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Acceleration of Pupal-Adult Development by Fenoxycarb in the Silkworm, *Bombyx mori*

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ABSTRACT—Treatment by fenoxycarb (> 0.1 µg/animal) during a short developmental period in the pharate pupal stage of the silkworm, *Bombyx mori*, induced alterations in the normal pupal-adult development. All fenoxycarb-treated animals showed accelerated development and precocious adult cuticle deposition very early in the pupal stage. Various internal and external organs of the developing adults underwent abnormal differentiation and were extensively malformed. Consequently, pupal-adult development and adult eclosion behaviour were disturbed. Similar effects were induced by injections of high doses (≥ 4 µg/animal) of 20-hydroxyecdysone (20E) only around the time of pupal ecdysis. Treatment with 1 µg of fenoxycarb in the pharate pupal stage did not affect haemolymph ecdysteroid titers early in the pupal stage. Our results, therefore, suggest that fenoxycarb, a potent juvenile hormone mimic, can imitate the developmental effects of excess 20E and behave as an ecdysteroid mimic after its application during the short developmental period of the pharate pupal stage of *B. mori*. These results shed light on the timely and coordinated increase of endogenous juvenile hormone titer before pupal eclosion.

INTRODUCTION

Investigations in the cecropia silkworm, *Hyalophora cecropia* (Williams, 1961) and the tobacco hornworm, *Manduca sexta* (Kiguchi and Riddiford, 1978) revealed that the increase in endogenous juvenile hormone (JH) titer during the pharate pupal stage is necessary to prevent precocious adult development of imaginal structures. Application of excess JH or JH analogues (JHAs) into lepidopteran pupae prevents normal adult development and results in various gradations of pupal-adult intermediates in a dose-dependent manner (Sehnal, 1983). Treatment with JH or JHAs induces morphological alterations in the adult cuticle, reproductive organs, wings, and compound eyes (Williams, 1961; Sehnal, 1983). Furthermore, studies by Williams (1968) and Judy and Gilbert (1970b) showed that excess amounts of moulting hormone cause pupae to differentiate into animals resembling the JH-induced pupal-adult intermediates. Treatment of *M. sexta* (Truman et al., 1983; Schwartz et al., 1983) and the mealworm beetle, *Tenebrio molitor* (Slama, 1980) with ecdysteroids early in the pupal stage resulted in acceleration of adult differentiation, while administration in the pharate adult stage delayed the developmental program and adult eclosion.

In the silkworm, *Bombyx mori*, the JH titer increases late in the 5th instar and reaches a peak during the pharate pupal stage (Akai and Rembold, 1989; Niimi and Sakurai, 1997) and it is eliminated very soon after pupal ecdisys. This increase in the JH titer coincides with the major increase in the ecdysteroid titer at the pharate pupal stage of this insect, driven by the increased secretory activity of the prothoracic glands (Dedos and Fugo, 1996). It is generally assumed that this increase of the JH titer is necessary to prevent precocious adult development of imaginal structures as has been suggested for *H. cecropia* (Williams, 1961) and *M. sexta* (Kiguchi and Riddiford, 1978). Thus the current belief is that the coordinated increase in the JH titer in the pharate pupal stage antagonizes the action of ecdysteroids. This led us to assume that excess JH will check or delay the timing of pupal-adult differentiation if applied at a time when the endogenous JH titer is increasing (Akai and Rembold, 1989; Niimi and Sakurai, 1997).

To test hypothesis, we have investigated the effects of high doses of fenoxycarb, a potent JH mimic (Dorn et al., 1981; Grenier and Grenier, 1993), on the pupal-adult development. Our main focus was on the developmental morphology of the treated animals. We expected that fenoxycarb treatment in the pharate pupal stage would check the progress of the pupal-adult development. On the contrary, we observed that an acceleration of the pupal-adult development with extensive morphological abnormalities in *B. mori*.
MATERIALS AND METHODS

Animals

Bombyx mori larvae of the hybrid J106xDAIZO were used in all experiments. The insects were reared on mulberry leaves under a 12:12 L:D photoperiod at 25±1°C with 60% relative humidity (Dedos and Fugo, 1996). All the larvae initiated wandering behaviour about 62 hr before pupal ecysis. Pupal ecysis occurred about 208 hr after the 4th larval ecysis. In this study, we defined the pupal stage as the developmental period between pupal ecysis and adult eclosion. This developmental period lasted about 204 hr.

