Comparison of Ecdysteroid Production in Drosophila and Manduca: Pharmacology and Cross-Species Neural Reactivity

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Abstract:

In both *Manduca sexta* and *Drosophila melanogaster,* metamorphic events are driven by ecdysteroids whose production in prothoracic gland (PGs) is stimulated periodically by neural factors. Differences in the life cycle of moths and flies have made it difficult to compare the regulation of ecdysteroid biosynthesis in these two species.

As in *Manduca,* at least two neural factors in the larval *Drosophi/a* BVG complex were separable by molecular weight, and they stimulated increased ecdysteroid biosynthesis from the ring gland, a composite organ that includes PG cells. *Drosophi/a* neural extracts accelerated ecdysteroid biosynthesis in *Manduca* PGs and, conversely, partially purified *Manduca* PTTH preparations elevated ecdysteroid biosynthesis in *Drosophila* ring glands, suggesting that the two species may share structurally similar prothoracicotropic factors. *Drosophila* ring glands required the presence of calcium ions to respond to neural extracts, but the phosphodiesterase inhibitor MIX and cAMP analogues exerted little, if any, positive effect on production.

Mean ecdysteroid production rates of BVG-ring gland complexes taken from *Drosophila* larvae during various phases of the wandering period were often submaximal and highly variable, suggesting that they fluctuate widely prior to pupariation. Based on available data in *Drosophila* and the *Manduca* model for the control of ecdysteroid biosynthesis, a developmental scheme for neuroendocrine control in *Drosophila* is proposed.

Key words: prothoracicotropic hormone, steroidogenesis, cAMP, calcium-dependent

Abbreviations used: BVG = brain-ventral ganglion; EGTA = Ethylene glycol bis(I3-aminoethyl ether)-n,N,n',N'-tetraacetic acid; MIX = 1-methyl-3-isobutylxanthine; PG-prothoracic gland; PTTH = prothoracicotropic hormone; RIA = radioimmunoassay; SD = standard deviation; SEM = standard error of the mean; SG = salivary gland.

Article:

INTRODUCTION

Considerable recent progress has been made concerning the molecular basis of ecdysteroid response in *Drosophila melanogaster* (Andres and Thummel, 1992; Talbot et al., 1993; Yao et al., 1993; Thomas et al., 1993), but less is known about the regulatory mechanisms that underlie surges of ecdysteroid production occurring during pre-metamorphic development. This issue is crucial for understanding the molecular mechanisms associated with hormonal regulation because numerous lines of evidence in *Drosophila* indicate that ecdysteroid synthesis and response are coordinated and interdependent activities (Karim and Thummel, 1992; Pak et al., 1992; Sehnal and Bryant, 1993; Hurban and Thummel, 1993).

Most current insights about the regulation of ecdysteroid biosynthesis have come from studies conducted in the Lepidoptera, notably *Manduca sexta* (Gilbert et al., 1988). Several features of the Lepidoptera have made them particularly amenable for insect endocrine studies. First, their large size has made it possible to purify the neuropeptidergic PTTHs* responsible for stimulating ecdysteroid biosynthesis (Bollenbacher et al., 1984; Nagasawa et al., 1986; Kataoka et al., 1987). Secondly, their larval and pupal stages are marked by clearly discernible changes that allow precise staging. The accuracy of staging possible with Lepidoptera has, in turn, facilitated the assembly of a detailed ecdysteroid and juvenile hormone titer profile during development (Riddiford, 1994). Third, the PG of Lepidoptera is a homogeneous tissue whose ecdysteroid-synthesizing capability and response to chemical stimuli can be tested in vitro (Bollenbacher et al., 1979; Gilbert et al., 1988). Consequently, it is possible not only to evaluate the effects of purified biological factors and pharmacological agents on ecdysteroid synthesis, but also to analyze their intracellular biochemical effects.

In both *Bombyx* and *Manduca,* two PTTH forms (big and small) have been identified which can evoke heightened levels of in vitro ecdysteroid biosynthesis (Bollenbacher et al., 1984; Kataoka et al., 1987). The gene encoding the *Bombyx* big PTTH has been cloned and the complete peptide sequence deduced (Kawakami et al., 1990). It bears no resemblance to any other reported peptide sequence and has so far not served successfully as a molecular probe to identify a homologous neuropeptide in other insect species. The insulin-like bombyxin, formerly known as *Bombyx* small PTTH, is not generally regarded as a true prothoracicotrope (Ishizaki et al., 1983). The partial amino acid sequence of *Manduca* big PTTH resembles the vertebrate retinol binding and retinoic acid binding proteins, CRBP and CRABP, and forms a homodimer (Muehleisen et al., 1994). The *Manduca* small PTTH remains uncharacterized molecularly.

