Release of neurosecretory granules within the corpus allatum in relation to the regulation of juvenile hormone synthesis in Diploptera punctata

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Abstract

The release of neurosecretory granules within the corpora allata (CA) of the viviparous cockroach Diploptera punctata has been compared in glands with intact nerves from the brain (Brain–CA) and those detached from the brain. Measurements of juvenile hormone (JH) synthesis in vitro, comparing these two conditions of the CA at several stages of vitellogenesis in adult females, showed lower production of hormone in Brain–CA complexes than in CA alone. Glands treated with tannic acid to trap exocytotic granules before fixation for electron microscopical examination showed, in sample sections, 10 times more exocytotic profiles in the glands with intact nerves to the brain than in the isolated glands. Sections treated with antibody against allatostatin I (Dip 7), a member of the neuropeptide family that inhibits JH synthesis by CA in vitro, showed neurosecretory granules in allatostatin immunoreactive nerves to be 75±4% of the granules in the sample of sections of CA. Because the total quantity of allatostatin in CA was found by ELISA not to vary significantly with changes in JH synthesis, it is concluded that the lower rates of JH synthesis by glands with intact nerves to the brain are most likely due to the release of small amounts of allatostatin within the CA. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Cockroach; Neurosecretory exocytosis; Allatostatin; Juvenile hormone synthesis; Immunocytochemistry; ELISA

1. Introduction

Juvenile hormone plays a major role in insect metamorphosis and reproduction. The changes in juvenile hormone (JH) titer that are associated with these physiological processes require regulation of JH production by the retrocerebral glands, the corpora allata (CA) (Tobe and Stay, 1985). Among the many factors that contribute to this regulation, an important one is the influence of the brain (Tobe and Stay, 1985). The paired CA are connected to the brain by nerves, containing axons of medial and lateral neurosecretory cells, and considerable experimental evidence indicates that the integrity of these nerves contributes to the normal functioning of the CA (Tobe and Stay, 1985).

A family of neuropeptides, allatostatins, was purified from brains of the cockroach Diploptera punctata on the basis of their ability to inhibit JH production by CA in vitro (Woodhead et al., 1989, 1994; Pratt et al., 1989, 1991). Subsequently, immunocytochemical studies on D. punctata showed allatostatin-immunoreactivity in lateral neurosecretory cells and their axonal projections to and arborization within the CA (Stay et al., 1992a). Previous studies had shown that inhibition of JH production in unmated D. punctata females could be removed by severing the nerves between the CA and the brain (Stay and Tobe, 1977). These findings suggest that at times of decreased JH synthesis in vivo, one should expect to see lateral neurosecretory cells releasing allatostatins at their nerve terminals within the CA. Experiments presented in this paper demonstrate that the quantity of allatostatin contained within the nerves in the CA remains constant during a cycle of JH synthesis, which suggests that release cannot be predicted from overall content of allatostatin in the CA. Therefore, to quantitate release of allatostatins, a direct measurement of exocytosis of neurosecretion within the CA is necessary. Visualization of exocytotic profiles has been demonstrated at the electron
microscopic level of resolution by incubating tissue in buffered tannic acid prior to and during glutaraldehyde fixation. Tannic acid, which does not penetrate the plasma membrane, binds to the neurosecretory material as it is discharged and renders it electron dense (Buma et al., 1984; Pow and Golding, 1987).

In the present study, exocytosis in nerve branches and terminals within the CA has been visualized in glands removed from females either with nerves to brain intact or as isolated CA, conditions in which they show considerably different rates of JH synthesis in vitro. Rankin et al. (1986) found for unmated female D. punctata that brain–corpus cardiacum–CA complexes (Brain–CA) with intact nerves between the brain and the CA, produced much less JH in vitro than did CA alone and hypothesized that this was due to more allatostatin release in the Brain–CA complexes than in isolated CA. In this paper, we have shown that this difference in JH production exists also for mated females during the vitellogenic cycle. We have used CA from mated females at the end of the vitellogenic cycle to compare exocytosis in CA alone and Brain–CA complexes after exposure to tannic acid in vitro. In addition, the proportion of neurosecretory granules in nerves in the CA that occur in profiles of nerves that are immunoreactive for allatostatin has been estimated from immunoreacted sample sections in order to establish the probability that decreased JH synthesis in the Brain–CA is due to increased exocytosis of allatostatin.

2. Materials and methods

2.1. Insect rearing

Female D. punctata mated on the day of adult emergence (day 0) were kept at 27°C and given laboratory chow (Purina, St Louis, MO) and water. Under these conditions vitellogenesis in the ovary begins on day 3, is completed by about day 6, and oviposition occurs between days 7 and 8.