Reagents

Fenoxycarb (ethyl [2-(p-phenoxy-phenoxy)ethyl]carbamate; C_{17}H_{19}O_{4}N MW:301.3) of analytical grade (99% purity) was a gift from Ciba-Geigy (Basel, Switzerland). All treatments were done by injection of various doses of fenoxycarb in 50% acetone in distilled water. Control animals received 5 µl of 50% acetone in distilled water. Recombinant PTTH (rPTTH) (Ishibashi et al., 1994) was a gift from Dr. Hiroshi Kataoka (The University of Tokyo). It was dissolved in aliquots of Grace’s medium (GIBCO-BRL, Grand island, NY, USA) and stored at −20°C. 20-hydroxyecdysone (20E) was purchased from Sigma (St. Louis, MO, USA).

Surgical manipulations

To determine the developmental profiles of each stage, animals were dissected and various developmental traits were recorded. ovaries from 3 or 4 animals were dissected, blotted on filter paper and immediately weighed. Haemolymph was collected in chilled Eppendorf tubes, containing a few crystals of phenylthiourea, and subsequently stored at −20°C until assayed.

In vitro prothoracic gland assay

Prothoracic glands were dissected from pupae in sterile saline solution (0.85% NaCl). The glands were pre-incubated in Grace’s medium for 15–30 min. A paired gland design was used in all the experiments. One gland of the pair was incubated in 20 µl of Grace’s medium containing 1 ng rPTTH; the other gland of the pair was incubated in 20 µl Grace’s medium alone and served as the control. Incubations were carried out at 25±1°C in high humidity in 96-well microplates (Iwaki, Tokyo, Japan) for 6 hr. At the end of the incubation period, the medium was removed and aliquots were subjected to radioimmunoassay for the quantification of the secreted ecdysteroid.

Radioimmunoassay

The amount of ecdysteroid in the haemolymph or in the incubation medium was quantified by radioimmunoassay (RIA) as described previously (Dedos and Fugo, 1996). Radiolabeled ecdysone, [23,24-³H]ecdysone (sp. act. 53 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA, USA).

Statistical analyses

Statistical significance of the results was determined by analysis of variance (ANOVA) or Student’s t-test. For some experiments, ANOVA was followed by Tukey multiple comparisons test. The statistical analyses were done with computer software (GraphPad Prism™ 2.0).

RESULTS

Developmental acceleration of pupal-adult development after the application of fenoxycarb in the pharate pupal stage

In preliminary experiments, we observed that treatment with doses of fenoxycarb higher than 0.1 µg and up to 5 µg/animal at 58, 34 or 10 hr before pupal ecysis, produced pupae that displayed abnormal developmental characteristics. These animals underwent pupal-adult differentiation at a greatly accelerated developmental pace compared to the untreated pupae.

To better understand the mode of action of fenoxycarb at such doses, we investigated its effect on the morphological characteristics of the animals during pupal-adult development. Fenoxycarb (1 µg/animal) was injected 34 hr before pupal ecysis and various developmental traits were recorded until shortly before the adult eclosion of control animals (204 hr of the pupal stage). These observations are shown in Table 1.

The physiological stages of normal adult development appeared earlier in the treated animals (Table 1). Precocious deposition of the cuticle occurred by 60 hr of the pupal stage in the treated animals. Injection of fenoxycarb (1 µg/animal) induced a precocious and abnormal eye pigmentation as early

