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In vitro activation of insect prothoracic glands by the prothoracicotropic hormone

(*Manduca sexta*/neurohormone/ecdysone synthesis/endocrine regulation/metamorphosis)

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ABSTRACT An *in vitro* assay for the prothoracicotropic hormone has been developed that utilizes an ecdysone radioimmunoassay to quantify the increase in the rate of ecdysone synthesis elicited by the neurohormonal activation of the prothoracic glands. The rapidity, reproducibility, and accuracy of the assay were maximized by using one member of a gland pair as the control and the other as the test gland. This was possible because the basal rates of ecdysone synthesis by the members of a gland pair were equivalent. Activation was demonstrated to be dose dependent and specific, with prothoracicotropic hormone activity present only in homogenates of brain. The *in vitro* activation of the prothoracic glands was verified with the *Manduca* bioassay for the prothoracicotropic hormone in which the morphological responses to the hormone were correlated with increased *in vivo* ecdysone titers. These results provide unequivocal evidence that the activation of the prothoracic glands by the prothoracicotropic hormone is direct and suggest that activation represents an increase in a basal rate of ecdysone synthesis.

The endocrine function of the insect brain in the process of metamorphosis was first demonstrated in 1922 (1), but nearly 2 decades passed before it was demonstrated that this function involved the control of molting (2, 3). The initial observations on the role of a cerebral neurohormone in the molting process have since been confirmed by various implantation, extirpation, and ligation experiments (4–6). On the basis of these *in vivo* studies, it has been assumed that this brain hormone, now termed prothoracicotropic hormone (PTTH), acts directly on the prothoracic glands (PGs) to stimulate the synthesis of the steroid prohormone ecdysone (7). Hydroxylation of ecdysone at C-20 to form ecdysterone (20-hydroxyecdysone) then occurs in tissues peripheral to the PGs, and it is ecdysterone that initiates the molting process (7).

Although PTTH was the first hormone to be discovered in insects, of the three major hormones involved in metamorphosis it alone remains to be chemically characterized. A necessary prerequisite to the chemical characterization of this putative peptide hormone is the unequivocal demonstration that PTTH directly activates the PGs. Recent attempts have not been successful due in part to the assay methods used to measure PTTH activity (8–10). Current PTTH assays are biological in nature and indirect by definition, in that they measure secondary morphological responses presumably elicited by a PTTH-stimulated increase in ecdysterone titer (10–12). However, with the availability of a radioimmunoassay for ecdysteroids (13) and culture techniques for maintaining prothoracic glands *in vitro* (14), it is now possible to demonstrate the direct activation of the PGs by PTTH.

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This study details the development of an assay for PTTH that utilizes these two methods and proves that PTTH acts directly on the PGs to stimulate ecdysone synthesis.

MATERIALS AND METHODS

Animals. The tobacco hornworm (*Manduca sexta*) was used for the development of the *in vitro* assay for PTTH activation of the PGs because the endocrine basis of its larval–pupal metamorphosis has been extensively studied (15), especially with respect to the timing of PTTH release and the subsequent activation of the PGs to synthesize ecdysone (16, 17). Larvae were reared on an artificial diet with a 16-hr photophase and were staged as described (18).

Radioimmunoassay. The radioimmunoassay (RIA) used for quantifying ecdysone *in vitro* and ecdysteroids *in vivo* made use of an antibody prepared in collaboration with D. H. S. Horn (7). Ecdysterone was obtained from Rhoto Pharmaceutical (Osaka, Japan) and ecdysone was a gift from D. H. S. Horn. The labeled ligand used was [23,24-^{3}H]ecdysone [57 Ci/mmole (1 Ci = 3.7×10^{10} becquerels)], provided by D. Schooley. The purity of the ecdysteroids was verified by high-pressure liquid chromatography (7). The RIA procedure has been described (13, 17).

