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Morphometric analysis of corpus allatum cells in adult females of three cockroach species

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Summary

The number of cells and their sizes in the corpus allatum (CA) of adult female *Blattella germanica*, *Supella longipalpa* and *Diploptera punctata* were determined during oocyte maturation. Cell number and size were directly measured in cell suspensions following enzymatic dissociation of freshly excised CA. Cell numbers were verified by total cell counts in whole-mount CA monolayers and by hemocytometric sampling. In all three species, cell number did not change during the period of CA activation, averaging ca. 2000 cells per gland in *B. germanica*, 3500 cells per gland in *S. longipalpa* and 11 000 cells per gland in *D. punctata*. Cell diameter increased significantly in all three species during this period from a mean value of 8.9 μm to 11.7 μm in *B. germanica*, from 9.2 μm to 14.6 μm in *S. longipalpa* and from 10.0 μm to 15.6 μm in *D. punctata*. During a 4 h incubation period, dissociated CA cells incorporated L-[*methyl*-^{3}H]-methionine into juvenile hormone-III at rates comparable to intact glands. These data suggest that CA activation in the first ovarian cycle of these species is associated mainly with an increase in cell size with minor changes in cell number.

Introduction

Cyclic changes in corpus allatum (CA) volume in the adult female are related to the ovarian cycle in many insect species (Engelmann, 1970) and in several species, the rates of juvenile hormone-III (JH-III) biosynthesis in vitro have been related to cyclic changes in CA volume and oocyte maturation (Feyereisen, 1985). The increase in CA volume during oocyte maturation in the first gonotrophic cycle has been attributed mainly to increases in cell number and in cell volume (Cassier, 1979; Tobe and Stay, 1985). However, both parameters were estimated by indirect methods with inherent and at times untested assumptions. For example, the number of CA cells was estimated from the number of nuclei found in representative sections of fixed organs (Scharrer and von Harnack, 1958; Szibbo and Tobe, 1981; Tobe et al., 1984). Counts of nuclei in sections of fixed thickness do not consider variations in nuclear size and shape although such changes are related to different developmental stages of the CA of adult female *Diploptera* punctata (Johnson et al., 1985) and nuclear swelling may accompany activation of the CA in other insects (Cassier, 1979). Therefore, this method may underestimate or overestimate cell number in different developmental stages.

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The size of insect CA cells has not been measured directly. It is usually estimated in fixed glands by the ratio of nuclei to total gland volume, first suggested by Engelmann (1957) as the 'nucleocytoplasmic ratio'. In *Leucophaea maderae*, this ratio was shown to be the best indicator of CA activity, which in turn was estimated by oocyte development (Engelmann, 1957). In *D. punctata* the rates of JH-III synthesis in vitro are similarly related to the nucleocytoplasmic ratio during the gonotrophic cycle (Szibbo and Tobe, 1981), although this relation does not hold under certain experimental conditions (Feyereisen, 1985).

In this paper, we describe methods for the direct measurement of cell number and size in fresh dissociated CA. In contrast to previous reports of significant increases in CA cell number during oocyte maturation in *D. punctata* (Szibbo and Tobe, 1981; Tobe et al., 1984), we demonstrate that cell number does not increase during this period in three cockroach species including *D. punctata*. We also show that cell size increases significantly during activation of the CA in all three cockroach species. Finally, we demonstrate de novo synthesis of JH-III in a suspension of CA cells, which has important implications to future preparation of primary cell cultures.

Materials and methods

Insects

Blattella germanica, *Supella longipalpa* and *D. punctata* were reared at 27°C under a 12 h light: 12 h dark photoperiodic regime and supplied with pelleted dog food and water ad libitum. Low-activity CA for all three species were from newly emerged (day 0) adult females. High activity CA were from 6-day grouped virgin *B. germanica* with basal oocytes 1.6–1.9 mm long (Gadot et al., 1989), 7- to 8-day mated *S. longipalpa* with basal oocytes 1.6–1.9 mm (Smith et al., 1989), and 5-day *D. punctata* (mated on day 0) with basal oocytes 1.1–1.5 mm (Tobe and Stay, 1977).

Total cell count in whole-mount CA monolayers

The CA were dissected in cold cockroach saline solution A (BG-SSA; Kurtti and Brooks, 1976), separated, and thoroughly cleaned from any adjacent tissue. The isolated CA was subjected to partial digestion by 0.1% collagenase (Type IA, Sigma) in 250 μl BG-SSA at 28°C with gentle shaking for 20 min. The desheathed gland, which kept its integrity, was transferred with a stainless steel loop to a drop of 0.01% crystal violet in 0.1 M citric acid on a gelatin subbed slide and left in the drop for 3–5 min. The gland was then spread into a cell monolayer under a coverslip which was sealed with vaseline. The total number of cells was counted in individual CA from *B. germanica* and *S. longipalpa* under a light microscope with an ocular square grid.