| Table 1. Developmental profile of Bombyx pupae in control, fenoxycarb-injected and 20E-injected animals |
|-------------------------------------------------|-----------------|-----------------|
| Events in pupal-adult development                | Control         | Fenoxycarb      | 20E             |
| Initiation of wing retraction                     | ~ 56 hr         | ~ 30 hr         | ~ 36 hr         |
| Ovarian growth in mass                           | ~ 80 hr         | N. D.           | ~ 80 hr         |
| Abdominal cuticle deposition                      | ~ 102 hr        | ~ 60 hr         | ~ 72 hr         |
| Precocious eye pigmentation                       | N. D.           | ~ 72 hr         | ~ 60 hr         |
| Completion of eye pigmentation                    | ~ 122 hr        | ~ 132 hr        | ~ 120 hr        |
| Rectum dilation                                  | ~ 130 hr        | N. D.           | N. D.           |
| Differentiation of external genitalia            | ~ 132 hr        | ~ 92 hr         | ~ 132 hr        |
| Retraction of abdominal cuticle                   | ~ 136 hr        | ~ 100 hr        | ~ 108 hr        |
| Malformation of follicles                        | N. D.           | ~ 108 hr        | N. D.           |
| Formulation of legs                              | ~ 156 hr        | ~ 130 hr        | ~ 130 hr        |
| Complete abdominal scale formation                | ~ 172 hr        | ~ 156 hr        | ~ 156 hr        |
| Antenna pigmentation                             | ~ 178 hr        | ~ 160 hr        | ~ 160 hr        |
| Wing Pigmentation                                | ~ 186 hr        | ~ 174 hr        | ~ 174 hr        |
| Resorption of molting fluid                      | ~ 194 hr        | N. D.           | N. D.           |
| Adult eclosion                                   | ~ 204 hr        | N. D.           | N. D.           |

Fenoxycarb (1 µg/animal) or 5 µl acetone (control) was injected into the pharate pupae (34 hr before pupal ecysis). Twenty-Hydroxyecdysone (20E) was injected immediately after pupal ecysis. The indicated hours correspond to the occurrence of each developmental event taking as standard the pupal stage of control animals and as reference (0 hour) the pupal ecysis. N. D.: Not Detected.
as 72 hr (Fig. 1) into the pupal stage and in some cases as early as 48 h. This abnormal eye pigmentation occurred at the same time (48 or 72 hr) of the pupal stage irrespective of the dose of fenoxycarb (≥0.5 µg/animal). It was characterized by pronounced ommochrome accumulation at the lateral side of the compound eyes while the other hemisphere had no conspicuous pigment (Fig. 1). Such precocious pigmentation of the compound eyes was never observed even when high doses (≥ 1 µg/animal) of fenoxycarb were injected after pupal ecdysis (data not shown).

In fenoxycarb-treated animals (1 µg/animal) the voluminous meconium did not accumulate in the rectum. The rectum of these animals was malformed and the meconium appeared as a gradually increasing leakage underneath the old pupal exuviae at the pharate adult stage. This meconium leakage first appeared about 120 hr after pupal ecdysis at the time when the rectum began to increase in size in control animals (Table 1). First it appeared as a small leakage but it quickly developed into a promiscuous stain at the ventral tip of the abdomen.

The data in Fig. 2 show that injection of 1 µg of fenoxycarb strongly inhibited the development of the ovaries. The ovaries of the treated animals became largely malformed. Follicle development was initiated at about the same time as that of control animals, but only a small number of follicles developed, which made the ovary/whole body weight ratio very low (Fig. 2). The follicles that grew into full size were irregularly shaped with abnormal elongations and constrictions.

**Fig. 1.** A: Normal pigmented compound eyes (arrows) in pharate adult *Bombyx mori* (×40 magnification). B: Partially pigmented compound eyes (arrows) in pharate adult *Bombyx mori* injected with fenoxycarb (1 µg/animal) 34 hr before pupal ecdysis (×30 magnification). See text for details.
Fig. 2. Effect of fenoxycarb injection on ovarian development in the pupal stage of Bombyx mori. Fenoxycarb (1 µg/animal) or 5 µl acetone (control) was injected 34 hr before pupal eclosion. Ovaries were dissected every day of the pupal stage and their weights were recorded. Each value is the mean±SEM of 4 animals. Results of Tukey multiple comparisons tests revealed that the means were not significantly different between groups at 0, 24 and 48 hr of the pupal stage. (P>0.05).