Based primarily on in vitro tests of *Manduca* PG responsiveness to pharmacological reagents, Gilbert and his colleagues have determined that big PTTH acts via a cAMP-mediated, calcium / calmodulin-dependent pathway to increase the rate of ecdysteroid synthesis (Smith et al., 1985; Gilbert et al., 1988). Exposure of PGs to neurohormonal factors is also associated with changes in transcriptional and translational activity (Keightley et al., 1990; Rybczynski and Gilbert, 1994), as well as phosphorylation of the ribosomal protein, S6 (Song and Gilbert, 1994). Moreover, the response characteristics of *Manduca* PGs undergo developmental changes in the final larval instar that may reflect regulatory features important for the normal onset of metamorphosis (Smith and Pasquarello, 1989; Meller et al., 1990).

The PGs of *Drosophila melanogaster* comprise a portion of the ring gland lying at the base of the larval brain (Dai and Gilbert, 1991). Despite the difficulties intrinsic in testing this small, composite gland, the genetic technology available in *Drosophila* has motivated the development of an in vitro bioassay for ecdysteroid synthesis as a means to identify proteins that disrupt this process when mutated (Henrich et al., 1987a). Ring glands from mutants homozygous for two conditional mutations, *dre4* and *ecd*^{1}, exhibit reduced ecdysteroid synthesis at their restrictive temperature (Sliter and Gilbert, 1992; Henrich et al., 1987b), although these mutations do not completely eliminate protein function. Mutant *ecd* larvae also exhibit phenotypes that reflect the gene's role in other developmental processes (Henrich et al., 1993) and the regulatory model of ecdysteroid production proposed for *Manduca* includes several proteins that likely play important roles in nonendocrine tissues (Gilbert et al., 1988).

The validity of comparisons between the neuroendocrine axes of Lepidoptera and Diptera will depend ultimately on whether the functional steroidogenic components identified genetically in *Drosophila* resemble the proteins characterized biochemically in Lepidoptera. Presently, the degree of similarity between these systems is unclear. Antibodies raised to lepidopteran PTTH bind to sites in cells of the *Drosophila* brain lobe that correspond with the locale of similar cells in the lepidopteran brain, and these antibodies also recognize cells in the ventral ganglion (Zitnan et al., 1993), the major source of prothoracicotropic activity from the *Drosophila* central nervous system as determined by in vitro assay (Henrich et al., 1987a). The late larval brain lobes of *Drosophila* also contain prothoracicotropic activity, but the effect of brain extracts appears to be

modulatory (Henrich et al., 1987a). As in *Manduca*, at least two peptides with several possible isoforms have been purified partially from *Drosophila* BVGs (Pak et al., 1992).

These experiments provide a basis of comparison between the *Drosophila* and *Manduca* neuroendocrine axes by testing the cross-species reactivity of *Manduca* and *Drosophila* neural extracts. This study also reports the ability of *Drosophila* ring glands to produce ecdysteroids when challenged with pharmacological agents such as those that have led to the formulation of the *Manduca* model. Finally, individual ring glands were dissected from *Drosophila* late third instar larvae and tested for ecdysteroid synthesis in vitro. A fraction of these ring glands produced ecdysteroids at levels that resemble those induced by neural extracts in vitro suggesting that neurohormonally elevated synthesis occurs in vivo.

MATERIALS AND METHODS

Drosophila and *Manduca* Rearing Conditions

A Canton-S strain of *Drosophila melanogaster* was maintained at 25°C in a 12L:12D regime according to protocols described previously (Henrich et al., 1987b). *Manduca* larvae were reared in a non-diapause inducing regime (16L:8D) at 25°C on an artificial diet and coordinated developmentally by established methods (Vince and Gilbert, 1977).

Staging of *Drosophila* Larvae

Wandering *Drosophila* larvae were classified into one of four categories based on salivary gland morphology and wandering behavior. Category I larvae refer to individuals showing active wandering behavior whose SGs show no signs of lumen expansion or cellular swelling. These SGs possessed a "grape cluster" appearance and ring glands from these larvae were employed for the in vitro assay (Henrich et al., I987a). Ring glands from this stage were also utilized for pharmacological and cross-species tests described below. Category II larvae showed wandering behavior and their SGs were partially swollen with a slightly dilated lumen. Category III larvae displayed more sluggish wandering activity, their SG cells were fully bloated, and the SG lumen was fully expanded. Category IV individuals were selected as they entered the white prepupal stage. They did not display wandering activity, their bodies had begun to contract, and partial anterior spiracle eversion had occurred. Ring glands were dissected and tested from Category IV larvae that still possessed partially swollen salivary glands indicating that glue protein had not been extruded completely.

In Vitro Assay Conditions

The preparation of *Drosophila* BVG extracts, methods for in vitro incubation of larval ring glands and BVG-ring gland complexes (Henrich et al., 1987a,b), as well as procedures for competitive RIA for ecdysteroids (Warren and Gilbert, 1988) have been described previously All ecdysteroid measurements were based on alpha-ecdysone equivalents using the H-22 antibody In vitro incubations were conducted for 2 h at 25°C with single ring glands isolated from Category I larvae after which the culture medium was measured by RIA. These ring glands did not contain measurable ecdysteroid titers. Reported levels of ecdysteroid production evoked by extracts were adjusted by subtracting the amount of endogenous ecdysteroids detected in each extract.