PTTH Assay. For the *in vitro* PTTH assay, PGs were extirpated in a lepidopteran saline (19) and then rinsed for 15–30 min in Grace's medium (GIBCO) to remove hemolymph ecdysteroids. After the wash, individual glands were incubated (<12 hr) or cultured (>12 hr) in 0.025-ml standing drops of Grace's medium, with or without brains or tissue homogenates, in multiwell culture plates. One PG from an animal served as the control and the contralateral PG was the test gland. The *in vitro* conditions were simplified from those previously published (14, 17) in that the Grace medium used was not supplemented with macromolecules and the incubations or cultures were carried out at 24°C under high humidity in the dark and in an air atmosphere. The period of *in vitro* maintenance varied from minutes to days, depending upon the particular experimental design. When long-term cultures were necessary, sterile conditions were maintained. To determine a time course of ecdysone synthesis, the same population of glands was used for each time point by replenishing the culture medium with an equal volume of Grace's medium after an aliquot was taken for RIA.

At the termination of a culture or incubation, 0.01 ml of the medium was extracted with 0.3 ml of methanol and the ecdysone content of the extract was then determined by RIA. RIA activity was expressed in nanograms of ecdysone because this

Abbreviations: PTTH, prothoracicotropic hormone; PG, prothoracic gland; A_r, activation ratio; RIA, radioimmunoassay.

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is the only ecdysteroid synthesized by the PGs *in vitro* (14). It was not necessary to assay the medium plus the glands for ecdysone because the PGs release newly synthesized hormone immediately (14). For determination of ecdysteroid levels in hemolymph, 0.01-ml samples were extracted with 0.3 ml of methanol, and two different concentrations of the extract were assayed in duplicate. This assay approach was valid because it has been shown that direct RIA of methanol extracts of culture medium or hemolymph is specific and quantitative for both ecdysone and total ecdysteroids (14, 17).

Because a less complex medium was used for the incubation and culture of the PGs, it was possible that gland activity *in vitro* no longer would reflect *in situ* activity (17) and thus preclude use of the assay. To demonstrate that physiological gland activity persisted for the length of time the PGs would be maintained *in vitro*, the relative amounts of ecdysone synthesized *in vitro* by PGs from larvae throughout the last larval instar were compared with the hemolymph ecdysone titers at the same stages of development. The resulting close temporal and quantitative correlations between changes in *in vitro* rates of synthesis and changing ecdysteroid titers, as were derived with the more complex medium (17), demonstrated that the simplified *in vitro* protocol could be used for investigating PTTH activation of the PGs.

Animal Selection. Day 6 last-instar larval brains were selected as a source of PTTH for demonstrating the activation of the PGs. The choice was based on the supposition that PTTH levels in the brain would be high at that time in preparation for release and the subsequent activation of the PGs at day 7–7½ (16, 17). In addition, the hemolymph titer of PTTH at day 6 presumably would be negligible so that tissues other than brain could serve as controls for studies on the localization of PTTH activity and the specificity of PG activation.

Brains, thoracic ganglia, abdominal ganglia, and muscle of day 6 larvae were extirpated and washed by the same protocol as that for the PGs. For assays of whole tissue, approximately equal masses of the different tissues were precultured for 24 hr and then cocultured with the PGs for 6 or 24 hr. In the standard assay, tissues were homogenized in Grace's medium, heat-treated at 100°C for 2 min, and centrifuged at 10,000 × *g* for 10 min. PGs were then incubated in the resulting supernatant, which is termed "tissue homogenate" for the purpose of this study. To ensure that any apparent activation of the PGs by a tissue homogenate was not due to an ecdysteroid contaminant, the ecdysteroid contents of the different homogenates were quantified by RIA. In all cases the RIA activity present in the homogenates was not significantly above background.

Activation of the PGs by PTTH was demonstrated with a dose–response protocol. Activation was expressed as an activation ratio (A_r) which represents the quantity of ecdysone synthesized by the experimental gland during a given incubation period (E_{pg}) divided by the amount synthesized by the control (contralateral) gland during the same period (C_{pg}). The final A_r for each concentration of brain homogenate assayed was the mean of the A_rs obtained from each of three to six assays.