Cell dissociation

Corpora allata were dissected, separated and cleaned as described above, and partially digested by 0.1% trypsin (from porcine pancreas, 1000–1500 BAEE units per mg, also containing chymotrypsin and elastase activities; Sigma) in 250 μl BG-SSA at 28°C with gentle shaking. The relatively small glands of *B. germanica* and *S. longipalpa* were incubated for 40 min, while the larger CA from *D. punctata* was first incubated on ice for 20 min to ensure the diffusion of trypsin throughout the gland, followed by a 40 min incubation at 28°C. Trypsinization was stopped by adding 250 μl cockroach saline solution B (BG-SSB) containing 20% fetal bovine serum (Kurtti and Brooks, 1976). The trypsinized CA was transferred in a small amount of solution to an Eppendorf tube and dissociated by gentle vortexing for 2 min. The number of cells in the suspension was determined by hemocytometry. Cells were randomly chosen by the hemocytometer grid and their maximum diameters were measured under a compound microscope with an ocular filar micrometer.

In vitro synthesis of JH-III by dissociated CA cells

Following trypsinization, individual CA were transferred with a stainless steel loop to 100 μl modified TC 199 (Pratt and Tobe, 1974; with modifications as detailed in Gadot et al., 1989) with 10% fetal bovine serum and 1 μCi L-[*methyl*-^{3}H]methionine (NEN, specific activity 200 mCi/mmol) in an Eppendorf tube and vortexed as described above. The cell suspension was then incubated for 4 h at 28°C and JH-III synthesis was assayed from an isooctane extract. The sus-

pension was examined under a microscope before extraction to estimate the degree of dissociation.

High performance liquid chromatographic (HPLC) analysis of radiolabeled products was performed on a Varian 5500 Vista by reconstitution of an aliquot from the isooctane phase of the incubation extract in acetonitrile which was injected into a 10 μm RP-18 column (Lichrosorb, Merck). Elution was performed with a linear gradient of acetonitrile in water (60–70%) at a flow rate of 1 ml/min for 15 min. Fractions were collected every 12 s and radioassayed in 20 ml of Scintiverse II (Fisher). As a marker, we used JH-III (Sigma, 80% pure) monitored by absorbance at 230 nm.

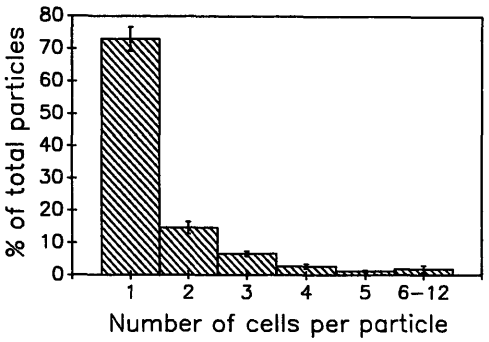

Fig. 2. Degree of dissociation of the CA of *B. germanica* females with basal oocyte length of 1.7–1.9 mm. Any undissociated clump of cells was considered as one particle. Bar above each column is the SEM of six glands.

Results

Dissociated CA cells are largely globular in suspension. Therefore, the maximum diameter of each cell was used as an indicator of cell size. In all three species, the frequency distributions of cell sizes in the CA approximated normal distributions (Fig. 1). The average cell diameter of low-activity CA (mean ± SEM = 8.9 ± 0.1 μm, 9.2 ± 0.1 μm and 10.0 ± 0.1 μm for *B. germanica*, *S. longipalpa* and *D. punctata*, respectively) was significantly smaller than that of high-activity glands (11.7 ± 0.2 μm, 14.6 ± 0.1 μm and 15.6 ± 0.2 μm, respectively in each of the three species (*t*-test, $p < 0.05$). Cells from low-activity CA were similar in size in all three species while cells from high-activity CA were largest in *D. punctata* and smallest in *B. germanica*.

