CELLULAR CHANGES IN THE PROTHORACIC GLANDS OF DIAPAUSING PUPAE OF *MANDUCA SEXTA*

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SUMMARY

Prothoracic glands from diapausing pupae of the tobacco hornworm, *Manduca sexta*, synthesize markedly less ecdysone *in vitro* in response to prothoracicotropic hormone (PTTH) than do glands from non-diapausing pupae. Impaired steroidogenesis is also observed in glands from diapausing animals exposed to agents that enhance ecdysone synthesis in non-diapausing pupal glands by increasing intracellular levels of cAMP (1-methyl-3-isobutylxanthine, dibutyryl cAMP, and the calcium ionophore A23187). In contrast, prothoracic glands from diapausing pupae synthesize significantly more cAMP in response to PTTH and A23187 than do those from non-diapausing pupae. These observations indicate that the PTTH-refractoriness characteristic of prothoracic glands during diapause results from a lesion in the steroidogenic pathway occurring beyond the level of the PTTH receptor-adenylate cyclase system. The diapause condition of the prothoracic glands (reduced ecdysone synthesis accompanied by enhanced cAMP formation) can be mimicked by extirpation of the brain of a non-diapausing pupa. Thus, cellular changes in the prothoracic glands associated with diapause may arise as a result of the absence of some factor produced by the pupal brain (e.g. PTTH).

INTRODUCTION

When reared under a short-day photoperiod, larvae of the tobacco hornworm, *Manduca sexta*, enter diapause shortly after pupation. Recent evidence indicates that the neuroendocrine system plays a primary role in regulating the onset of pupal diapause in this insect (Bowen, Bollenbacher & Gilbert, 1984*a*; Bowen, Saunders, Bollenbacher & Gilbert, 1984*b*). Following exposure to diapause-inducing conditions during larval development, the release of prothoracicotropic hormone (PTTH) from the pupal brain-retrocerebral complex is suppressed. In the absence of circulating PTTH, haemolymph ecdysteroid titres remain below the threshold required to initiate adult development. In addition to altered PTTH release, the target organs for the neuropeptide, the prothoracic glands, are functionally impaired during diapause. Although the glands of diapausing pupae synthesize low basal levels

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of the steroid prohormone, ecdysone, they became increasingly refractory to stimulation by PTTH *in vitro* as diapause progresses (Bowen *et al.* 1984*a*). Thus, while the onset of diapause appears to result from events occurring in the larval brain-retrocerebral complex, changes in the prothoracic glands may contribute to the maintenance of chronically reduced ecdysteroid levels.

In the present study, the phenomenon of prothoracic gland refractoriness and its relationship to diapause are explored. Specifically, the following questions are addressed. (1) Is steroidogenic refractoriness a result of atrophy of the glands, i.e. a manifestation of a general reduction in synthetic processes, or alternatively, is ecdysone synthesis altered at specific cellular locations? (2) Does refractoriness arise as an autonomous response of the prothoracic glands to environmental cues, or, as recent data suggest (M. F. Bowen, W. E. Bollenbacher & L. I. Gilbert, un-published), is it a consequence of a reduction in the activity of the neuroendocrine system? Recent studies of the cellular basis of PTTH-stimulated ecdysone synthesis in non-diapausing pupae indicate that the neurohormone acts to stimulate cyclic AMP (cAMP) synthesis in a calcium-dependent manner, with cAMP in turn enhancing the rate of ecdysone synthesis (Smith, Gilbert & Bollenbacher, 1984, 1985). Using this information, the present study probes the cellular basis of PTTH-refractoriness in the prothoracic glands of diapausing pupae.