2.2. Tissue dissection, tannic acid treatment, fixation, embedding

The CA and brain retrocerebral complexes, which include the corpora cardica and the CA, were dissected from 6-day-old mated females chilled on ice for 5–15 min. The brain was cut at the optic lobes and circumesophageal connectives; care was taken not to damage the nerves that leave the brain and travel through the corpus cardiacum to the CA. No fluids were employed during dissection. In order to establish the stage of the vitellogenic cycle, ovaries were dissected and basal oocytes were measured with an ocular micrometer and staged as vitellogenic or chorionogenic.

Before fixation the tissues were incubated for 1 h in 0.5% tannic acid adjusted to pH 8.6 in a Ringer’s solution containing 130 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 6 mM NaH₂PO₄, 4 mM NaHCO₃, 2.5% glutaraldehyde, 120 mM Sorensen’s phosphate buffer (pH 7.3) and 0.3 mM CaCl₂ to which was added 0.5% tannic acid for 3 h. Postfixation was in 1% osmium tetroxide in Sorensen’s buffer containing 0.3 mg/ml potassium ferrocyanide for 1 h. During dehydration in an ethanol series, CA were separated from the brains. Treatment with propylene oxide preceded infiltration in Polybed 812 (Polysciences, Warrington, PA) for 48 h and polymerization for 40 h at 60°C.

2.3. Sectioning, immunolabeling, staining

Silver sections were cut from individually imbedded CA (240–400 μm in length) at three positions on the long axis: the center, and 50 μm on either side of the center. At each position, two ribbons were cut 3 μm apart so that the same neurosecretory granules would not occur in the two ribbons. (The maximum diameter of 900 granules within the CA was 250 nm.) Pairs of ribbons were placed on 1000 mesh nickel grids with 55% open area.

For immunolabeling, sections were etched with 4% sodium metaperiodate for 1 h and treated overnight with monoclonal antibody against allatostatin I (5F10, Stay et al., 1992a) 1:4 dilution in Tris buffered saline (TBS, 50 mM Tris, 0.35 M NaCl, pH 7.2) followed by 10 nm gold-conjugated goat-anti-mouse antibody in TBS for 1 h. Sections were then stained with 3% aqueous uranyl acetate and Reynold’s lead citrate. Because immunolabeling was variable, only sections judged to be well labeled were used. This was assessed by dividing the number of gold particles in a nerve profile by the number of granules in that profile after subtracting the background. Background, which was low with the monoclonal antibody, was estimated by counting the gold particles in an area adjacent to and of similar size to a labeled profile. Profiles with a ratio of less than 0.3 were considered unlabeled. Analysis of all of the profiles in three sections from each of three CA showed a mean ratio of gold particles to secretory granules between 1 and 2; with a range between 0.3 and 6.1. This analysis established that a subjective scoring sufficed to determine whether glands were labeled adequately for further analysis.

2.4. Counting procedures

One section from each of six ribbons from each corpus allatum studied was viewed with a Philips 300 electron
microscope at 60 kV. For quantifying the neurosecretory granules in allatostatin-immunoreactive and non-immunoreactive nerve profiles in each of seven CA, photographs were taken at 16,000x of all nerve profiles in each of the six sections (with the exceptions noted in Table 1). The number of granules in the two types of profiles were counted from the micrograph negatives.

Exocytoses in CA, of isolated CA (n=11) and Brain–CA complexes (n=7), were also counted in micrographs of the profiles in the six sample sections. In addition, for the tissue that had not immunoreacted sufficiently for allatostatin quantification, exocytoses were recorded directly at the microscope.

2.5. Assay of JH synthesis

CA and Brain–CA complexes were removed as for electron microscopy, cleaned of extraneous tissue with care not to damage the nerves from the brain to the CA. Rates of JH synthesis were determined by in vitro radiolabeling assay (Feyereisen and Tobe, 1981) and controls for adsorption and degradation of JH by the brain were carried out as previously described (Rankin et al., 1986). Percent inhibition of JH synthesis in Brain–CA complexes compared with isolated CA was calculated as follows:

\[
\% \text{ inhibition} = 1 - \left( \frac{\bar{X}}{\bar{X}} \text{JH synthesis rate of Brain} - \frac{\bar{X}}{\bar{X}} \text{JH synthesis rate of CA alone} \right) \times 100
\]

2.6. ELISA

Brains and CA pairs with corpora cardiaca removed were dissected from chilled animals and transferred to a small quantity of 0.9% NaCl in a 1.5 ml Eppendorf tube on ice. Tissue from five animals was homogenized, heated at 100°C for 10 min and centrifuged at 5000g for 20 min. The supernatant was stored at −20°C. To minimize sample loss, mini sep-paks were prepared from C18 packing material (Waters, Milford, MA) and used as described in Yu et al. (1993). The 500 μl 40% ACN eluate was stored at −20°C until time of assay.