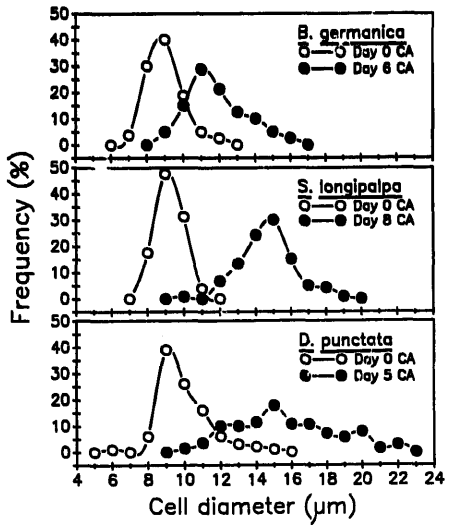

Fig. 1. Distributions of cell diameters of low-activity (○) and high-activity (●) CA in three cockroach species. Each distribution was generated from a sample of ten cells per gland from eight different glands taken from four insects. Sampled cells were grouped into size categories of 1 μm each.

The degree of dissociation of the CA was evaluated by counting the number of cells associated with each particle (or clump) in both chambers of the hemocytometer. Following the cell dissociation procedure, the majority of the particles consisted of single cells, and ca. 94% of the particles contained 1–3 cells (Fig. 2).

The reliability of the sampling method, by which the frequency distributions of cell diameters were generated, was confirmed in larger samples, which were drawn from the same cell suspensions (Fig. 3). The small sample (80 cells per eight CA) and the larger sample (400 cells per eight CA) do not differ significantly in their means (*t*-test, $p > 0.05$) or their distributions (Kolmogorov-Smirnov, $p > 0.05$).

The validity of the dissociation procedure with regard to possible changes in cell size was confirmed by monitoring the JH-III biosynthetic activity of dissociated cells during a 4 h incubation. Dissociated CA cells of 6-day virgin female *B. germanica* synthesized JH-III at 2.16 ± 0.19 pmol per gland per h (average of five samples with 2–9 glands each). This corresponds to 78% of the activity of similarly treated but untrypsinized intact glands (2.77 ± 0.22 pmol per gland per h,

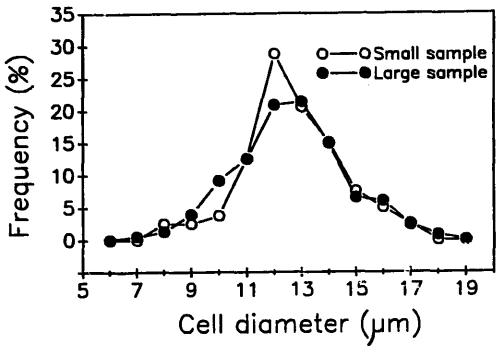

Fig. 3. Distributions of cell diameters derived from small samples of 80 cells (o) and large samples of 400 cells (●) from the same population of cells from eight CA of *B. germanica* with basal oocyte length of 2.0–2.1 mm.

average of three samples with 1–6 glands each). HPLC analysis confirmed that JH-III was the only JH produced by these cells. Approximately 80–90% of the radioactivity in the eluted fractions corresponded to the JH-III peak in incubations of both intact and dissociated CA. These results indicate that the partial digestion by proteolytic enzymes and mechanical dispersion of the cells did not impair their JH-III biosynthetic activity.

Whole-mount monolayers of CA provided counts of the absolute number of cells in individual desheathed glands. However, this method was less practical for glands with more than 4000 cells, and the more convenient method of sampling cell suspensions by hemocytometry was used in this case. Applying both methods to *B. germanica* CA indicated no significant differences between either low- and high-activity CA or between the two methods ($p > 0.05$; Duncan's multiple range test) Table 1), thus confirming the reliability of both methods.

In each of the three species, cell number was the same in low-activity and high-activity CA (*t*-test, $p > 0.05$) (Fig. 4). However, the number of CA cells differed significantly among the three species, averaging ca. 2000 cells per gland in *B. germanica*, 3500 cells per gland in *S. longipalpa* and 11 000 cells per gland in *D. punctata* (Fig. 4).

Discussion

We present a simple enzymatic dissociation method to determine the size and number of cells

TABLE 1

NUMBER OF CELLS IN PAIRS OF *B. germanica* CORPORA ALLATA DETERMINED BY TWO METHODS

Low-activity CA were from newly emerged (day 0) adult females. High-activity CA were from 6-day grouped virgin females with basal oocytes 1.6–1.9 mm long. Mean ± SEM, $n = 4$ CA pairs.