The methods employed for in vitro assay of *Manduca* fifth instar, Day 3 PGs with *Drosophila* neural extracts were based on established methods (Bollenbacher et al., 1979). Both PGs were dissected from each *Manduca* larva and placed into either 25 µl of insect Grace's medium or 25 µl of medium containing a *Drosophila* neural extract whose concentration was expressed as the number of BVG equivalents per 10 µl Differences between the *Manduca* and *Drosophila* neural dosage levels that are necessary to stimulate a maximal response arise from the greater molar quantity of PTTH activity in each *Manduca* brain. PGs were incubated for 2 h at 25°C and the incubation medium measured for ecdysteroids by RIA.

Preparation of Tissue Extracts

A neural extract of *Drosophila* was prepared by homogenization, boiling, and centrifugation of BVGs dissected from wandering third instar larvae as described (Henrich et al., 1987a,b) except that the homogenization was performed in distilled water. A portion of the extract was separated into two fractions by centrifugation at 4°C

for 45 min at 5,000g through a Centricon-10 ultrafilter membrane (Amicon, Danvers, MA). The resulting filtrate (less than 10 kD) and retentate (more than 10 kD) fractions were lyophilized with heat, then adjusted to a concentration of 8 BVG per 10 μl by adding an appropriate volume of Grace's insect medium to each fraction. Ring glands from Category I larvae reach a maximal rate of ecdysteroid synthesis when challenged with this dosage of neural extracts (Henrich et al., 1987a). A second portion of the neural extract, treated identically but not separated by ultrafiltration, was also prepared for testing as a positive control. The fractions and control extract were then tested with *Drosophila* ring glands for their effect on ecdysteroid biosynthesis in vitro. A third portion of the crude neural homogenate was treated with 0.5 μg of trypsin (Sigma Chemical Co., St. Louis, MO) for 3 h at 45°C prior to boiling, centrifugation, and collection of the supernatant for incubation.

Partially purified *Manduca* big and small PTTH extracts were also prepared by ultrafiltration through a Centricon-10 membrane with the use of previously employed methods (O'Brien et al., 1986). The concentration of *Manduca* PTTH activity used for this study was expressed in units per 10 μl, one unit corresponding to the amount of PTTH activity in a Day One pupal brain and calculated as described by Bollenbacher et al. (1984). The concentrations tested, 0.625 U/10 μl small PTTH and 0.10 U/10 big μl PTTH cause maximal stimulation of larval *Manduca* PGs (Bollenbacher et al., 1984).

Pharmacological Experiments

All pharmacological tests were performed with ring glands dissected from Category I larvae and incubated for 2 h, as described earlier. For tests of calcium ion dependence, *Drosophila* neural extracts were prepared as described previously except that homogenization was performed in a calcium-free Grace's medium (Gibco, Grand Island, NY) supplemented with 100 RM EGTA. Calcium ion was then added back to either calcium-free Grace's medium or the calcium-free neural extract to a final concentration of 10 mM, using a 1 M CaC12 stock solution. Ring glands were tested with each of these incubation media as well as calcium-free Grace's medium. For another experiment, incubation media were prepared by dissolving MIX (Sigma) and/ or benzoyl cAMP (Sigma) salts in Grace's medium to a final concentration of 500 1..t.M MIX and/or 1 mM 8-benzoyl cAMP. These concentrations of calcium ion, MIX, and 8-benzoyl cAMP evoke a maximal effect on ecdysteroid synthesis from *Manduca* PGs (Smith et al., 1985). MIX and 8-benzoyl cAMP also exert maximal allatostatic effects on juvenile hormone production in the *Drosophila* ring gland at these concentrations (Richard et al., 1990). None of the reagents distorted the measurements of the ecdysteroid competitive RIA.

RESULTS

PTTH Activity from *Drosophila* Neural Extracts Involves Two or More Factors

Ventral ganglion extracts evoke a maximal response from *Drosophila* ring glands, but the amount of ventral ganglion extract needed to attain a maximal response is less when brain extracts are also added (Henrich et al., 1987a). Brain extracts alone evoke a relatively weak response, raising the possibility that at least two factors play a role in stimulating ecdysteroid biosynthesis in *Drosophila.* When a neural extract was separated by ultrafiltration into fractions of more and less than 10 kD, both the filtrate and retentate elicited an increased rate of ecdysteroid synthesis (Fig. 1a—c). The filtrate, containing molecules of less than 10 kD, exhibited an activating potency reminiscent of the effect of ventral ganglion extracts, while the retentate elicited a less robust response whose level of activity corresponded closely to those associated with brain extracts. The sum of effects above the basal level induced by the two separated fractions was roughly equal to the effect of an unseparated neural extract (Fig. 1b—d). Incubation of the neural homogenate with trypsin prior to the boiling step resulted in an extract whose activity was reduced by 48% (Fig. 1e—g). The failure to eliminate activity may indicate that the activating peptide was resistant to trypsin digestion or that one or more peptide fragments resulting from trypsin digestion retained partial function. The differences observed in the activation caused by the two different neural preparations (Fig. 1d and f) reflected an uncontrolled source of variability that presumably stems from differences in the activity of neural extracts and/or responsiveness of dissected ring glands in separate experiments. Similar differences have been observed among identically treated control groups producing ecdysteroids at basal rates. For this reason no comparisons have been drawn between the values gathered from separate experiments.