The thoracic legs were quickly sclerotized in the pupal stage, but were non-functional and shorter than those of the normal adults. The wings had rather pronounced pigmentation and a curled appearance. All treatments with fenoxycarb (>0.1 µg/animal) at the pharate pupal stage prevented adult eclosion. Removal of the old pupal exuviae revealed that the fenoxycarb-treated animals were inert and unable to respond to external stimuli.

20E-induced morphological alterations in the pupal-adult development

In a previous study (Dedos and Fugo, 1999), we have observed that injection of fenoxycarb (1 µg/animal) to Bombyx mori pupae immediately after the pupal eclosion produces a higher ecdysteroid titer in the pupal stage than control. To determine whether the fenoxycarb-induced effects are specific for this JH mimic or whether they are arising from excess ecdysteroid in the haemolymph, doses of 20E (1–10 µg/animal) were injected at pupal eclosion or the pharate pupal stage. Injections of 20E (≥4 µg/animal) shortly before or at pupal eclosion prevented adult eclosion (Table 1). Precocious deposition of the adult cuticle occurred by 72 hr of the pupal stage only when doses of ≥4 µg/animal of 20E were injected at pupal eclosion (Table 1). Such treatment also induced precocious partial pigmentation of the compound eyes (Table 1). This effect occurred very rapidly (within 60 h of the pupal stage), and was similar to that recorded with fenoxycarb, but it was dose-dependent, transient, and lasted until the 120h of the pupal stage. Malformation of the rectum followed by meconium leakage was observed in 20E-injected animals (≥ 4 µg/animal at pupal eclosion) only at 144 or 168 hr of the pupal stage (Table 1). Those animals that showed precocious deposition of the adult cuticle had external organ-abnormalities similar to those observed in fenoxycarb-treated animals. Similar morphological effects were produced with injections of 5 or 10 µg/animal of 20E shortly before or at pupal eclosion but lower doses of 20E (1 or 2 µg/animal) did not produce any of the above-mentioned morphological effects and the animals progressed normally in the pupal-adult development (data not shown).

Effects of fenoxycarb on ecdysteroid titer in the haemolymph and the ecdysteroid secretory activity of the prothoracic glands in the pupal stage

Since the 20E-injected animals at the pharate pupal stage had some morphological characteristics that were similar to the fenoxycarb-injected ones (Table 1), it was important to clarify how the fenoxycarb-mediated effects are brought about. It was critical to determine whether the fenoxycarb-induced effects are mediated via an excess ecdysteroid titer that results from the fenoxycarb injection in the pharate pupal stage. Thus, we determined the effects of fenoxycarb injection (1 µg/animal) on the ecdysteroid titer in the haemolymph. Injection of fenoxycarb resulted in similar titers to the control animals until 96 hr of the pupal stage (Fig. 3). From 48 until 96 hr of the pupal stage, the ecdysteroid titer of the fenoxycarb-treated animals declined similarly to the control. The titer then remained constantly high (Fig. 3). Analysis by high performance liquid chromatography of haemolymph ecdysteroids late in the pupal stage of control and fenoxycarb-treated animals (168 hr of the pupal stage) revealed that conjugated forms of ecdysteroids constituted a similarly large portion of the total ecdysteroid pool in the haemolymph in both groups (data not shown).

Next, we determined the effects of fenoxycarb on the secretory activity of the prothoracic glands (PGs) after pupal eclosion. In control pupae, degeneration of the PGs begins as early as 24 to 30 hr after pupal eclosion. The PGs of fenoxycarb-treated animals did not begin to degenerate soon after pupal
ecdyis. As a consequence, intact prothoracic glands could be extirpated even until 192 hr after pupal ecdisis. PGs could secrete ecdysteroid until 48 hr after pupal ecdisis (Fig. 4). Ecdysteroid secretion started declining at 72 hr and then became very low or undetectable beyond 96 hr (Fig. 4). The rPTTH-stimulated ecdysteroid secretion from these non-degenerating PGs was significantly lower than that of control at 0 and 24 hr and was significantly higher from basal ecdysteroid secretion only at 0 hr of the pupal stage (P<0.05; Fig. 4).