Bioassay. Under long-day rearing conditions (16-hr photophase), more than 95% of all fourth-instar larvae release PTTH during the second photophase after ecdysis to this instar (gate I). Ligation data revealed that PTTH release had not yet occurred in day 2 fourth-instar larvae weighing less than 0.9 g at 13:00 AZT. Therefore, for the bioassay of PTTH in whole brains and brain homogenates, day 2 fourth-instar larvae weighing just less than 0.9 g (0.835–0.899 g) were anesthetized and neck-ligated and the portion of the head containing the brain was then removed (12). The implantation of brains from day 6 last-instar larvae or the injection of homogenates (5 or 10 μl) was made through an anterior proleg of an anesthetized, ligated larva. Control larvae were injected with an equal volume of distilled water or Grace's medium. Implanted or injected larvae were kept at 25°C and scored at 24-hr intervals over 4 days for either spiracle apolysis or gut purge and dorsal vessel exposure.

RESULTS

Gland Selection. To demonstrate that PTTH directly activates ecdysone biosynthesis by the PGs, a specific, sensitive, and reproducible assay for the hormone is required, and an *in vitro* approach was chosen for its development. In establishing this assay, the initial and most critical feature to be considered was the developmental stage to serve as donor for the PGs. It was reasoned that PGs may be competent to respond to PTTH only at specific developmental stages. Therefore, suitable PGs for the assay might be obtained at a time just prior to PTTH release—i.e., just before the normal time of gland activation. Based on the results of ligation experiments (16) and ecdysone titer determinations (17), days 3 and 6–7 of the last larval instar and day 0–1 of the pupal period represented such times.

To determine on which of these days the PGs would be most responsive to activation by PTTH and therefore be in an optimal state for the assay, *in vitro* time courses of ecdysone synthesis by PGs from these three developmental stages were determined (Fig. 1). The rate of ecdysone synthesis by day 6–7 larval PGs was unexpectedly high (≈18 ng per gland per 6 hr), suggesting that these glands may already be partially activated. This would explain both the rapid rate of ecdysone synthesis and the apparent substrate limitation effect at 12 hr. Therefore, day 6–7 PGs did not appear to be suitable for the PTTH assay. Day 3 larval PGs synthesized ecdysone at a very low rate (≈2 ng per gland per 6 hr), with a plateau of synthesis at approximately 24 hr. These data indicated that day 3 PGs would be ideal for the assay because even slight activation of the glands would be detectable. However, subsequent efforts to activate these PGs failed because the glands apparently contain minimal amounts of substrate. By contrast, day 0 pupal glands exhibited synthesis kinetics that were essentially a compromise between the kinetics of day 3 and of day 6–7 larval glands. The rate of synthesis was relatively low (≈4 ng per gland per 6 hr) but

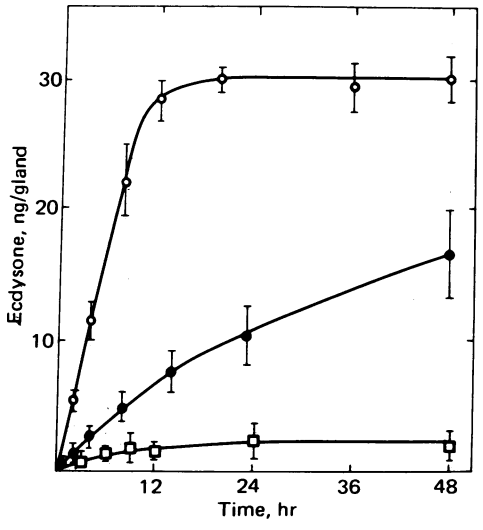

FIG. 1. Time course of ecdysone synthesis by *M. sexta* PGs at day 3 (□) and day 6–7 (○) of the last larval instar and at pupal day 0 (●). Each datum point represents the mean (±SEM) of four separate determinations.

virtually linear up to 48 hr. Approximately 16–20 ng of ecdysone was normally synthesized by these glands during a 48-hr period, demonstrating that a significant quantity of substrate was present and thus eliminating the problem of substrate limitation exhibited by day 3 or day 6–7 larval glands. On this basis, day 0 pupal PGs were selected for the PTTH assay. It is important to note that, regardless of the developmental stage of the donor, PGs always appear to synthesize some ecdysone *in vitro* (17). Thus, the *in vitro* assay for PTTH will monitor changes in the rate of ecdysone synthesis rather than measure the activation of completely inactive glands.