The ELISA method, a variation of Kingan’s assay (Kingan, 1989), is described in Skinner et al. (1997). An affinity purified polyclonal antibody against allatostatin I was used for the ELISA. The antibody showed less than 10% cross-reactivity with allatostatins II, III, and IV (Yu et al., 1993).

2.7. Statistical analysis

Student’s t-test was used.

3. Results

3.1. Quantity of allatostatin in CA and in brains

The quantity of allatostatin found in the CA and in the brains of females on days 2–6 of the first vitellogenic cycle was determined by ELISA using antibody against allatostatin I (Fig. 1). Pairs of CA contained between 0.30 and 0.35 pmoles of allatostatin I equivalents during this time course; the brains contained between 2.24 and 3.28 pmoles. Although it appears that allatostatin increases in the brain between day 3 and 6, there is no significant difference in the allatostatin content of the brains or the CA.

3.2. Rates of JH synthesis by Brain–CA compared to CA alone

JH synthesis was measured in vitro for CA with nerves to the brain intact and for CA alone taken from mated females on days 2, 4, and 6. On day 4, the basal

<table>
<thead>
<tr>
<th>Insect no.</th>
<th>No. of sections counted</th>
<th>Allatostatin positive</th>
<th>Allatostatin negative</th>
<th>% of total positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>1655</td>
<td>494</td>
<td>77</td>
</tr>
<tr>
<td>2A</td>
<td>6</td>
<td>3376</td>
<td>1052</td>
<td>76</td>
</tr>
<tr>
<td>2B</td>
<td>6</td>
<td>2702</td>
<td>848</td>
<td>76</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>2872</td>
<td>331</td>
<td>90</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
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</tr>
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<td>5</td>
<td>6</td>
<td>2837</td>
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<td>86</td>
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<td>1263</td>
<td>1086</td>
<td>54</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>2402</td>
<td>811</td>
<td>75</td>
</tr>
</tbody>
</table>

\(\bar{X} \pm 4\)

\* For insects 1–4 and 5–7, the CA were from Brain–CA complexes and CA alone, respectively.
\* Both CA were counted.
\* Sections not counted: insect 1, one at 2nd location; insect 4, one at each location.
Allatostatin (AST) contents of corpora allata (CA) and brain determined by ELISA with antibody against allatostatin I (Dip 7) shown in relation to basal oocyte length of the mated females on days 2–6. Data points are means of the number of samples assayed (shown below symbols). Bars show standard errors of means (SEM) where larger than the symbols. Each sample was an extract of five CA pairs or five brains. Each sample was assayed two or three times.

Oocytes showed considerable variation in stage of vitellogenesis and therefore measurements of JH production were grouped according to length of basal oocytes in the ovary. On day 6, the smaller oocytes were vitellogenic and the larger ones were early or mid-chorionogenic. Rates of JH synthesis were higher for isolated CA than for CA with intact nerves to the brain, although both groups showed a marked increase in JH synthesis with increasing basal oocyte length, a decrease in JH synthesis before the end of vitellogenesis and low rates when oocytes were forming chorion (Fig. 2A).

When the data in Fig. 2A are expressed as percent inhibition of JH synthesis occurring in the CA connected to the brain compared to isolated CA, it can be seen that inhibition of JH synthesis decreased through day 4 and was lowest when JH synthesis was highest. On day 6, inhibition increased markedly when JH synthesis declined at the end of vitellogenesis (Fig. 2B). These results indicate that glands from 6-day-old females would be suitable for investigating immunolabeling and exocytosis within CA attached to the brain and isolated CA.

3.3. Allatostatin-immunolabeling of nerves in the CA

Immunohistochemical localization of allatostatin in the CA shows an extensive arborization of nerves within the glands (Fig. 3). Sections for electron microscopy, two in each of the locations shown in Fig. 3, showed various shapes and sizes of neurosecretory nerve profiles which could be interpreted as sections of nerve branches, swellings along the branches, or as nerve terminals. Since it is not possible to distinguish with certainty among these alternatives, the sectioned neurosecretory elements will be referred to as profiles. A preliminary analysis of the six sections from each of five different single CA contained a mean of 187±21 profiles. Although the area of the profiles in this preliminary
study varied greatly from small (with one neurosecretory granule) to large (with a mean of 94±10 granules), the mean number of granules per profile in these CA varied less (from 15.4±0.9 to 21.9±2.1). From these observations we decided that the relative amount of allatostatin immunoreactive neurosecretion in the CA could most easily be estimated from the number of neurosecretory granules in immunolabeled profiles. It is of interest to know what proportion of granules in neurosecretory profiles within the CA contain allatostatin because the CA are innervated by both medial and lateral neurosecretory cells, and only lateral cells and their axons in the NCC II are allatostatin immunopositive. The identity of the material in other neurosecretory neurons is unknown at this time.