MATERIALS AND METHODS

Animals

Eggs from a strain of *Manduca* with a high incidence of diapause were supplied by Dr James Buckner (USDA, Fargo, ND, U.S.A.). Diapausing pupae were obtained by rearing larvae under a short-day photoperiod (LD 12:12) at 25 °C on an artificial diet (Bell & Joachim, 1976). Under this regime, approximately 60 % of the pupae entered diapause. Non-diapausing pupae were obtained from our 16-year-old colony, and were reared identically with the exception of photoperiod (LD 16:8). None of the long-day-reared animals entered diapause. Ten-day-old diapausing pupae (D_{10}) were selected as the experimental group as: (a) diapausing pupae at this time are easily distinguished from short-day-reared, non-diapausing cohorts by the absence of pharate-adult characters, such as dissociated fat body in the haemolymph, and eye and wing pad development, and (b) while reduced responsiveness to PTTH can be observed as early as the second day of diapause, refractoriness is maximal in glands from D_{10} animals (Bowen *et al.* 1984*a*). Newly-emerged, non-diapausing pupae (ND_0) were chosen as controls, as they represent a stage prior to the initiation of a metamorphic increase in ecdysone synthesis. That is, they comprise a non-dia-pausing pupal stage analogous to the pre-active condition of the diapausing animals. Once enhanced ecdysone synthesis is initiated, the prothoracic glands undergo pharate-adult development changes (including the onset of cell death) that render them inappropriate as pupal controls.

Brainless pupae were obtained by placing ND_0 animals on ice, removing a small square of cuticle from the dorsum of the head, and extirpating the brain. The cuticle

was replaced and the edges sealed with melted Tackiwax (Cenco, Chicago, IL). Such pupae remain alive, in a state of arrested development, for several months.

Chemicals, radionuclides and antibody

The calcium ionophore A23187 was purchased from Calbiochem (San Diego, CA). A23187 was prepared as a stock solution in dimethylsulphoxide (Fisher, Pittsburgh, PA) and diluted in Grace's culture medium (GIBCO, Grand Island, NY) just prior to use. Dibutyryl cyclic AMP (dbcAMP) and 1-methyl-3-isobutyl-xanthine (MIX) (Sigma, St Louis, MO) stock solutions were prepared in Grace's culture medium. [^{3}H]Ecdysone (specific activity $60 \, \text{Ci mmol}^{-1}$) and [^{3}H]adenine (specific activity $29 \, \text{Ci mmol}^{-1}$) were obtained from New England Nuclear Corp. (Boston, MA) and ICN (Irvine, CA), respectively. The ecdysone antibody used was produced in rabbits against an ecdysone-22-succinyl thryoglobulin synthesized by Dr D. H. S. Horn (C.S.I.R.O., Canberra, Australia) and characterized as described previously (Gilbert, Goodman & Bollenbacher, 1977). All other reagents were obtained from Sigma.

PTTH

Of the two prothoracicotropic peptides found in the pupal brain of *Manduca sexta* ($M_r = 7000$ and $28\,500$; Bollenbacher *et al.* 1984), the larger form was used in this study. The neurohormone was purified approximately 200-fold by gel-filtration and ion-exchange chromatography as previously described (Bollenbacher *et al.* 1984). One unit (U) of PTTH, as defined by Bollenbacher, O'Brien, Katahiri & Gilbert (1983), is the amount of prothoracicotropic activity present in a day-1 pupal brain. This quantity is functionally equivalent to the amount of hormone required to half-maximally stimulate $16.7 \, \text{ND}_0$ pupal prothoracic glands using the *in vitro* assay described below.

In vitro *assay*

Pairs of prothoracic glands were dissected in lepidopteran saline and maintained for up to 1 h in Grace's medium prior to use. Assays were conducted using randomly distributed individual glands, each gland being placed in $25 \, \mu\text{l}$ of Grace's medium containing the experimental or control treatment. Incubations were carried out at $25\,°\text{C}$ for the times indicated, and duplicate $10\text{-}\mu\text{l}$ samples of the culture medium were assayed for ecdysone directly by radioimmunoassay (RIA). Details of the dissection, incubation, RIA procedures and data analysis by computer were the same as those described previously (Bollenbacher *et al.* 1983; Warren, Smith & Gilbert, 1984). None of the agents employed (MIX, dbcAMP, PTTH, A23187) interfered with the RIA.