Time of Incubation. The activation of an endocrine gland is usually an immediate biosynthetic event; therefore, a critical feature of the PTTH assay was the incubation time. The duration of the assay must be sufficient to permit observable activation but not so long as to result in substrate depletion of the responding gland. Substrate depletion would result in a decreased rate of synthesis and an apparently low A_r. The time course of ecdysone synthesis by day 0 pupal PGs revealed that a basal rate of synthesis (≈4 ng) could be accurately determined at 6 hr (Fig. 1). Because the glands have sufficient endogenous substrate to synthesize at least 16–20 ng of ecdysone, maximal activation during a 6-hr incubation could theoretically yield an A_r of at least 4–5. A 6-hr assay period would also obviate the need for sterile assay conditions.

Right vs. Left PG. The basal levels of ecdysone biosynthesis by day 0 pupal PGs from different animals varied considerably, generally from 2 to 10 ng per gland per 6 hr. Such variability would preclude the conclusive demonstration of PG activation by PTTH if glands from one animal served as the experimentals and the glands from another as the controls. Fortunately, the PGs are paired and groups of right and left PGs synthesize ecdysone at similar rates for up to 48 hr (Fig. 2), the difference between the rates of ecdysone synthesis by members of a single pair being consistently less than 10%. Therefore, the basal rate of ecdysone synthesis of both members of the pair could be determined precisely with one gland (control) and the degree of activation in the presence of PTTH with the contralateral gland. This experimental design permitted statistical analysis of the resulting data with a two-tailed paired-sample *t* test. This test treats the difference between each control and experimental member of a pair as a single datum point and uses the mean of the differences obtained from a population of similarly treated pairs as the basis of the analysis. This type of analysis was found to improve greatly the statistical significance and reproducibility of the assay.

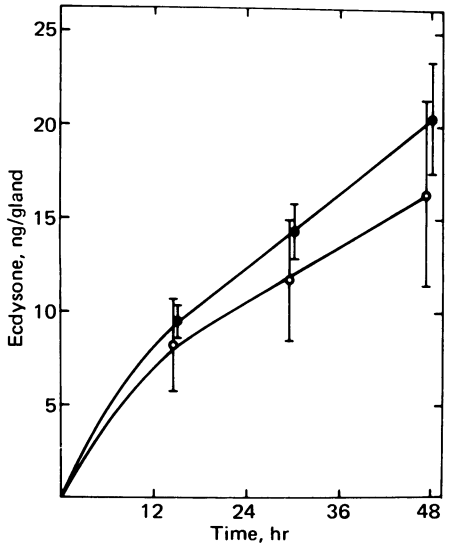

FIG. 2. Time course of ecdysone synthesis in right (○) and left (●) PGs of *M. sexta*. Each datum point is the mean (±SEM) of four separate determinations. The mean of the difference in the rates of synthesis between gland pairs was always less than 10%.

PTTH Activation. *In vitro* activation of PGs was initially attempted by using whole brains, either precultured for 24 hr before incubation with the PGs or coincubated with the glands immediately. A dose–response protocol to demonstrate activation of PGs by the brain revealed that an increase in ecdysone synthesis could be obtained with either approach. However, due to the heterogeneity of the A_r data and the low level of activation (A_r = 2.0–2.5), a dose–response effect was not demonstrable even with an incubation time of 12 hr. Although activation was not consistently obtained with whole brains, the data did indicate the presence of a substance having PTTH activity. It was concluded that dose-dependent activation could not be demonstrated because the whole brain released PTTH at varying and often negligible rates. Perhaps this is the reason that day 6 last-instar larval brains failed to elicit a molting response when implanted into larvae used for the *in vivo* PTTH assay.

To circumvent this possible problem of release, brain homogenates (supernatant fractions) were used to demonstrate a dose–response of activation. PGs cultured with either fresh or frozen day 6 larval brain homogenates synthesized ecdysone in a dose-dependent manner indicative of hormonal activation (Fig. 3). A maximal A_r of 4 was obtained, approximating the A_r predicted on the basis of the time course of ecdysone synthesis by day 0 pupal PGs. The amount of PTTH activity in the day 6 brain homogenate was measured by using the A_{50} value—i.e., brain equivalents necessary to elicit half-maximal activation. This value was 0.16 brain equivalent for both fresh and frozen brains. Heat treatment of the homogenates was used to demonstrate that activation was not caused by nonspecific factors such as proteases released by homogenization. Heat-treated homogenates elicited virtually the same dose–response as did unheated homogenates, with an A_{50} of 0.15 brain equivalent. These data demonstrate the presence of a heat-stable and cold-stable substance, presumably PTTH, that directly effects an increase in the rate of ecdysone biosynthesis by the PGs.