For this study only nerve profiles internal to the stromal sheath around the CA were considered. An example of immunolabeling for allatostatin in an electron micrograph is shown in Fig. 4. The lower, partial profile has no immunolabeling (10 nm gold particles). The profile above it, with 10 neurosecretory granules and nine gold particles, was scored positive as were the three profiles at the top of the figure.

The quantities of neurosecretory granules in allatostatin immunopositive and immunonegative profiles were determined in cross-sections cut from one of the CA from each of five 6-day-old females and both CA from one insect. Two sections 3 μm apart from each of three locations in each gland (Fig. 3) were scored with the exception of insect 1 (one section from location 2) and insect 4 (one section from each location). The results for each gland are shown in Table 1. A total of 17,812 granules were in allatostatin positive profiles and only 5433 granules were in negative profiles. Thus, 77% of the granules were in allatostatin positive nerves. The percentage for each gland is given in Table 1. The mean value was 75±4% for the eight glands. Profile numbers were also recorded; the mean number of allatostatin positive and negative profiles was 137±19 and 44±3, respectively. For complete sets of sample sections the total number of profiles found per corpus allatum ranged from 109 to 212. However, in an individual section the range of profiles found was from 8 to 53. From these data it is clear that allatostatin-bearing nerves predominate in the CA. A comparison of the total number of neurosecretory granules in the six sections of CA analyzed from three isolated CA (Table 1, insects 2A, 2B, 3) and three Brain–CA (Table 1, insects 5–7) showed no statistical difference between the two conditions (2960±307 vs 3727±364) showing that no massive
release of secretion occurs under either of these conditions in vitro.

3.4. Quantification of exocytosis

Exposure of CA to 0.5% tannic acid for 1 h prior to fixation and during fixation trapped sufficient neurosecretory granules in exocytosis for quantitative analysis, although the numbers were low. The extent of penetration of tannic acid into the glands was calculated by plotting the exocytotic figures within six sections of five glands according to the grid square in which they were found. Exocytoses were found in the outer 1/4 to 1/3 of the glands. Examples of exocytotic profiles are shown in Figs. 5 and 6. The number of granules in exocytosis from neurosecretory profiles was significantly greater in CA from Brain–CA complexes than in CA alone (p<0.001, Table 2). Also significantly more profiles showed exocytosis and more exocytoses occurred per profile in the former than the latter (p<0.001 for both comparisons, Table 2).

To determine whether or not differential release of granules occurred from allatostatin positive and negative profiles, three glands that reacted well for both immunolabeling and trapping of exocytoses were analyzed (Table 3). More exocytoses were seen on allatostatin positive profiles than on negative ones. However, when exocytosis from positive profiles is expressed as a percentage of total exocytoses, the values are similar to those that show the number of granules in allatostatin positive profiles as a percentage of total granules (Table 1). The mean for these three glands is about 80% in both analyses. Thus, there does not appear to be more release from allatostatin positive profiles than from negative ones in these Brain–CA complexes incubated in tannic acid in vitro.

4. Discussion

This study has shown by ELISA that no major change occurs in the quantity of allatostatin I in brains or CA within the first vitellogenic cycle. To the small extent that the antibody used reacts with other members of the allatostatin family (Yu et al., 1993), this is only a slight overestimate of allatostatin I content. Since other allatostatins have been isolated from brains and CA, and these occur in about equal amounts (Woodhead et al., 1989; Stay et al., 1991b), the values for allatostatin I content reported here are an underestimate of the total allatostatin present.