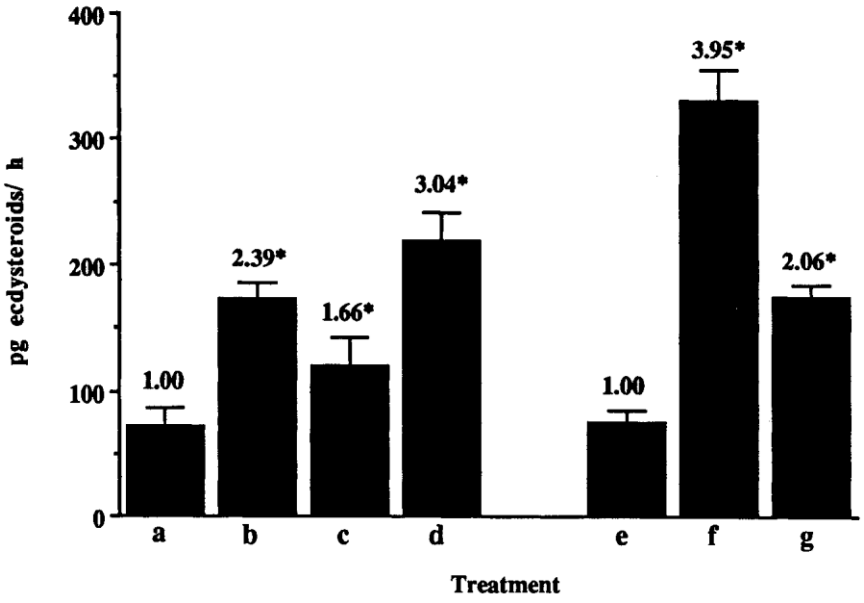

Fig. 1. Effect of fractionation by molecular weight and trypsin treatment on neural extract-induced in vitro ecdysteroid synthesis by individual Category I larval ring glands of *Drosophila melanogaster*. N = 5 for each group and error bars designate SEM. Numbers above each column designate fold-activation over control group and asterisk (*) designates a significant increase in ecdysteroid synthesis compared to basal control group (t-test; $P < 0.01$, except (c), $P < 0.05$). **a:** Grace's medium; **b:** filtrate (< 10 KDa) adjusted to 8 BVG/10 µl after ultrafiltration and lyophilization; **c:** retentate (> 10 KDa) treated same as b; **d:** neural extract (8 BVG/10 µl) treated identically but not fractionated by ultrafiltration; **e:** Grace's medium; **f:** neural extract (8 BVG/10 µl); **g:** same as f except incubated with trypsin prior to boiling, centrifugation, and incubation with ring glands. Columns a–d and e–g represent two different experiments.

Cross-Species Tests of PTTH Activity

The ability of partially purified *Manduca* big and small PTTH to activate *Drosophila* ring glands was also tested with doses that induce a maximal response from Manduca Day 3 PGs (Bollenbacher et al., 1984). Both *Manduca* PTTHs activated *Drosophila* ring glands to levels of ecdysteroid synthesis associated with maximal activation (Fig. 2).

In a reciprocal experiment, *Drosophila* neural extracts were tested for their ability to evoke elevated ecdysteroid synthesis in vitro among PGs dissected from prewandering (Day 3) fifth instars of *Manduca.* A dose-dependent response was noted that indicates the ability of one or more *Drosophila* factors to increase ecdysteroid production from *Manduca* PGs (Fig. 3) more than fourfold, although the maximal response was highly variable. The RIA antibody used did not detect 3-dehydroecdysteroids, which are released from *Manduca* PGs and converted into ecdysone by a heat-labile keto-reductase that would have been destroyed in the preparation of the *Drosophila* neural extract during the boiling step (Warren et al., 1988).