Measurement of cAMP

The accumulation of cAMP in prothoracic glands was assayed by the method of Shimizu, Daly & Creveling (1969), as modified by Meeker & Harden (1982). Glands

were pre-incubated individually in $25 \mu l$ of Grace's medium containing $1 \mu Ci$ [^{3}H]adenine at 25°C for 90 min, rinsed in fresh Grace's medium, and placed in $25 \mu l$ of medium containing PTTH or other agents of interest. Following incubation at 25°C for 20 min, a period of time previously found to coincide with enhanced levels of both [^{3}H]cAMP and steroidogenesis (Smith *et al.* 1984), glands were placed in $200 \mu l$ ice-cold 25 % trichloroacetic acid ('TCA) and maintained at 4°C overnight. Chromatographic separation of [^{3}H]cAMP from [^{3}H]ATP in the TCA extract was accomplished by the method of Salomon, Londos & Rodbell (1974), as described previously (Smith *et al.* 1984). Accumulation of cAMP in the prothoracic glands was expressed as a percentage of conversion of [^{3}H]ATP to [^{3}H]cAMP or $\{[^3H]cAMP/[^3H]ATP+[^3H]cAMP\} \times 100$. Results obtained with this method correspond to those obtained with a cAMP RIA (Smith *et al.* 1984).

RESULTS

Ecdysone synthesis by ND_0 and D_{10} prothoracic glands

Previous investigations indicated that prothoracic glands removed from 10-day-old diapausing pupae (D_{10}) show an almost total absence of response *in vitro* to a single, maximal, dose of pupal brain (PTTH) extract (Bowen *et al.* 1984*a*). In the present study, the responses of glands from D_{10} animals to a range of doses of partially purified big PTTH were examined. Following a 2-h period of incubation, D_{10} prothoracic glands were essentially unresponsive to all doses of PTTH employed (Fig. 1A). Although basal levels of ecdysone synthesis were slightly, but significantly, reduced in the D_{10} glands, as compared to non-diapausing day 0 (ND_0) controls ($P < 0.02$, Student's *t*-test), this difference was not sufficient to account for the reduced level of synthesis observed in response to PTTH (increased stimulation at 0.1 U PTTH: D_{10}, ×1·28; ND_0, ×4·24). The protein contents of prothoracic glands of D_{10} and ND_0 pupae were not statistically different ($ND_0 = 21 \pm 1 \mu g$ gland^{-1}, $D_{10} = 18 \pm 2 \mu g$ gland^{-1}). Reduced responsiveness of the D_{10} glands appeared to result from a reduction in the maximal levels of ecdysone synthesis, rather than a reduced sensitivity to PTTH which would have shifted the dose-response curve to the right. It is unlikely that the D_{10} glands would have produced much more ecdysone if the period of incubation had been extended, as PTTH-stimulated ecdysone synthesis is markedly lower in prothoracic glands from diapausing pupae than in glands from ND_0 controls for at least 6 h (Bowen *et al.* 1984*a*).

Knowing that cAMP and calcium are involved in the response of the prothoracic glands to PTTH (Smith *et al.* 1984, 1985), the following agents were examined for their effects on steroidogenesis in glands from diapausing and non-diapausing pupae: (1) the cAMP analogue, dbcAMP; (2) MIX, a potent phosphodiesterase inhibitor; and (3) the calcium ionophore A23187. Each of these agents stimulates ecdysone synthesis up to five-fold in ND_0 prothoracic glands, and, although acting at different cellular sites, exerts its steroidogenic effect by increasing intracellular levels of cAMP (Smith *et al.* 1984, 1985).

As shown in Fig. 1B–D, D_{10} prothoracic glands exhibited a reduced response to ,ach agent. In every case, the reduction in ecdysone synthesis as compared to ND_0 controls occurred as a result of an impaired maximal response. These findings indicate that the loss of sensitivity to PTTH observed in the prothoracic glands of diapausing pupae may result, at least in part, from alterations in hormone-stimulated ecdysone synthesis beyond the level of cAMP formation. To determine whether such alterations indicated a general reduction in synthetic activity by the glands or a specific steroidogenic lesion, the ability of PTTH to stimulate cAMP formation was assessed.