Although PG activation had been demonstrated, it was possible that the rate of synthesis by an activated gland would not be linear during the 6-hr incubation period due to substrate depletion. This would be manifested as a decrease in the A_r as well as lower assay sensitivity and reproducibility. A time course of ecdysone synthesis by PGs maximally activated with 1.0

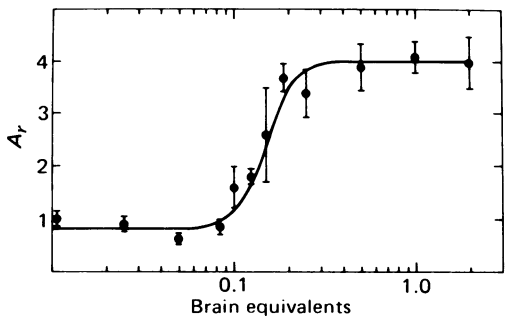

FIG. 3. Dose–response of activation of PGs by homogenates of day 6 last-instar larval *M. sexta* brains. The homogenate was defined as the supernatant after centrifugation of a crude homogenate at 10,000 × *g* for 10 min. Each datum point represents the mean (±SEM) of the A_rs for three to six separate activation assays.

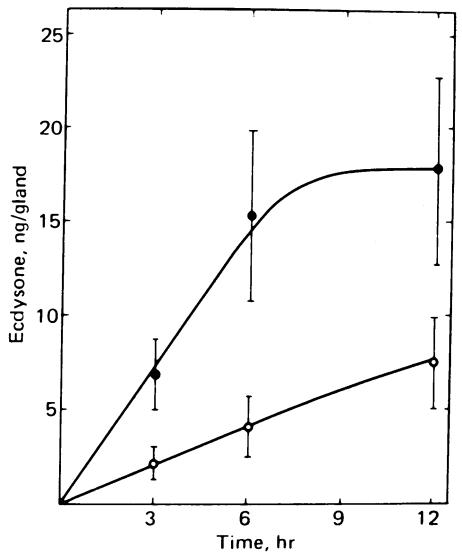

FIG. 4. Time course of ecdysone synthesis for PTTH activated (●) and nonactivated (○) day 0 pupal PGs. Each datum point represents the mean (±SEM) of four separate determinations. The A_rs (±SEM) for each time point derived by a two-tailed paired sample *t* test are 3.7 ± 0.99, 4.5 ± 0.58, and 2.5 ± 0.26 for 3, 6, and 12 hr, respectively.

brain equivalent revealed that synthesis remained linear for approximately 7–8 hr and plateaued at 10–12 hr (Fig. 4). Not only was sufficient substrate present at 6 hr to support linear synthesis but also the greatest A_r was obtained at this time (4.5 ± 0.68; see legend, Fig. 4). Thus, 6 hr was the optimal incubation time for the assay. The rate of ecdysone synthesis by day 0 PGs activated *in vitro* (≈17 ng per gland per 6 hr) approximated that of day 7½–8 glands activated *in vivo* (20–25 ng per gland per 6 hr), suggesting that the degree of activation observed *in vitro* was indeed physiological.

Specificity of Activation. Although the heat stability of the putative PTTH and its dose-dependent activation of the PGs strongly suggested hormonal activation, various nonspecific factors conceivably could have elicited the same effect. Therefore, several corroborative studies were conducted to demonstrate conclusively that activation of the PGs had occurred in response to a prothoracicotropic neurohormone. If day 0 PGs were activated *in vitro* by PTTH, then glands that had been maximally activated *in vivo* should exhibit no change in the rate of ecdysone synthesis when incubated with PTTH *in vitro* ($A_r = 1$). Conversely, if PGs were stimulated nonspecifically *in vitro*, then the glands activated *in vivo* should exhibit a further increase in the rate of ecdysone synthesis in the presence of brain homogenate *in vitro* ($A_r > 1$). When day 7½–8 larval PGs, which are maximally active (17), were incubated *in vitro* with heat-treated day 6 larval brain homogenates, the A_r was ≈1. This supported the view that PTTH was indeed responsible for activation of the day 0 pupal PGs.

