Regulation of prothoracic gland ecdysteroidogenic activity leading to pupal metamorphosis

Keiko Takaki a, Sho Sakurai b,∗

a Division of Life Sciences, Graduate School of Science and Technology, Kanazawa University, Kakumamachi, Kanazawa 920-1192, Japan
b Department of Biology, Faculty of Science, Kanazawa University, Kakumamachi, Kanazawa 920-1192, Japan

Received 20 January 2003; received in revised form 28 May 2003; accepted 28 June 2003

Abstract

The prothoracic glands of early last (fifth) instar larvae of the silkworm are inactive with regard to ecdysteroidogenesis and unresponsive to prothoracicotropic hormone (PTTH) [J. Insect Physiol. 31 (1985) 455]. In an attempt to elucidate the hormonal mechanisms that cause the inactivity, we compared the effects of PTTH, dibutyryl cyclic AMP (dbcAMP), a cAMP phosphodiesterase inhibitor (IBMX), juvenile hormone analogue (JHA) and 20-hydroxyecdysone (20E) on secretory activity of the third, fourth and fifth instar glands. Among the factors examined, feedback inhibition by 20E was indicated to be the most likely factor. Inhibition was moderate in the third and early fourth instars while 20E strongly inhibited the glands of middle fourth instar larvae. The inhibitory effect of 20E was reduced by removal of the brain and corpora allata. Once the glands were suppressed by 20E to the degree of exhibiting neither secretory activity nor responsiveness to PTTH, dbcAMP or IBMX did not elicit ecdysone secretion at all. Thus the feedback inhibition may shut down ecdysteroidogenesis although it is obscure whether it affects the intracellular transductory cascade from the PTTH receptor through cAMP. Taken together, this evidence suggests that inactivity of the gland in the early fifth instar is brought about by feedback inhibition of the glands by 20E occurring in the late fourth instar, and that this inactivity is maintained by the juvenile hormone found in the early fifth instar.

© 2003 Elsevier Ltd. All rights reserved.

Keywords: Prothoracic gland; Prothoracicotropic hormone; Ecdysteroidogenesis; dbcAMP; IBMX; Silkworm; Bombyx mori

1. Introduction

The last larval instar of lepidopterans is the stage when the developmental program is switched from larval growth to pupal metamorphosis, which is associated with various developmental events such as cell proliferation, cellular commitment, pupal differentiation and cell death, all of which are elicited by the principal molting hormone, 20-hydroxyecdysone (20E). Thus, one of the main concerns of insect endocrinologists has been the mechanisms that give rise to the hormonal environment characteristic to the last instar and trigger each event associated with pupal metamorphosis.

Preparation for pupal metamorphosis starts from the beginning of the last instar. The early last instar is characterized by hormonal conditions that differ from those in younger instars. The JH titer in the hemolymph decreases to an undetectable level after ec dysis (Baker et al., 1987; Niimi and Sakurai, 1997), followed by inactivation of secretory activity of the corpora allata (CA) (Granger et al., 1982; Gu and Chow, 1996), in contrast with that in the early last instar, the prothoracic glands do not secrete ecdysone (Sakurai, 1984; Okuda et al., 1985), and therefore the ecdysteroid titer declines to a very low level (Gu and Chow, 1996; Sakurai et al., 1998), and simultaneously the prothoracic glands lose their responsiveness to prothoracicotropic hormone (PTTH) (Okuda et al., 1985).

The glands retain their secretory activity and responsiveness to PTTH after ec dysis to the fourth instar, and therefore the hemolymph ecdysteroid titer remains at a detectable level (Kiguchi and Agui, 1981). After ec dysis, the JH titer declines but remains at a level sufficient to induce a stationary molt, in contrast with that in the early
fifth instar (Niimi and Sakurai, 1997). These characteristic hormonal conditions in the early fifth instar thus appear to be necessary to switch the insect developmental program from larval growth to pupal metamorphosis.

The secretory activities of the CA and prothoracic glands are affected reciprocally. JH stimulates the pupal prothoracic glands (Gilbert and Schneiderman, 1959; Williams, 1959) while it suppresses the larval glands (Sakurai and Imokawa, 1988), although it has not been demonstrated unequivocally that JH directly affects the prothoracic glands. In addition, an artificial decrease in the JH titer effected by removing the CA from young instar larvae induces precocious pupation (Fukuda, 1944). This suggests that control of CA activity is responsible for the switchover.