Accumulation of cAMP by D_{10} and ND_0 prothoracic glands

We tested whether cAMP formation was different in D_{10} and ND_0 prothoracic glands. Since pupal prothoracic glands accumulate little cAMP in the absence of a phosphodiesterase inhibitor (Smith *et al.* 1984), glands were incubated in the
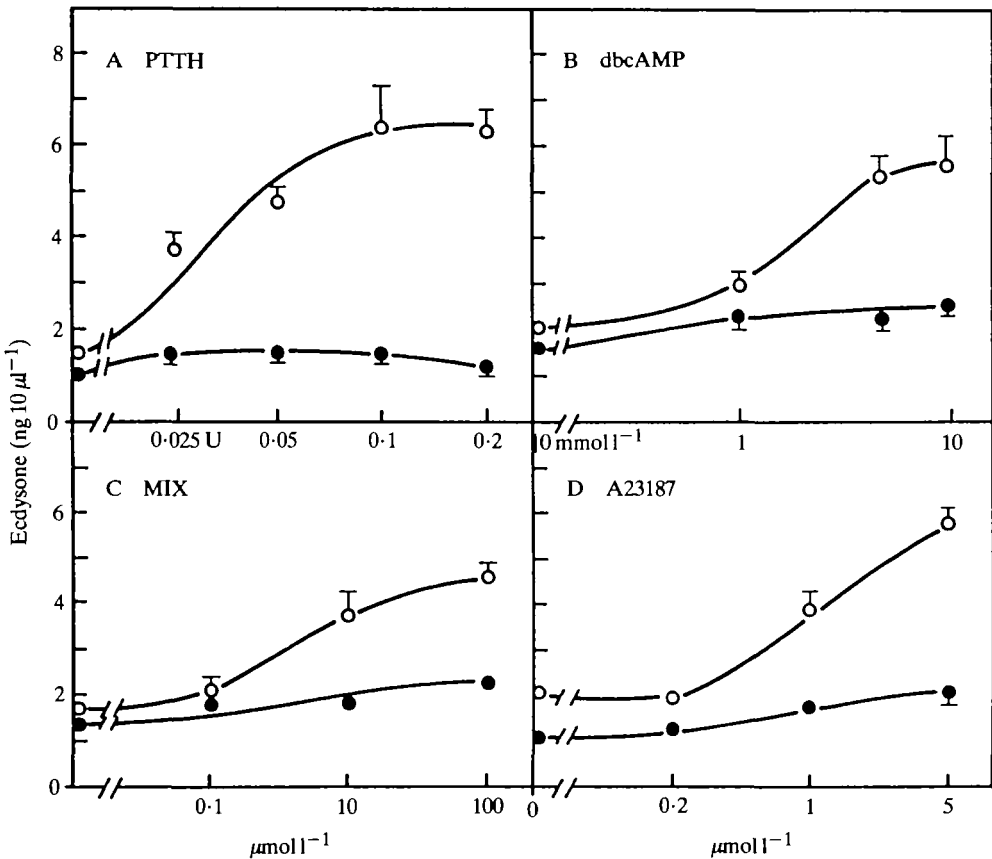