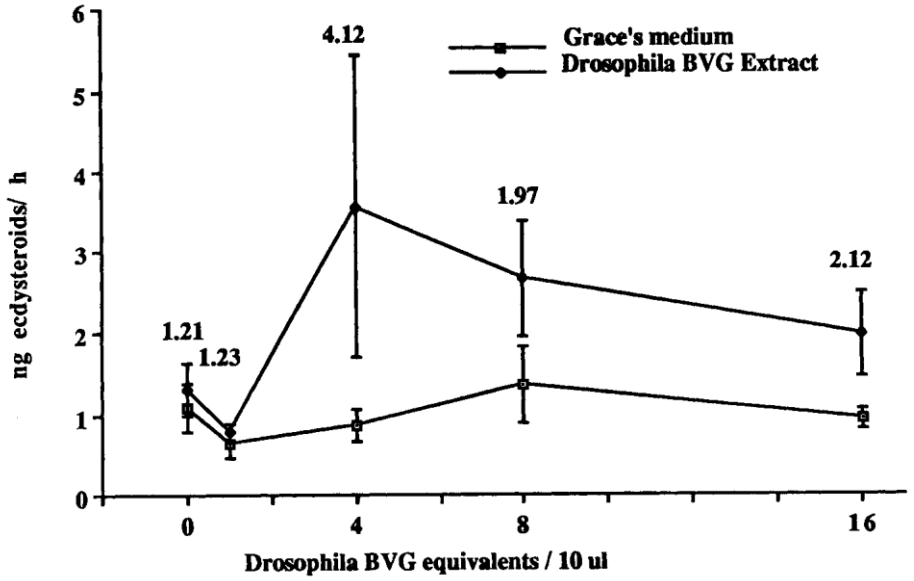

Fig. 2. The effect of *Drosophila* BVG extracts of various dosages on ecdysteroid production in vitro by PGs dissected from Day 3, fifth instar larvae of *Manduca sexta*. Error bars designate SEM based on N = 5 for each data point. Numbers above each pair of points indicates fold-activation.

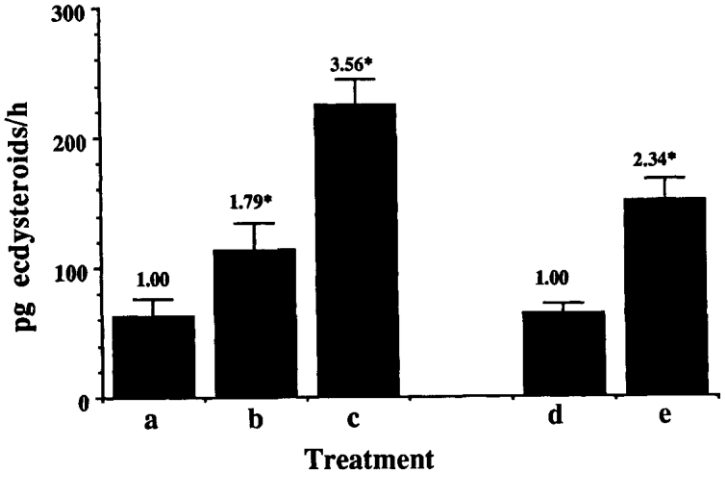

Fig. 3. Effect of *Manduca* small PTTH and *Manduca* big PTTH on ecdysteroid production by the ring gland of *Drosophila melanogaster*. **a:** Grace's medium; **b:** 0.313 units/10 µl *Manduca* small PTTH; **c:** 0.625 U/10 µl *Manduca* small PTTH; **d:** Grace's medium; **e:** 0.1 U/10 µl *Manduca* big PTTH. Error bars designate SEM and N = 5 for each group; numbers above each column indicate fold-activation over control group and asterisk designates statistically significant difference as determined by t-test ($P < 0.01$). Columns a–c and d–e represent results from two different experiments.

Drosophila Ring Gland Response to Neural Extracts Is Calcium-Dependent But May Not Be cAMP-Dependent

Based on the importance of cAMP and calcium for elevating rates of in vitro ecdysteroid synthesis in *Manduca* PGs (Smith et al., 1985), similar pharmacological experiments were repeated using *Drosophila* ring glands.

The response to brain-ventral ganglion neural extracts was calcium-dependent although basal levels of ecdysteroid production were unaffected by the absence of extracellular calcium. Neural extracts prepared in calcium-free Grace's medium stimulated no significant increase of ecdysteroid production and/or secretion from ring glands (Fig. 4a—b). However, when the same extract was supplemented with calcium ion to a concentration of 10 mM the response was restored to nearly normal levels (Fig. 1 and Fig. 4c—d), In other experiments, additions of calcium ion that brought its final concentration to 1 and 5 mM positively affected the rate of ecdysteroid synthesis to about the same level (data not shown).

In order to evaluate the effect of increasing intracellular cAMP levels on ecdysteroid synthesis, the phosphodiesterase inhibitor, MIX, was added to the incubation medium. At a concentration of 500 gIVI, the treatment resulted in a statistically significant elevation of ecdysteroid synthesis ($P < 0.05$, t-test; Fig. 5a—b) although it was much lower (about 1.3-fold) than the greater than two-fold activation normally associated with *Drosophila* neural extracts. Lower concentrations of MIX failed to evoke any significant response, nor did medium supplemented with 1 mM 8-benzoyl cAMP or dibutyryl cAMP alter ecdysteroid production (data not shown). In fact, when both MIX and 8-benzoyl cAMP were added together to the incubation medium, ring gland production of ecdysteroids was dramatically reduced compared to a control group (Fig. 5c-d). It is not clear whether these are toxic effects or reflect a biologically relevant form of down-regulation.