DISCUSSION

In a previous study with B. mori, we showed that when fenoxycarb is administered, at doses ≤0.1 µg/animal after the pupal ecdisis, the animals did not exhibit any disturbance or acceleration of pupal-adult development and did not show extensive morphological abnormalities as developing adults (Dedos and Fugo, 1999). The most sensitive period was determined to be at pupal ecdisis when the animals are very sensitive in relation to the ability of fenoxycarb to disturb adult ecdisis (Dedos and Fugo, 1999). In the present study, we showed that doses of fenoxycarb higher than 0.1 µg administered specifically at the pharate pupal stage could disturb pupal-adult development in B. mori.

The fenoxycarb-treated developing animals had adult-like external characteristics but they did not exhibit the stereotypical behaviours of adults. Similar morphological and behavioural abnormalities were observed with the 20E-injected animals. However, some of the characteristics of the fenoxycarb-injected animals such as the abdominal scales on the adult cuticle, the pronounced and unpatterned wing pigmentation, the malformations in the external genitalia and the developmental inhibition of the ovarioles, were not observed in the 20E-injected animals (Table 1).

In general, JHAs-mediated effects in the pupal stage have been interpreted as an ability of JH to oppose the ecdysteroid-mediated expression of genetic information for forming adult structures (Williams, 1968). Similar conclusions have been reached for H. cecropia (Williams, 1961), M. sexta (Kiguchi and Riddiford, 1978) and Mamestra brassicae (Hiruma, 1980). However, the induction mechanisms of the morphological abnormalities cannot be readily explained if one assumes that JHAs or mimics modulate the action of ecdysteroids. One would have expected that fenoxycarb would have checked or delayed the pupal-adult differentiation in B. mori, but our results showed that it behaved in a similar way as 20E (Table 1). Presumably, the accelerated deposition of the adult cuticle (Table 1) has disturbed the proper chain of events leading to the coordinated deposition of the adult cuticle and as a consequence caused the extensive malformations (Table 1). This line of thought agrees with the conclusion by Wigglesworth (1964) who suggested that the secretion of cuticle is of particular importance because the various events that occur from its initiation until its completion become possible only during a certain critical phase of the moult. The timing of fenoxycarb-mediated precocious cuticle deposition dictates the point at which the development of the various tissues is arrested. Thus, the animals find themselves trapped in a rapidly deposited cuticle that prevents further development of the various organs (Williams, 1968).

Several lines of evidence suggest that fenoxycarb, when administered at the pharate pupal stage of B. mori, mimics the 20E-induced effects. For example, the precocious partial pigmentation of the compound eyes in both fenoxycarb-treated (Fig. 1) and 20E-treated pupae is very similar to that described in allatectomized pupae of H. cecropia (Williams, 1961), M. sexta (Kiguchi and Riddiford, 1978) and M. brassicae (Hiruma, 1980). Furthermore, injection of exogenous ecdysteroids resulted in similar abnormal eye pigmentation in pupae of Samia cynthia (Williams, 1968).

Moreover, research on the morphology and histology of the rectum in H. cecropia showed that this organ is sensitive to exogenous JHAs and ecdysteroids (Judy and Gilbert, 1969; 1970a, b). They suggested that JH may play a role in inhibiting the processes involved in reducing the sheath around the rectal epithelium (Judy and Gilbert, 1970b). However, the abnormal development of the rectal epithelium after injection of 20E was reported to be qualitatively different from that following injection of juvenile hormone (Judy and Gilbert, 1970b).

Warren and Gilbert (1986) showed that the rectum functions as a repository for the ecdysteroids cleared from the haemolymph. Malformation of the rectum prevents sequestration of haemolymph ecdysteroids and this results in the presence of a high ecdysteroid titer in the late pupal stage of the fenoxycarb-treated animals (Fig. 3; Dedos and Fugo, 1999). Accumulation of meconium underneath the old pupal exuviae at the pharate adult stage of fenoxycarb-treated ani-
mals is not due to a failure of the excretory system to absorb wastes but results from the inability of the malformed rectum to dilate. The same malformation of the rectum was reported in Samia injected with ecdysteroids (Williams, 1968). We also observed leakage of the meconium in animals injected with high doses (4–10 µg/animal) of 20E shortly before or at pupal ecdisis.