The quantity of allatostatin I in the CA with respect to the other unknown secretory materials present was estimated by a morphometric immunological analysis at the electron microscopic level of resolution. It showed

Table 2

<table>
<thead>
<tr>
<th>Numbers of</th>
<th>Brain–CA (n)</th>
<th>CA alone (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exocytotic granules</td>
<td>33.0±6.1 (11)</td>
<td>3.4±1.7 (7)</td>
</tr>
<tr>
<td>Profiles with exocytoses</td>
<td>18.7±3.0 (11)</td>
<td>2.9±1.4 (7)</td>
</tr>
<tr>
<td>Exocytoses per profile</td>
<td>1.69±0.08 (11)</td>
<td>1.10±0.06 (5)</td>
</tr>
</tbody>
</table>

a Tissue was exposed to 0.5% tannic acid for 1 h before fixation and during fixation.

b Counts were taken from electron micrographs of the sections from 6 CA; for other CA, counts were taken directly from the electron microscope screen.

c Two of these CA showed no exocytosis in the sampled sections. Comparisons between Brain–CA and CA alone were all significantly different (p<0.001).
that the neurosecretory granules in allatostatin immunoreactive nerves were about three times more numerous than the neurosecretory granules in nerves that did not react with antibody against allatostatin. Thus, allatostatin innervation predominates in the CA.

Allatostatins are effective inhibitors of JH synthesis when applied to CA in vitro (Tobe et al., 1994). However, the extent to which allatostatins are physiological regulators of JH production in vivo has yet to be clearly demonstrated. In vivo experiments with male D. punctata showed that JH synthesis was more effectively inhibited by exogenous application of JH analog when nerves to the CA were intact and hemolymph allatostatin was low and considerably less inhibited when nerves to the brain were severed and hemolymph titers of allatostatin were elevated (Stay et al., 1994).

Rankin et al. (1986) found lower rates of JH synthesis in vitro in virgin, non-vitellogenic females in the Brain–CA compared to isolated CA. In the present study we have shown that Brain–CA complexes at all stages of the vitellogenic cycle showed lower rates of JH production than CA alone. Inhibition was greatest when JH synthesis was low and lowest when JH synthesis was high. This response is similar to the sensitivity of CA to allatostatin treatment in vitro. CA with low rates of synthesis are more strongly inhibited by the same concentration of allatostatin than CA at stages of high JH production (Pratt et al., 1990; Stay et al., 1991a). Because the sensitivity of the CA changes with the rate of JH production, it would appear that the pattern of change in inhibition of JH production by Brain–CA demonstrated in the present experiments results from this change in sensitivity of the CA rather than a change in the amount of allatostatin released. Thus, these results suggest that CA with intact nerves to the brain are responding to the release of a constant amount of allatostatin within the glands. The level of inhibition corresponds to that obtained by incubation of CA in 10^{-8} M allatostatin I (Stay et al., 1991a) — a physiological concentration.

The analysis of the number of neurosecretary granules in exocytosis in the nerves within the CA that were visualized in the electron microscope as a result of tannic acid treatment of Brain–CA and CA alone, showed 10 times more exocytoses in the former than in the latter. This strongly supports the hypothesis that JH production by the CA is inhibited as a result of local release of neurosecretion when nerves to the brain are intact. The release that was captured in our experiment did not show greater allatostatin release relative to other release because the number of exocytoses from allatostatin positive and negative profiles was in proportion to the distribution of such granules in the nerves of the CA. However, significantly more granules were released per profile from allatostatin positive nerves (Table 2). That the exocytosis of neurosecretion observed in Brain–CA under these experimental conditions represents the normal activity of these nerves is questionable because the brain may be traumatized. However, it does demonstrate that the release of neurosecretion is associated with inhibition of JH production. Khan and Buma (1985) analyzed exocytosis in nerves of the CA of the Colorado potato beetle, in which Brain–CA complexes from insects reared under short- and long-day photoperiods were treated with tannic acid in vitro. More exocytosis was found in glands from short-day animals which are known to have lower rates of JH synthesis than CA from animals reared in long-day conditions. They suggest that the differential release is a reflection of the in vivo condition in which CA are restrained by release of neurosecretion. However, the material released was not identified.

Stimulation of exocytosis of neurosecretion in vivo in the corpora cardiaca of locusts was demonstrated by injecting tannic acid into the necks of the animals before a 10 min flight test (Pow and Golding, 1987). These results suggest that it may be possible to monitor exocytosis in vivo in D. punctata to determine whether allatostatin release changes during the vitellogenic cycle. Another question that should be addressed in future studies is the nature of the material in non-allatostatin positive terminals. Immunostaining has identified met-enkephalin in nerves within the CA and in medial neurosecretory cells (Duve et al., 1991), however, no action of this peptide could be demonstrated on JH production in vitro (Stay et al., 1992b).

This study has shown that the inhibition of JH production seen in Brain–CA complexes in this in vitro
experiment is associated with exocytosis from nerves within the CA and suggests that release of allatostatin predominates over any other substances that might also be released.

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References