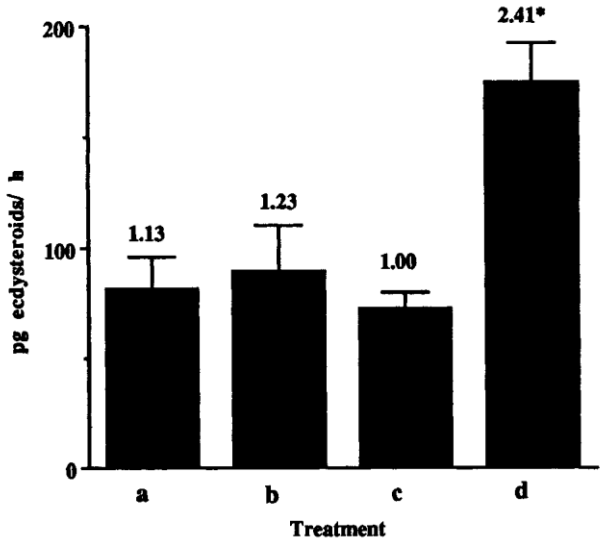

Fig. 4. Effect of calcium on ecdysteroid production by individual Category I ring glands of *Drosophila melanogaster*. All media and extracts contain 100 µM EGTA and 10 mM Na_2HPO_4. **a:** Grace's medium without Ca^{++}; **b:** BVG extract (8 BVG/10 µl) with no added Ca^{++}; **c:** Grace's medium with Ca^{++} added to a final concentration of 10 mM; **d:** BVG extract (8BVG/10 µl) with Ca^{++} added to a final concentration of 10 mM. Error bars designate SEM and N = 6 for each group. Numbers above each column denote fold-activation over control group and asterisk (*) designates a statistically significant difference as determined by t-test ($P < 0.01$).

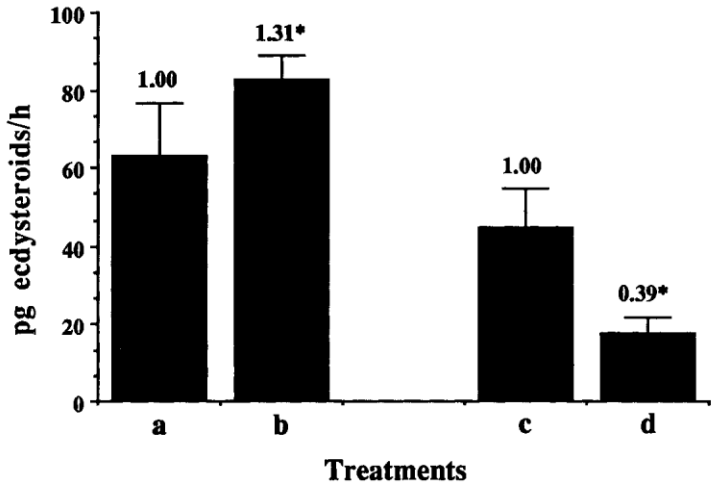

Fig. 5. Effect of pharmacological reagents on ecdysteroid production by individual ring glands of *Drosophila melanogaster*. **a:** Grace's medium; **b:** Grace's medium with 500 µM MIX; **c:** Grace's medium; **d:** Grace's medium with a concentration of 500 µM MIX and 1 mM 8-benzoyl cAMP. Error bars designate SEM and N = 5 for each group and numbers above each column indicate fold-activation over control group. Asterisks (*) designate a statistically significant difference as determined by t-test ($P < 0.05$). Columns a–b and c–d represent results from two different experiments.

Ecdysteroid Synthesis by BVG-Ring Gland Complexes Dissected From Wandering Third Instars
In order to assess whether ring glands produce ecdysteroids at stimulated levels during the late third instar in vivo, BVG-ring gland complexes were dissected from larvae which had been selected during wandering based on criteria described in Materials and Methods.

Ecdysteroid production did not vary significantly in BVG-ring gland complexes among any of the four experimental categories (I-IV). Closer examination of the individual points revealed that ring gland ecdysteroid synthetic activity ranged widely in Categories II through IV and that the large SD encountered in the late wandering groups obscured any possible mean differences. Moreover, only some complexes displayed rates of biosynthetic activity approaching the levels of synthesis caused by neural extracts (Fig. 6).

The mean and standard deviation (88.4 ± 22.6 pg/ring gland/h) of ecdysteroid synthesis among the Category I ring glands tested in this experiment is typical. Based on the normalized mean and standard deviation values observed here and the application of standard statistical procedures, it was calculated that approximately 30% of Category I ring glands would be expected to produce more than 100 pg ecdysteroids per h over a 2-h period. Only about 8% of these ring glands would produce more than 120 pg per h, this being a level of synthesis minimally associated with neural activation (Henrich et al., 1987a,b). Applying the same analysis to Category IV ring glands (96.1 ± 35.3 pg/h), approximately 46% would be expected to produce more than 100 pg per h and 26% of these ring glands would produce more than 120 pg per h. Just as importantly, the proportion of Category II—IV ring glands producing less than 50 pg/h was greater in these groups than in Category I ring glands. Therefore, while mean levels of ecdysteroid production were not significantly different between early and late wandering ring glands, the variability of ecdysteroid production was higher as pupariation approached.