Fenoxycarb injections prevented the degeneration of the PGs of the treated animals. The fenoxycarb-treated animals had well structured PGs that exhibited a gradually increasing secretory activity until the 48 hr of the pupal stage and showed marginal response to rPTTH thereafter (Fig. 4). Wigglesworth (1955) showed in Rhodnius prolixus that the degeneration of the prothoracic glands in the adult stage is prevented by juvenile hormone. This JH-mediated effect was reported in H. cecropia and S. cynthia (Gilbert, 1962). The secretory titer early in the pupal stage was not significantly different between control and fenoxycarb-treated animals (Fig. 3). Furthermore, the basal secretory activity of the PGs of the fenoxycarb-treated animals (Fig. 4) was similar at 0 and 24 hr of the pupal stage to the basal secretory activity of the control PGs (Fig. 4). Prothoracic glands of B. mori initiate their degeneration process very early in the pupal stage (Fig. 4; see also Dedos and Fugo, 1999). However, Dai and Gilbert (1997) showed that PGs of M. sexta in the pupal stage exhibited increased ecdysteroid secretion well after pupal ecdisis. Further research by these authors showed that injection of JH into young pupae of M. sexta prevented apoptosis of the PGs later in the pupal stage (Dai and Gilbert, 1998). The prothoracic glands from JH-injected Manduca pupae remained intact and their ability to synthesize ecdysteroid was maintained at a fairly active level (Dai and Gilbert, 1998).

The results in this study showed that fenoxycarb disturbs the developmental pace of pupal-adult differentiation of B. mori only when it was applied during a short developmental “window” (the pharate pupal stage). This may suggest that the small increase in the endogenous JH titer during this stage has to be carefully coordinated and precisely timed, so that the subsequent pupal-adult differentiation can proceed at its normal pace. In other words, a higher and more sustained plateau of JH titer would have elicited an accelerated development upon entrance in the pupal stage and would have produced an abnormal developmental pace in B. mori.

Contrary to our expectations, fenoxycarb induced acceleration of adult cuticle deposition and extensive abnormalities in the internal and external organs of the developing animals (Table 1, Fig. 1 and 2). Although similar morphological abnormalities were observed with injections of 20E around the time of pupal ecdisis, the application of fenoxycarb at the pharate pupal stage did not induce a higher ecdysteroid titer early in the pupal stage (Fig. 3). This indicates that higher ecdysteroid levels in the developing animals do not mediate the mode of action of fenoxycarb.

The similarities between fenoxycarb and 20E in the morphological effects they produce (Table 1) suggest that this JH mimic can act also as a 20E mimic at the particular developmental stage. Research in the mode of action of JH is hampered by the lack of knowledge on, either nuclear or intracellular, JH receptors (see Davey, 2000). However, the discovery that JH binds to the nuclear receptor USP (Jones and Sharp, 1997), the heterodimeric partner of the ecdysone receptor (Yao et al., 1993), and the subsequent finding that JH downregulates the expression of one USP isofrom in Manduca sexta epidermis resulting in prevention of pupal commitment of the epidermal cells (Hiruma et al., 1999), reveal that there is a complex manner of modulation by JH of the ecdysteroid receptor complex. Thus, we propose that this JH mimic, fenoxycarb, may act by interference with the ecdysone receptor complex, as suggested for JH by Jones and Sharp (1997), resulting in accelerated switching on of the ecdysteroid-controlled developmental pathway and the subsequent induction of stage-specific ecdysteroid-regulated genes that are responsible for the pupal-adult differentiation. This can explain our observation that the fenoxycarb-mediated effects were brought about by its application during a very short developmental stage and at a time that preceded the induction of similar effects by excess 20E (Table 1). This notion adds a new dimension to the pathways of JH-mediated gene expression. Further research is in progress to identify the cascade of events leading to fenoxycarb-mediated acceleration of pupal-adult differentiation of B. mori.

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