Fig. 1. *In vitro* ecdysone synthesis by D_{10} (filled circles) and ND_0 (open circles) prothoracic glands, in response to: (A) partially purified big PTTH, (B) dibutyryl cAMP (dbcAMP), (C) 1-methyl-3-isobutylxanthine (MIX), (D) calcium ionophore (A23187). Glands were challenged with indicated concentrations of each agent for 2 h. Each point represents the mean ± S.E.M. of 10–12 glands.
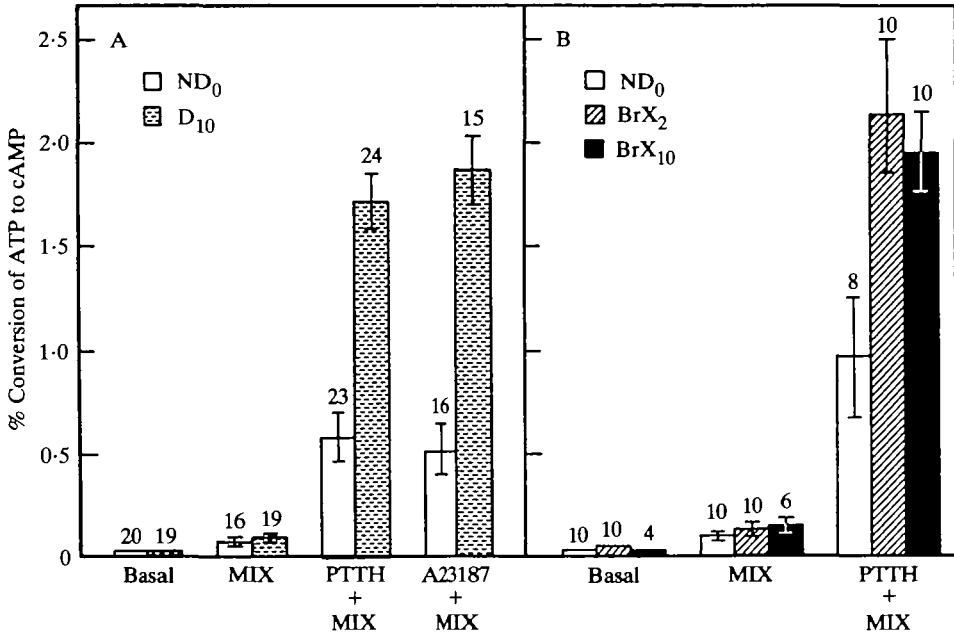
Fig. 2. *In vitro* accumulation of cAMP by prothoracic glands during a 20-min exposure to: Grace's medium (Basal); 5×10^{-5} mol l^{-1} 1-methyl-3-isobutylxanthine (MIX); 0·1 U PTTH+MIX; or 5×10^{-5} mol l^{-1} A23187+MIX. (A) Responses of glands from ND_0 pupae (white bars) *vs* D_{10} pupae (stippled bars). (B) Responses of glands from ND_0 pupae (open bars) *vs* ND_0 pupae 2 days (BrX$_2$, cross-hatched bars) or 10 days (BrX$_{10}$, filled bars) after brain removal. Bars represent mean ± S.E.M.; sample size indicated above bars.

presence of 5×10^{-5} mol l^{-1} MIX (Fig. 2A). The data reveal that low basal levels of cAMP accumulation were slightly but significantly enhanced ($P < 0.01$) by MIX alone, approximately three-fold in both D_{10} and ND_0 prothoracic glands (Fig. 2A). In ND_0 glands, the addition of MIX plus 0·1 U PTTH, a dose previously shown to elicit maximal stimulation of ecdysone synthesis, led to an approximately 10-fold stimulation of cAMP accumulation as compared to MIX alone. This level of cAMP accumulation was similar to that seen in previous experiments with non-diapausing pupal prothoracic glands (Smith *et al.* 1985). Surprisingly, glands removed from D_{10} pupae accumulated significantly *more* cAMP in response to PTTH (17-fold over MIX alone) than did those from non-diapausing pupae ($P < 0.01$). Similar results were obtained using MIX plus the calcium ionophore, A23187, which was previously shown to stimulate cAMP formation in prothoracic glands from non-diapausing pupae at levels similar to those elicited by PTTH (Smith *et al.* 1985). The addition of A23187 led to an enhancement of cAMP formation in D_{10} prothoracic glands that was significantly greater than that observed in ND_0 glands ($P < 0.01$).

Ecdysone synthesis during the same 20-min period is shown in Table 1. Steroidogenesis was clearly reduced in the D_{10} glands, corroborating the results shown in Fig. 1. In the ND_0 glands, similar levels of ecdysone synthesis were elicited by MIX, PTTH+MIX, or A23187+MIX, despite markedly different levels of

cAMP accumulation. This result indicates that while cAMP synthesis is likely to play an important role in pupal steroidogenesis, the dramatic *accumulation* of cAMP obtained with PTTH or ionophore in the presence of MIX is not essential for glandular activation. Such an observation is in keeping with the ability of PTTH alone to stimulate ecdysone synthesis in ND_0 glands in the absence of a detectable increase in cAMP accumulation (Smith *et al*. 1984).

Of particular relevance to the present study, the results shown in Fig. 2 indicate that prothoracic glands from diapausing pupae are indeed capable of synthesizing cAMP in response to both receptor-mediated (PTTH) and non-receptor-mediated (A23187) secretagogues. Thus, refractoriness is unlikely to be a consequence of a general reduction in anabolic activity. Further, the glands from diapausing animals appear to be hypersensitive to both PTTH and ionophore as far as cAMP formation is concerned.