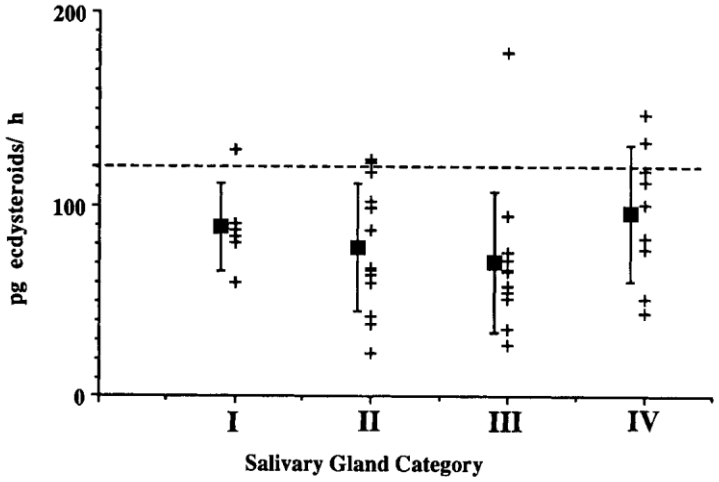

Fig. 6. Rates of ecdysteroid production by individual BVG-ring gland complexes isolated from *Drosophila* third instars during four phases of wandering period (I through IV). Individual data points indicate rates of synthesis observed in individual ring glands and points with error bars designate mean rate and SD for each category. Dashed line indicates 120 pg/h, a rate of synthesis associated with minimal in vitro activation.

DISCUSSION

Some features of ecdysteroid biosynthesis in the *Drosophila* ring gland resemble those already described for *Manduca.* In both species, at least two heat stable neural factors evoked increased ecdysteroid production. Moreover, late larval *Drosophila* neural extracts stimulated prewandering *Manduca* larval prothoracic glands. Conversely, partially purified *Manduca* big and small PTTH stimulated higher levels of ecdysteroid synthesis from *Drosophila* ring glands significantly above the basal rate of production. Together, these results leave open the possibility that both factors are conserved between these two species although tests with molecularly characterized peptides will be necessary to substantiate this inference.

Based on the amount of starting BVGs, extracts either prepared from the larval ventral ganglion or recovered from the BVG filtrate increased ecdysteroid synthesis to about the same maximal level at a given dosage (Henrich et al., 1987a). It can be inferred, therefore, that a *Drosophila* "small PTTH" accumulates in the late larval ventral ganglion and upon release, acts by a calcium-dependent mechanism to evoke the ecdysteroid peak associated with pupariation in *Drosophila.* By comparison, the *Manduca* small PTTH has been implicated in the stimulation of an ecdysteroid peak associated with larval wandering (Bollenbacher et al., 1984), although its activity is also detected later in pupal brains (O'Brien et al., 1986).

A fraction of *Drosophila* ecdsyteroid-stimulating activity was recovered after ultrafiltration as a retentate (> kD) and likely is the 14-17 kD prothoracicotropic factor described previously (Pak et al., 1992). It may also prove to be the antigen detected in brain lobes and ventral ganglia by antibodies to *Bombyx* big PTTH (Zitnan et al., 1993). The sum of retentate and filtrate activation roughly equalled the activation caused by a combined extract and could indicate that the same activating factors exist in both tissues. Nevertheless, earlier studies have demonstrated that a *Drosophila* brain factor showing relatively little activity by itself disproportionately enhances the stimulatory effects of a ventral ganglion factor and may represent the convergence of multiple activation pathways (Henrich et al., 1987a).

Since the characteristics of the *Drosophila* "small PTTH" resemble those of the ventral ganglion factor, it also follows that the retentate fraction harbors the brain factor described previously (Henrich et al., 1987a). In fact, both brain extracts and retentate fractions stimulated a submaximal increase in ecdysteroid production from larval ring glands at similar concentration levels (8 larval brains per 10 µl of extract). By contrast, an extract containing 8 ventral ganglia per 10 µl induces a much greater response. The relatively low titer of brain activity could result if this factor is depleted because of its release prior to wandering and/or repeatedly during late larval development, as may be the case for *Manduca* big PTTH (O'Brien et al., 1988). It is less likely that the low level of stimulation caused by the brain factor involved ring gland refractoriness, since both chromatographically purified *Drosophila* "big PTTH" and *Manduca* big PTTH can induce a higher response from larval ring glands when tested in vitro (Pak et al., 1992).

BVG-ring gland complexes dissected from wandering larvae almost never produced ecdysteroids at the high rate induced by incubating ring glands in vitro with neural extracts. Possibly, therefore, the *Drosophila* nervous system releases submaximal dosages of prothoracicotropic factors to regulate ecdysteroid biosynthesis during most of the wandering phase of the late third instar. The in vivo effect of these *Drosophila* factors may simulate the synergistic activity seen in vitro when submaximal dosages of each factor are mixed (Henrich et al., 1987a).