On the other hand, 20E is involved in maintaining CA activity because the CA becomes inactive under a very low ecdysteroid titer in the hemolymph while 20E stimulates the CA in both the penultimate and last larval instars (Gu and Chow, 1996; Whisenton et al., 1987a, b). Thus, the low ecdysteroid titer of the early fifth instar may bring about CA inactivation and thereby initiate pupal metamorphosis. Accordingly, elucidation of the hormonal mechanisms that control prothoracic gland ecdysteroidogenesis may have primary importance in understanding the hormonal control underlying the switchover of the endocrine program to directing pupal metamorphosis. What is described above has been elucidated during the past three decades, but it remains to be seen how the prothoracic glands are inactivated specifically in the early fifth instar.

During the first three days of the last instar in Bombyx mori, the prothoracic glands are inactive with respect to secretory activity and also responsiveness to prothoracicotropic hormone (PTTH) (Okuda et al., 1985; Gu et al., 1997). On the second to third day of the instar, PTTH activity appears in the hemolymph with a sharp peak (Shirai et al., 1993), indicating that inactivity of the glands is not due to the lack of PTTH but that the glands do not produce any ecdysone. Rather, the inactivity may be caused by an interruption of the PTTH transductory cascade. PTTH stimulates ecdysone production through binding to PTTH receptors on the plasma membrane of prothoracic gland cells, and receptor activation elicits an increase in intracellular Ca²⁺ (Smith et al., 1993; Birkenbeil and Dedos, 2002). Although Ca²⁺ is involved in the PTTH transductory cascade, an increase in the Ca²⁺ level results in stimulating Ca²⁺/CaM and thereby activating glandular adenylyl cyclase. Accordingly, Ca²⁺ signals converge on the cAMP signalling cascade (Smith et al., 1984, 1985; Gilbert et al., 1988, 1996, 2002). Thus, one possible mechanism to bring about loss of responsiveness to PTTH could be the disappearance of PTTH receptors from the plasma membrane and/or deficiency of one or more factors involved in the cAMP transductory cascade. On the basis of this idea, responses of the glands to a cell-permeable cAMP analogue, dibutyryl cyclic AMP (dbcAMP), have been examined in the fourth and fifth instars (Smith et al., 1984; Gu et al., 2000). In the early fifth instar, when secretory activity of the glands is undetectable, neither PTTH nor dbcAMP restored.

Prothoracic gland cells contain cAMP phosphodiesterase, and its activity changes through the fifth instar in Manduca sexta with high activity early and late in the instar (Smith and Pasqurello, 1989). Therefore, inhibition of phosphodiesterase activity by an inhibitor such as 1-methyl-3-isobutylxanthine (IBMX or MIX) results in an increase in ecdysteroidogenesis of the glands in the middle to late fifth instar (Smith et al., 1984; Gu et al., 1996). In the early fifth instar, however, IBMX does not elicit gland ecdysteroidogenesis although it does increase the glandular cAMP level (Gu et al., 1996). These results imply that inactivity of the early fifth instar glands may be due to an interruption downstream of cAMP in the PTTH transductive cascade and/or the ecdysone biosynthetic pathway, although it is not conclusively known whether the PTTH receptor is lost at the last larval ecdysis.

JH is another factor in the control of secretory activity of the prothoracic glands. JH suppresses feedback activation of the glands in the feeding period of the last instar (Sakurai and Imokawa, 1988) as well as PTTH release from the brain (Hiruma et al., 1978; Rountree and Bollenbacher, 1986). Prothoracic glands of the silkworm begin to secrete ecdysone from day 3 of the last instar (Sakurai, 1984). Removal of the corpora allata at the beginning of the last instar restores secretory activity of the prothoracic glands while a topical application of JH prolongs the recovery of the gland activity (Sakurai et al., 1989). These are in contrast to the prothoracic glands and brain in the fourth instar because then the glands secrete ecdysone (Okuda et al., 1985; Gu et al., 2000) and the brain releases PTTH (Truman and Riddford, 1974; Sakurai, 1983) in the presence of JH. Thus the difference between the glands of the penultimate and last instar larvae may lie in the mode of response to JH.