Effects of brain removal on prothoracic gland function

The above data indicated that the refractoriness observed during diapause arose from an inability of the prothoracic glands to synthesize ecdysone in response to increased intracellular levels of cAMP. Of equal interest, however, was the relationship of this condition to the principal cause of pupal diapause in *Manduca*, the photoperiodically curtailed release of PTTH (Bowen *et al*. 1983, 1984). Conceivably, refractoriness of the prothoracic glands during diapause could be a consequence of the absence of PTTH release itself, and/or the absence of another cerebral neuropeptide necessary for sustained responsivity of the glands after pupation. To investigate these possibilities, brains were removed from non-diapausing day-0 pupae and the synthetic events in the prothoracic glands examined.

The ability of prothoracic glands from brainless pupae to synthesize ecdysone is illustrated in Fig. 3. When challenged with PTTH (0·1 U) 2 or 10 days after brain removal, these prothoracic glands showed a significant ($P < 0.01$) reduction in ecdysone synthesis. In addition, prothoracic glands from brainless pupae were significantly less responsive to a 10 mmol l^{-1} dose of dbcAMP ($P < 0.01$). Further, intracellular levels of cAMP following a 20-min exposure to PTTH+MIX were enhanced significantly in prothoracic glands from brainless pupae, as compared to

Table 1. *Ecdysone synthesis by prothoracic glands from diapausing and non-diapausing pupae concomitant with cyclic AMP production shown in Fig. 2A*

Treatment	Ecdysone synthesis (ng gland^{-1} 20 min^{-1})	
	ND_0	D_{10}
None (basal synthesis)	0·38 ± 0·05*	0·15 ± 0·03
MIX (10^{-5} mol l^{-1})	0·83 ± 0·23	0·18 ± 0·03
MIX (10^{-5} mol l^{-1})+PTTH (0·1 U)	0·70 ± 0·08	0·20 ± 0·03
MIX (10^{-5} mol l^{-1})+A23187 (10^{-5} mol l^{-1})	0·82 ± 0·05	0·20 ± 0·03

* Mean ± S.E.M., sample sizes as in Fig. 2A .
MIX, 1-methyl-3-isobutylxanthine.

intact ND_0 controls ($P < 0.01$, Fig. 2B). These results indicate that, as in the case of prothoracic glands from diapausing pupae, reduced responsiveness to PTTH following brain removal occurs at a point beyond cAMP formation. The similarity at the cellular level between prothoracic glands removed from diapausing and de-brained pupae strongly suggests that the refractory states of the two groups are functionally similar, and that the condition of the glands from diapausing pupae results from the absence of some factor originating in the pupal brain.

<center>DISCUSSION</center>

The present results indicate that prothoracic glands removed from D_{10} pupae of *Manduca sexta* synthesize less ecdysone in response to PTTH than do glands from ND_0 animals. Prothoracic glands from diapausing pupae are similarly unresponsive to agents that act by increasing intracellular levels of cAMP (MIX, dbcAMP and A23187). Paradoxically, D_{10} prothoracic glands synthesize more cAMP than ND_0 glands in response to PTTH and A23187. These data argue strongly for a post-receptor lesion in the prothoracic glands as the basis for cellular refractoriness to PTTH during diapause.
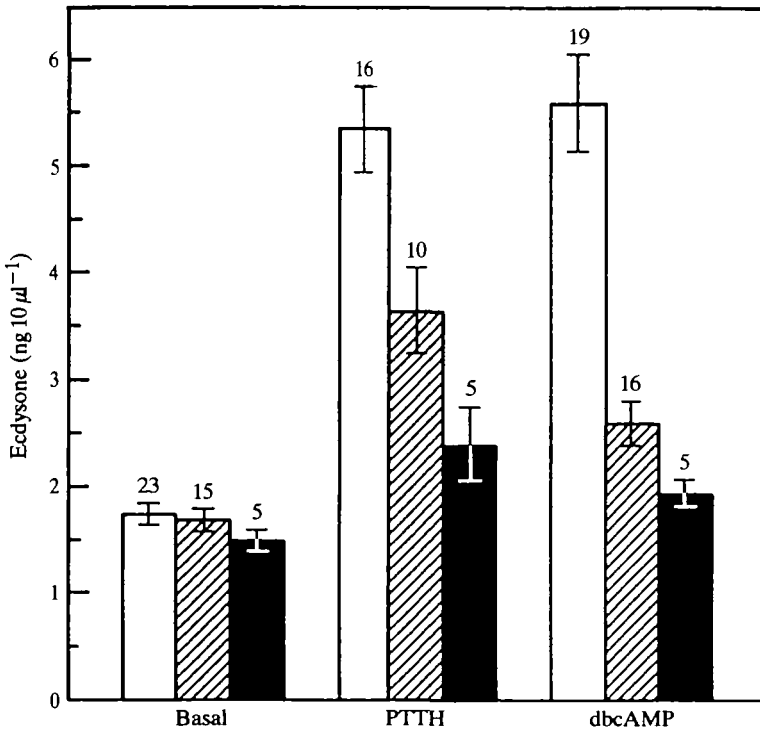