If PG activation occurred in response to nonspecific, heat-stable factors released from brains during homogenization, homogenates of other tissues should activate the glands as well. However, if activation were mediated by PTTH, activity would be associated only with brain (11, 12). Comparison of data from dose–response studies of homogenates of brains, subesophageal ganglia, abdominal ganglia, and muscle revealed that PTTH activity was present only in the brain (Fig. 5). In fact, it is localized in one neurosecretory cell in each hemisphere of the brain (unpublished data).

On the basis of the observed dose–response of PG activation

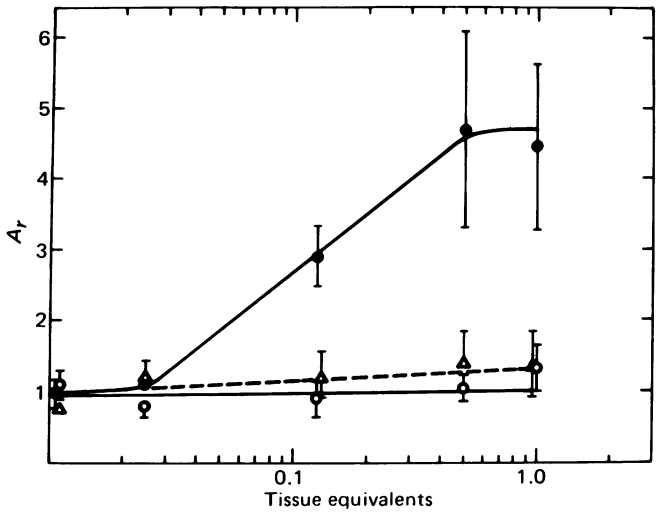

FIG. 5. Tissue specificity of the *in vitro* activation of *M. sexta* PGs. The tissues assayed were brain (●), subesophageal ganglion (○), and abdominal ganglion (△); they were from day 6 of the last-instar larva (muscle gave a response identical to that of ganglia). Each datum point represents the mean (±SEM) of the A_rs for three separate assays.

by brain homogenates, the heat stability of the activating moiety, the insensitivity of PTTH-activated glands to additional activation by PTTH, and the localization of PTTH activity to the brain, it was concluded that the day 0 pupal PGs were activated *in vitro* by PTTH.

Verification of *In Vitro* Activation. *In vitro* demonstration of hormonal activation of an endocrine gland requires verification *in vivo*. Therefore, the *in vivo* assay for PTTH developed for *Manduca* (12) was used to verify the conclusion that the PGs were activated directly by PTTH *in vitro*. Injection of brain homogenates previously determined by the *in vitro* assay to contain PTTH activity elicited one of two types of positive responses from the bioassay animals: (*i*) spiracle apolysis, which occurred within 24 hr of injection and was always followed by a black larval molt; and (*ii*) dorsal vessel exposure, which was observed between 48 and 72 hr after injection and was frequently coincident with purging of the gut. Control larvae exhibited dorsal vessel exposure and gut purge between 90 and 120 hr. The positive assay response in each case was the initiation of a molt, with the difference being the type of molt—i.e.,

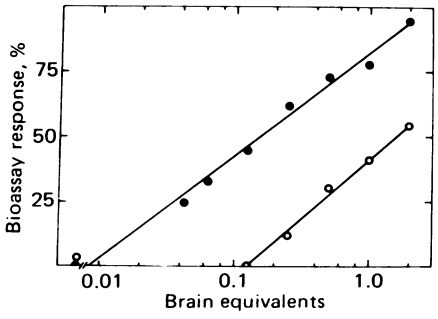

FIG. 6. Dose–response of bioassay activity of the day 6 last-instar larval brain homogenate having *in vitro* PTTH activity (Fig. 3). The two PTTH responses scored for each population assayed were spiracle apolysis (○) and spiracle apolysis, dorsal vessel exposure, and gut purge (●). At <0.2 brain equivalent assayed, the only response observed was dorsal vessel exposure and gut purge. A population of 20–25 animals was used for each brain equivalent assayed.