The third regulatory factor of gland activity is 20E. High secretory activity in the prothoracic glands is suppressed by 20E in vivo as well as in vitro in the late fifth instar of Manduca sexta (Sakurai and Williams, 1989; Song and Gilbert, 1998). The decrease in secretory activity of the prothoracic glands after peak activity is brought about by the high hemolymph ecdysteroid titer, or through feedback inhibition, and feedback inhibition could function as well in the decline in gland activity at the end of the fourth instar.

Thus, at least three possible factors are involved in inactivation of the prothoracic glands and loss of sensitivity to PTTH. With the use of the aforementioned differences between the fourth and fifth instars as clues to the solution for the problem of what hormonal mech-
organisms characterize the fifth instar as the last one, we examined the responses of the prothoracic glands of third, fourth and fifth instar larvae to PTTH, JH, 20E, dibutyryl cyclic AMP (dbcAMP) and IBMX.

2. Materials and methods

2.1. Animals

Larvae of the silkworm, Bombyx mori, were reared on an artificial diet under a 12L:12D photoperiod at 25 ± 0.5 °C. In our rearing regimen, second instar larvae molted in the late scotophase, and newly molted third instar larvae were fed from the beginning of the photophase, which was designated as 0 h of the third instar. The third ec dysis of the majority occurred during a photophase. Accordingly, third instar larvae were segregated at the end of a photophase and fed from the beginning of the following scotophase, which was designated as 0 h of the fourth instar. Since the fourth ec dysis to the fifth instar occurred during the scotophase, the newly molted fifth instar larvae were segregated at the beginning of the following photophase and immediately fed; the age was counted in hours from this point. The average period from the first feeding to the following ec dysis was 80 h for the third and 102 h for the fourth instar.

2.2. Hormones and chemicals

Ecdysone and 20E were obtained from Sigma (St. Louis). Both ecdysteroids were dissolved in water and stored at −20 °C until use. dbcAMP and IBMX were obtained from Sigma. dbcAMP was dissolved in water (50 mM) and stored at −30 °C. The stock solution was diluted to 5 mM with Grace’s medium immediately before use. IBMX was dissolved in DMSO (Nakalai, Osaka) at a concentration of 5 mM and diluted to 100 μM with Grace’s medium. S-Methoprene, a JH analogue (JHA: SBS Tokyo), was dissolved in acetone, and a 5 or 10 μl aliquot was applied to individual larvae. Although recombinant Bombyx PTTH is available, brain contains unidentified factor(s) that specifically stimulates the prothoracic glands in the early fifth instar (Dedos et al., 1999). Accordingly, brain extracts were used as PTTH samples and are referred to as PTTH in the present report. Brains of day 0, fifth instar larvae were homogenized in Grace’s insect culture medium and heated in boiling water for 5 min. The homogenate was centrifuged at 12 000 × g for 10 min, and the resulting supernatant was subjected to centrifugal filtration (Centricon-10, Gibco, Grand Island) to separate the prothoracicostatic peptide (MW 1090) from the extracts. The upper solutions on the filter were combined and stored at −80 °C. The extract was diluted with Grace’s medium to 1 brain-equivalent in 50 μl before use.

2.3. In vitro incubation and ecdysone radioimmunoassay

Prothoracic glands were incubated individually in 50 μl Grace’s medium (adjusted to pH 6.4 with NaOH) at 25 °C for 4 h. One of a pair of glands was incubated in plain medium (control gland) and the contralateral gland was incubated in medium containing PTTH, dbcAMP or IBMX (experimental gland). The amounts of ecdysone secreted by the glands were determined by radioimmunoassay (RIA) according to the method of Kirriishi et al. (1990). The detection limit was 0.03 ng per gland. Effects of PTTH, dbcAMP and IBMX are expressed as an activation ratio (Ar), the amount of ecdysone secreted by the experimental gland divided by the amount secreted by the control gland (Agui et al., 1979).

2.4. Ecdysteroid titer

Hemolymph ecdysteroid concentrations in third instar larvae were determined using RIA as described (Sakurai et al., 1998). Hemolymph collected from several larvae was combined and used as one sample. The titer is presented as ng ecdysone-equivalent per ml hemolymph.