Fig. 3. *In vitro* ecdysone synthesis by glands from ND_0 pupae (open bars), from ND_0 pupae 2 days (cross-hatched bars) or 10 days (filled bars) after brain removal. Glands were challenged for 2 h with Grace's medium (Basal), PTTH (0·1 U) or dibutyryl cAMP (10 mmol l^{-1}). Bars represent mean ± S.E.M.; samples sizes indicated above bars.

Removal of the brain mimics the diapause condition of the prothoracic glands with regard to both ecdysone synthesis and cAMP formation. Thus, the present study corroborates and strengthens the suggestion that changes in the prothoracic glands associated with diapause may be a consequence of the absence of an agent or agents produced by the pupal brain (Bowen *et al.* 1984*a*, and unpublished information). The possibility exists that PTTH itself may be the responsible factor. In rats, removal of adrenocorticotropin (ACTH) through hypophysectomy leads to adrenal atrophy, preceded or accompanied by reduced corticosteroid synthesis in response to ACTH (Grahame-Smith, Butcher, Ney & Sutherland, 1967; Sayers & Beall, 1972; Holmes, Neto & Field, 1980). Associated with a reduction in corticosteroid production, there is an increase in ACTH-stimulated cAMP accumulation (Sayers & Beall, 1972; Holmes *et al.* 1980) and in [^{125}I]ACTH binding to the adrenal glands (Golder & Boyns, 1972), suggesting an increase in either the affinity or number of ACTH receptors. Similar changes have been observed in the rat thyroid following removal of thyrotropin (TSH) *via* hypophysectomy (Zakarija & McKenzie, 1977; Friedman, Land, Levasseur & Burke, 1979, Holmes & Field, 1980). Reduced responsiveness to TSH may result from a decrease in cAMP-dependent protein kinase activity, as indicated by an approximately 50% decrease in the activity of this enzyme in hypophysectomized animals (Friedman *et al.* 1979; Combest & Russell, 1983). Studies on mammalian systems suggest that peptides which exert a trophic effect on their target tissues (maintenance of tissue weight and hormonal responsiveness) appear to do so *via* maintenance of enzyme activity beyond adenylate cyclase. In the absence of the trophic peptide, the activity of such enzymes is reduced, with a concomitant increase in peptide-stimulated second messenger production.

The present data are in remarkable accordance with the above findings for mammalian endocrine systems. We suggest that an absence of low (trophic) levels of PTTH during the first few days of pupal life, as a result of either larval exposure to short-day photoperiod (Bowen *et al.* 1984*a,b*) or brain extirpation, leads to decreased steroidogenic capacity accompanied by enhanced cAMP formation. The cellular basis of refractoriness to PTTH by the prothoracic glands occurs beyond cAMP formation, possibly at the level of cAMP-dependent protein kinase activity. Initial studies of protein kinase activity in the prothoracic glands indicate a 30–40% reduction in the cellular levels of cAMP-dependent protein kinase activity in glands from D_{10} as compared to ND_0 pupae (W. A. Smith, W. L. Combest & L. I. Gilbert, unpublished observation). However, although the results of the present study suggest a trophic role for PTTH in the regulation of prothoracic gland function, confirmation of this will require further experimentation. In particular, it will be important to determine whether administration of PTTH to day-0, de-brained pupae *via* brain implantation or infusion of purified hormone is capable of preventing a subsequent loss in steroidogenic capacity.