Table 1. Correlation of ecdysone titer with type of PTTH bioassay response

Response*	Animals, no.	Ecdysteroid titer, ng/ml hemolymph† Range	Mean ± SEM
Control	21	10–42	24 ± 2.0
DVE, GP	13	42–130	83 ± 11
SA	7	200–1200	560 ± 190

* A larval–larval type of molt is indicated by spiracle apolysis (SA), and a larval–pupal molt is indicated by dorsal vessel exposure (DVE) and gut purge (GP).

† Ecdysteroid titer is expressed in ecdysterone equivalents.

larval–larval as evidenced by spiracle apolysis or larval–pupal as typified by dorsal vessel exposure and gut purge. The initiation of both larval and pupal molts by PTTH occurred in a dose-dependent manner (Fig. 6), with lower concentrations of brain homogenate eliciting only dorsal vessel exposure and gut purging. Homogenates of muscle and thoracic ganglia, which had no effect on the activity of the PGs *in vitro*, also failed to elicit a positive response in the *in vivo* assay. Implantations of two brains into ligated larvae did not evoke a response different from that of control larvae, presumably due to the failure of the brains to release PTTH.

If the bioassay response had resulted from an increased ecdysteroid titer elicited by PTTH activation of the PGs, then the titer of ecdysteroids in the hemolymph of the injected larvae should correlate with the bioassay response (17). Thus, animals in which there was no response would have a very low ecdysteroid titer, those exhibiting dorsal vessel exposure and gut purge would have a slightly increased titer, and larvae undergoing spiracle apolysis would have a high titer. RIA quantification of ecdysteroids in methanol extracts of the hemolymph of assay larvae revealed the expected titer correlation (Table 1). These results therefore provide convincing evidence that activation of the PGs *in vitro* is mediated by PTTH.

DISCUSSION

The results of numerous *in vivo* and *in vitro* studies have indicated or demonstrated indirectly that insect PGs are activated by PTTH (8–10, 12, 16, 20). The present investigation provides unequivocal evidence that PTTH has a direct positive effect on PG activity—that is, PTTH elicits a significant increase in the rate of ecdysone synthesis by the glands and this increase occurs in a dose-dependent manner indicative of hormonal activation. The observation that activation is simply an increase in a basal rate of ecdysone synthesis directly contradicts the tenet that PTTH activates biosynthetically inactive glands (15). A developmental study contrasting the basal level of PG activity with gland activity in the presence of PTTH suggests that the larval PGs are always secreting a measurable amount of ecdysone and that modulation of this basal gland activity may be the actual mechanism by which regulation occurs (ref. 17; unpublished data). This concept is also supported by nerve-sectioning studies that indicate the absence of significant neural control of the PGs in two other species of Lepidoptera (21, 22). This type of regulation would also explain the results of several studies suggesting PTTH-independent activation of PGs as well as conclusions that spontaneously active glands can elicit a molting response (23–25).

The types of assays previously used to assess PTTH activity have provided little information about the chemistry, mechanism of action, and site of synthesis of this hormone (11, 15). These assays are biological and generally utilize lepidopteran pupae placed in a state of permanent diapause by brain extirpation. A positive response to PTTH is noted as the initiation of pupal–adult metamorphosis (20). The indirect manner in which the bioassays measure an apparently increased ecdysteroid titer and various other shortcomings (e.g., laborious and lengthy protocols, lack of specificity and sensitivity, and subjective interpretation) have probably been major reasons for the inconsistent results in the literature on PTTH. The development of alternative bioassay such as those using neck-ligated larvae (12, 25) did not eliminate these problems but only reemphasized the need for a sensitive and specific assay for PTTH with which the fundamental endocrinology of this hormone could be critically investigated.

The present study has yielded such an *in vitro* assay. The design of the *in vitro* assay is such that, depending on the conditions (incubation volume, brain donor, etc.), a single gland can detect as little as 0.01 brain equivalent compared to the many assay animal and brain equivalents required to achieve a sensitivity of 0.4 brain equivalents with the *Manduca* bioassay (12). In addition, the entire assay can be carried out in a single day.

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