3. Results

3.1. Changes in hemolymph ecdysteroid titer

Fig. 1 shows the changes in hemolymph ecdysteroids measured every 2 h throughout the third instar. Changes for the fourth and fifth instars are depicted from previous data (Koyama et al., 2003; Sakurai et al., 1998). In the third instar, the titer after the second larval ec dysis remained constant within a small range fluctuating between 10 and 20 ng/ml until 42 h. The titer increased to a peak at 52 h (602 ng/ml) and then decreased toward the third ec dysis but remained at a moderate level (24...
ng/ml) at ecdisis. The profile of the change in the fourth instar showed a single sharp peak similar to that in the third instar. Briefly, the titer remained at moderate levels (12–57 ng/ml) before the peak. After the peak, the titer decreased to a very low level (1.3 ng/ml) by 94 h and remained at this level within a range of 1.4–7.9 ng/ml until the ecdisis into the fifth instar. In the early fifth instar, the titer remained very low, ranging between 0.12 and 1.23 ng/ml for the first three days.

3.2. Basal secretory activity of prothoracic glands and responsiveness to PTTH

Secretory activity and responsiveness of prothoracic glands to PTTH were determined at selected time-points of the third, fourth and fifth instars (Fig. 2). The time-points in the third instar were 54 h, when the titer is high, and 78 h, which is after the peak point. In the fourth instar, the time-points examined were 18 h, when the titer is low, 42 h, immediately before the titer is about to increase, 66 h, immediately before the peak, 72 h, the time of peak value, and 90 h, when the titer is at a low level after the peak. For the fifth instar, 6 and 30 h were selected. In the mid-point of the intermolt period in the third (54 h) and fourth instars (18 and 42 h), the prothoracic glands exhibited low but significant secretory activity and the competence to respond to PTTH. The basal secretory activity and Ar at 54 h of the third instar and 18 and 42 h of the fourth instar were 1.9 ng/gland and 1.9, 0.4 ng/gland and 3.9, and 0.8 ng/gland and 7.7, respectively. At the time when the hemolymph ecdysteroid titer was high in the fourth instar, gland activity was 34 ng/gland at 66 h and 42 ng/gland at 72 h, and the Ar values were 1.2 and 1, respectively. At the end of the third and fourth instars, i.e., 78 h of the third instar and 90 h of the fourth instar, gland activities were 0.4 ng/gland and 4.6 ng/gland, respectively, but the Ar values were small (1.9 at 78 h of the third and 2.8 at 90 h of the fourth instar), indicating that gland activity had declined to a low level but still retained responsiveness to PTTH at the end of the third and fourth instars. By contrast, the glands in the early fifth instar did not exhibit secretory activity or respond to PTTH at all.

3.3. Responses of the glands to dbcAMP and IBMX

Since PTTH activation is mediated by the intracellular second messenger cyclic AMP (Gilbert et al., 2002), we examined the effects of an artificial increase in the glandular cAMP level using dbcAMP, a membrane-permeable cAMP analogue, and IBMX, an inhibitor of phosphodiesterase activity that thereby maintains or increases the intracellular cAMP level (Fig. 3). When examined at 42 and 90 h of the fourth instar, dbcAMP was sufficiently potent to increase secretory activity to Ar of 5.4 and 3.3, respectively. For IBMX, the Ar was 2.3 at 42 h and 8.5 at 90 h. Thus, the glands responded more strongly to dbcAMP than IBMX at 42 h, while at 90 h, the glands were more responsive to IBMX than to dbcAMP. The effect of dbcAMP was not pronounced when examined at 66 and 70 h, probably due to the high basal secretory activity at those time-points. By contrast, dbcAMP and IBMX elicited no ecdysone secretion by the glands of early fifth instar larvae.

3.4. Effects of JHA on basal secretory activity and PTTH response

The prothoracic glands are suppressed by JH during the feeding period of the fifth instar (Hiruma et al., 1978; Sakurai and Imokawa, 1988; Sakurai, 1990), but they may not be in the fourth instar because they secrete ecdysone in the presence of JH. In order to determine the time when sensitivity to JH is switched, we applied JHA 24 h before dissecting the prothoracic glands and incubated the glands in the presence or absence of PTTH, dbcAMP or IBMX (Fig. 4). In the fourth instar, JHA did not affect basal secretory activity or responsiveness to PTTH even at the end of the instar because the Ar at each time point was quite similar to that in intact larvae (see Fig. 2). This suggests that gland sensitivity to JH changes at the ecdisis from the fourth to the fifth
instar. The JHA-treated larval glands responded to dbcAMP (Fig. 4) like those of intact larvae (see Fig. 3), indicating that JHA had no effect downstream of the PTTH transductive cascade. Responses to IBMX were different from those in intact larvae. In glands at 42 h of the fourth instar, IBMX gave an Ar of 8.7 whereas dbcAMP gave 3.3. At the end of the fourth instar (90 h), the Ar value for incubation with IBMX (2.5) was half of that with cAMP. Presumably, JHA may increase phosphodiesterase activity, although it does not affect gland responsiveness to PTTH.