Interpretation of Pharmacological Experiments

As in *Manduca,* the *Drosophila* ring gland response to neural extracts was calcium-dependent. However, basal levels of ecdysteroid synthesis were observed even in the absence of extracellular calcium, suggesting that the presence of this ion is necessary for an elevation of ecdysteroid synthesis but not for sustaining ecdysteroid biosynthesis.

MIX and other cAMP analogues did not exert a strong positive impact upon ecdysteroid synthesis in the *Drosophila* ring gland, representing an important point of divergence between the two assays. This presents a problem for interpretation, since *Manduca* big and small PTTH act by one or more cAMP-mediated pathways (Watson et al., 1993; Smith et al., 1984, 1985) and also increase ecdysteroid biosynthesis from *Drosophila* ring glands. The poor response to MIX and cAMP analogues may reflect a technical problem, such as the failure of these agents to enter prothoracic gland cells. The small response itself may arise as a consequence of MIX's suppressive effects on juvenile hormone production from the corpus allatum portion of the ring gland (Richard et al., 1990), which in turn, might indirectly increase ecdysteroid synthesis (Richard and Gilbert, 1991). Moreover, ring glands dissected from early wandering larvae already produce a measurable level of ecdysteroids in vitro and already are partially stimulated at this developmental time. If the activation of ecdysteroid synthesis to the levels normally found in early wandering larvae was cAMP-mediated then the effects of MIX and cAMP analogues might be masked to some degree in ring glands from early wanderers.

Alternatively, the acceleration of ecdysteroid biosynthesis that leads to pupariation in *Drosophila* simply is not induced and may actually be inhibited by a cAMP-mediated pathway.

Ecdysteroid Production in Ring Glands During the Wandering Phase of the Late Third Instar

The large variability in ecdysteroid production among ring glands dissected from late third instars indicates that they rarely produce ecdysteroids in vivo at the sustained high rates induced by neural extracts. In fact, the lowest mean rate of ecdysteroid production was observed in BVG-ring gland complexes taken from late wandering (Category III) larvae, a developmental time when ecdysteroid titers are reaching relatively high levels. The increased frequency of moderate (i.e., greater than 100 pg/h) and high production (i.e., greater than 120 pg/h) ring glands in the latest phase of the wandering third larval instar (Category IV) can be interpreted to mean that periods of ecdysteroid production become longer, greater, and more frequent as the onset of pupariation nears. Nevertheless, a fraction of Category IV ring glands also produced very low levels of ecdysteroids, implying that a rapid deceleration in synthesis occurred at pupariation that, in turn, contributes to the rapid decline in whole body ecdysteroid titers seen after pupariation (Hodgetts et al., 1977; Pak and Gilbert, 1987). Interestingly, the unreliability of short *Drosophila* ring gland incubations (less than 1 h) often results from the failure to detect measurable ecdysteroid levels in a significant fraction of ring glands (Henrich, unpublished data), a logical observation if ecdysteroid production truly alternates between quiescent and active periods. Hourly measurements of in vitro production from individually cultured ring glands also have shown large fluctuations during a 4-h period, but experimental error caused by the manipulations necessary to take hourly readings has prevented an unassailable interpretation of those results (Henrich, unpublished data).

Proposed Steroidogenic Events in *Drosophila* During Late Larval Development

Considering all available data and drawing on elements of the *Manduca* system, the following course of neuroendocrine events is proposed for the late third instar of *Drosophila:* (1) ecdysteroid biosynthesis is stimulated by an unknown mechanism prior to wandering and (2) following this initial activation, the ring gland becomes relatively quiescent sometime during wandering; (3) the single or repeated release of a brain factor analogous to *Manduca* big PTTH "primes" the gland for its subsequent activation in the late wandering period and (4) as the wandering phase continues, high levels of ecdysteroid biosynthesis are triggered by the rapid release of a small peptide from the ventral ganglion that acts by a calcium-dependent mechanism; (5) the conversion of ecdysteroids into inactive metabolites remains low and as the hemolymph concentration of active ecdysteroids surges, specific transcriptional changes are induced; finally (6), the ecdysteroid concentration exceeds a threshold that induces molecular and cellular events associated with pupariation (Andres et al., 1993); (7) this is rapidly followed by a cessation of ecdysteroid synthesis and an increase in ecdysteroid metabolism (Pak and Gilbert, 1987; Hurban and Thummel, 1993). Presumably, neural factors also stimulate the pupal ecdysteroid peak of synthesis from the ring gland associated with the onset of metamorphosis (Redfern, 1983; Dai and Gilbert, 1991), as both big and small PTTH do in *Manduca* (O'Brien et al., 1986). The events proposed here represent an idealized situation that, even if correct, operates on a developmental continuum. However, the ability to construct "reporter flies" genetically in order to assess key molecular regulators of prothoracic gland function such as those identified in *Manduca* will expedite this process in the future.

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