Corpora allata	Number of cells	
	Total cell count [a]	Hemocytometric sampling [b]
Low activity	3744 ± 430	3729 ± 136
High activity	3954 ± 203	4025 ± 177

[a] Total cell count in whole-mount CA monolayers.
[b] Hemocytometric sampling of dispersed CA cells in suspension.

in fresh insect corpora allata. Although the dissociation of animal tissues with proteolytic enzymes in order to quantify changes in cell size and number has been used in ovine corpus luteum (Schwall et al., 1986; Farin et al., 1988), it has not been used for this purpose in insect tissue. Dissociated CA cells maintained their JH-III biosynthetic activity in vitro, indicating that the enzymatic treatment did not adversely affect the cells. Smith et al. (1986) similarly showed that enzymatically dissociated prothoracic gland cells remained responsive to prothoracicotropic hormone. The dissociated cells of *B. germanica* CA incorporated L-[*methyl*-^{3}H]methionine into JH-III at 78% of the rate of intact glands. As some loss of

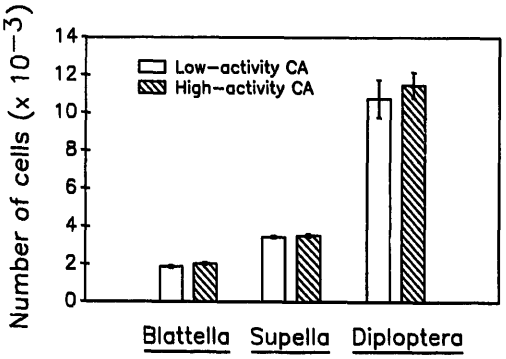

Fig. 4. Number of cells in individual CA of adult female *B. germanica*, *S. longipalpa* and *D. punctata*. The numbers of CA cells in *B. germanica* and *S. longipalpa* were determined by direct counts from whole-mount preparations. The number of CA cells in *D. punctata* was determined by hemocytometric sampling. Bar above each column is the SEM of eight glands.

cells is expected during the transfer of the trypsinized CA from saline to the incubation medium, the two rates are comparable (the latter step is not part of our routine dissociation procedure for morphometric analysis of CA cells). Homogenized CA cannot utilize methionine for JH biosynthesis (Judy et al., 1973; Schooley and Baker, 1985), so it is not plausible that released enzymes from disintegrated cells would account for this activity. Because trypsinization did not impair the activity of CA cells, this procedure may be useful in the preparation of primary CA cell cultures. Furthermore, these data suggest that the basal lamina of the CA of adult female *B. germanica* and intercellular junctions between CA cells are not required in the synthesis of JH-III, at least in short-term incubations.

Until the CA attain peak JH-III biosynthetic rates in vitro, their activity is highly correlated with CA volume and oocyte maturation in *B. germanica* (Gadot et al., 1989), *S. longipalpa* (unpublished data) and *D. punctata* (Szibbo and Tobe, 1981). For all three species we show that the size of CA cells also increases during this period. In contrast, changes in the number of Ca cells are insignificant during the same period. Our data therefore suggest that the growth and activation of the CA in adult female cockroaches are mainly achieved through increases in cell size rather than in cell number. These results are consistent with ultrastructural studies of the CA in *D. punctata* which show an increase in the cytoplasmic volume (Johnson et al., 1985), but they are in disagreement with reports of significant changes in cell number in the CA during oocyte development in *D. punctata* (Szibbo and Tobe, 1981; Tobe et al., 1984). Previous studies with *D. punctata* were based on counts of nuclei in fixed and sectioned CA. However, because the size of the nuclei (i.e. compact or swollen) and their shape (i.e. circular or irregular) fluctuate with cyclic changes in CA activity (Johnson et al., 1985), estimation of cell number by this method is less reliable. The lowest cell count in our study of *D. punctata* is higher than the highest estimation by Tobe et al. (1984). Because small pieces of nuclei in sections were not counted (Szibbo and Tobe, 1981), we suspect that sampling nuclei in paraffin sections underestimates the number of cell nuclei.

Tobe et al. (1984) reported that CA cell number increases more in the hypertrophic glands of ovariectomized *D. punctata* than during CA activation in intact females. Although we did not determine cell number in ovariectomized *D. punctata*, preliminary observations using the method described herein show that in the hypertrophic CA of ovariectomized *B. germanica* CA cell number does not change, whereas cell size increases dramatically.

In conclusion, we have shown that total cell number in the corpora allata of adult females of three cockroach species does not increase as juvenile hormone synthesis and corpora allata volume increase. Rather, cell size increases significantly during this period. We are currently investigating the factors which regulate cell size in the CA of the adult female cockroach throughout the gonotrophic cycle.

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