3.5. Inhibitory effect of 20E

Sensitivity of the glands to feedback control by 20E was examined at three selected time-points in the third and fourth instars by injecting 20E 24 h before the assay. In the third instar, 1 µg 20E effectively suppressed gland activity and 4 µg almost completely inhibited it (Fig. 5A). At those doses, the glands retained responsiveness to PTTH (Fig. 6A). A similar result was obtained when 20E was injected at 18 h of the fourth instar (Figs. 5B and 6B). By contrast, injection at 48 h of the fourth instar effectively suppressed gland activity at a dose as low as 0.4 µg (Fig. 5C), much less than the effective dose in the third and early fourth instars. Exogenous 20E suppressed basal secretory activity, but did not inhibit the glands from responding to PTTH (Fig. 6C). When relative secretory activity was plotted against the dose of 20E as expressed in µg per g body weight (Fig. 5D), the doses that effectively inhibited the glands (IC$_{50}$) in the third, and early and middle fourth instars were 1.8, 6 and 0.49 µg/g body weight, respectively. The IC$_{50}$ for the middle fourth instar was significantly smaller than those for earlier stages ($p < 0.01$), but there was no significant difference between the two curves for the early third and early fourth instars (analyzed with ANCOVA). Thus, sensi-
Fig. 5. Inhibition of prothoracic gland activity by exogenous 20E. Larvae were injected with various amounts of 20E, and basal secretory activity and responses to PTTH of the glands were examined 24 h after the injections. The time of injection was (A) 54 h of the third instar, (B) 18 h of the fourth, or (C) 42 h of the fourth instar, and the gland response to PTTH was examined 24 h thereafter. See the upper figure in Fig. 5 for the experimental manoeuvre. One of a pair of glands was incubated in plain medium to measure basal secretory activity (open bars) and the contralateral gland was challenged by PTTH (filled bars). Numbers above boxes indicate the activation ratio (Ar). Each value is an average ± SEM (N = 3–8). Letters above columns show results of ANOVA; points with no letter in common are significantly different from each other (p < 0.05). Points with no letter indicate no significant difference between the glands of a pair.

3.6. Effects of removal of brain and corpora allata at 18 h on the 20E inhibition

Effects of 20E were examined using larvae from which the brain and corpora allata had been removed (denoted as -BrCA) at 12–16 h of the fourth instar (Fig. 7). In intact larvae, 0.4 µg 20E suppressed gland activity at 24 h after injection, but the activity increased at 48 h. At doses of 1 and 4 µg, activity was reduced at 24 h and suppressed completely at 48 h after injection. If the glands retained basal secretory activity, they were capable of responding to PTTH, but if not, PTTH did not elicit secretory activity at all. In addition, when the glands of intact larvae that had been injected with 1 µg 20E were challenged with dbcAMP or IBMX at 48 h after injection, they did not produce any ecdysone.

In -BrCA larvae that were injected with water, basal activity at 24 h was 4.8 ng/gland and did not increase at 48 h after injection, which corresponds to 72 h of the fourth instar. At a dose of 0.4 µg, basal activity was reduced at 24 h in comparison with that at 0 µg, but the activity of the prothoracic glands to 20E feedback inhibition increases markedly from the early to middle fourth instar. This did not occur in the late third instar.