The cellular basis of increased cAMP accumulation by the prothoracic glands of diapausing animals is also conjectural at present. The presence of MIX during the incubation of both D_{10} and ND_0 glands argues against reduced phosphodiesterase activity in the D_{10} group as the cause of enhanced levels of cAMP. It thus appears

that cAMP synthesis is altered during diapause. Such a change may occur through an increase in the number of PTTH receptors or their affinity for the neurohormone, through enhanced interaction of the PTTH receptor with the catalytic unit of adenylate cyclase, or through an increase in the activity of the catalytic unit of the enzyme. The ability of the calcium ionophore to generate cAMP levels in diapausing glands equivalent to those generated by PTTH suggests that a change in the receptor cannot explain completely the enhanced capacity for cAMP production during diapause. This leaves the regulatory and/or catalytic unit of the adenylate cyclase as likely sites at which cAMP synthesis may be altered.

Since the prothoracic glands are refractory during diapause, the question arises as to how this condition is normally reversed to permit the resumption of development. Environmental cues, most probably an increase in temperature, must in some manner stimulate an increase in ecdysone synthesis, either by affecting the prothoracic glands directly or by stimulating the production of trophic factors (e.g. PTTH) that ultimately stimulate steroidogenesis. In the bollworm, *Heliothis*, PTTH is released in diapausing animals; however, the prothoracic glands remain inactive at low temperatures and become active as the temperature increases (*Heliothis zea*, Meola & Adkisson, 1977; *Heliothis punctiger*, Browning, 1981). Recent evidence indicates that, rather than responding directly to increased temperature, the glands are activated by a temperature-sensitive increase in levels of a humoral factor required for ecdysone synthesis (Meola & Gray, 1984). Similarly, in most insect species, a direct influence of environmental cues on prothoracic gland function has not been clearly demonstrated. However, activation of the prothoracic glands of diapausing pupae in a manner independent of the brain, particularly in response to temperature change and wounding, is well-documented (*Papilio*, Numata & Hidaka, 1984; *Antheraea*, McDaniel & Berry, 1967; McDaniel, Johnson, Saun & Berry, 1976; *Manduca*, Judy, 1972).

At present, the favoured mechanism for the restoration of prothoracic gland activity in *Manduca* is the resumption of PTTH release. Implantation of brains into brainless, non-diapausing pupae does re-establish synthetic competence of the prothoracic glands by the ninth day after implantation (M. F. Bowen, W. E. Bollenbacher & L. I. Gilbert, unpublished observations). Because the glands remain capable of producing cAMP in response to PTTH, the possibility exists that a chronic enhancement of intracellular levels of cAMP, as opposed to an acute increase, may be the mechanism by which synthetic capacity is eventually restored. In the rat adrenal glands, refractoriness arising from hypophysectomy can be reversed, at least in part, by prolonged (5-day) administration of dbcAMP (Ney, 1969). An additional mechanism by which ecdysone synthesis may be re-initiated is through the action of an agent that does not act *via* cAMP. One such agent, produced by the *Manduca* fat body in response to juvenile hormone (Gruetzmacher, Gilbert & Bollenbacher, 1984*a*; Gruetzmacher *et al.* 1984*b*) and apparently identical to an agent found in the haemolymph of developing *Manduca* larvae (Watson *et al.* 1985), stimulates ecdysone synthesis in both larval and non-diapausing pupal prothoracic glands without enhancing cAMP formation (Smith, Watson, Gilbert &

Bollenbacher, 1986). This compound, a 30 000 Da protein, is currently hypothesized to act as a carrier molecule for, or to enhance the transport of, a sterol substrate necessary for ecdysone synthesis (Watson *et al.* 1985). Although the cellular basis for the termination of pupal diapause is not known with certainty, the current study denotes the sites at which environmental or systemic cues may restore prothoracic gland function.

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