. In gravid females, mechanical stimuli from the egg case prevent the growth and activation of small inactive CA cells until the end of pregnancy. Thus, the ovary and the egg case regulate the cyclic pattern of JH synthesis by functioning jointly as a pacemaker which synchronizes the growth and regression of CA cells.

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