**Fig. 6. Effects of exogenous 20E on the gland responses to PTTH.** Selected doses of 20E were injected at (A) 54 h of the third, (B) 18 h of the fourth, or (C) 42 h of the fourth instar, and the gland response to PTTH was examined 24 h thereafter. See the upper figure in Fig. 5 for the experimental maneouvre. One of a pair of glands was incubated in plain medium to measure basal secretory activity (open bars) and the contralateral gland was challenged by PTTH (filled bars). Numbers above boxes indicate the activation ratio (Ar). Each value is an average ± SEM (N = 3–8). Letters in individual boxes show results of ANOVA: a, p < 0.05; b, p < 0.01. Points with no letter indicate no significant difference between the glands of a pair.
Fig. 7. Effects of removal of brain and corpora allata on feedback inhibition of prothoracic glands in the early fourth instar. Brain and corpora allata were removed prior to injection of various amounts of 20E at 18 h of fourth instar. Left panels: Effects of 20E on the glands of control larvae, which retained intact brain and corpora allata. Right panels: Effects of exogenous 20E on the prothoracic gland activity in larvae without brain and corpora allata. A dose of 0 µg indicates that the larvae were injected with 10 µl water. Prothoracic gland activity was assayed 24 and 48 h after injection. One of a pair of glands was incubated in plain medium while the contralateral gland was incubated in the presence of PTTH (1 brain-equivalent in 50 µl medium). In boxes where no data is shown, both glands did not produce detectable amounts of ecdysone at all. Numbers above boxes indicate the activation ratio (Ar). Notice that the scales on the ordinate of the top right panel are different from right one. Each value is an average ± SEM (N = 4–6). Letters in individual boxes show results of ANOVA: a, p < 0.05; b, p < 0.01. Points with no letter indicate no significant difference between the glands of a pair.

3.7. Effects of removal of brain and corpora allata at 66 h on 20E inhibition

Brain and corpora allata were removed at 60–62 h of the fourth instar, and the larvae were injected with various amounts of 20E at 66 h. Then, secretory activity of the prothoracic glands was examined 48 h after injection, which corresponds to 6 h of the fifth instar (Fig. 8). In the control larvae that were sham-operated and injected with water, the glands did not produce a detectable amount of ecdysone or respond to PTTH (data not shown). The glands of -BrCA larvae injected with water exhibited basal secretory activity of 0.4 ng/gland and responded to PTTH with an Ar value of 3.7. In addition, higher doses of 20E did not suppress but rather increased basal activity.

4. Discussion

We first determined the precise changes in the hemolymph ecdysteroid titer in the third instar. The titers in the early third and fourth instars are at a moderate level, similar to that at the onset of wandering, while the early fifth instar is characterized by a very low titer (Bollenbacher et al., 1981; Wolfgang and Riddiford, 1986; Sakurai et al., 1998). The most prominent difference was found at the end of the young instars. In the late third instar, the titer remained in a range between 20 and 30 ng/ml, similar to that in the early fourth instar, while in the late fourth instar, it declined to a very low level 12 h before ecdysis to the fifth instar. The low titer in the late fourth through the early fifth instars appears
maintaining basal JH secretory activity of CA while the very low titer of less than 1 ng/ml in the early fifth instar may cause the shut-down of CA activity (Gu and Chow, 1996). Therefore, the key factor that leads last instar larvae to undergo pupal metamorphosis may be in the control of prothoracic gland ecdysteroidogenesis, and the metamorphic shift in the control must begin from the late penultimate instar.

We confirmed the previous results (Okuda et al., 1985; Gu et al., 1996) that the glands lost their sensitivity to PTTH and dbcAMP in the early fifth instar, indicating, as first suggested by Gu et al. (1996), that inactivation of the glands in the early fifth instar is accompanied by a defect in the PTTH transductory cascade. By contrast, the fourth instar prothoracic glands retain their sensitivity to PTTH, and dbcAMP enhances ecdysteroidogenesis even at the end of the instar, an indication that the PTTH transductory cascade may be interrupted at the last-larval ecdysis.

Exogenous JHA inhibits ecdysteroidogenesis of the fifth instar prothoracic glands but not their responsiveness to PTTH (Sakurai and Imokawa, 1988). In the fourth instar prothoracic glands, JHA does not affect either basal secretory activity or responsiveness to PTTH. The latter was confirmed by the observation that Ar values for PTTH stimulation of JHA-treated larval glands were the same as those for intact larval glands. JHA however did affect the Ar for glands challenged by dbcAMP or IBMX. The Ar due to dbcAMP in the JHA-treated larval glands decreased when assayed at 42 h while it increased at 90 h as compared with those in intact larvae. By contrast, the Ar due to IBMX increased markedly at 42 h while it decreased at 90 h. This indicates that phosphodiesterase activity is low in the early fourth instar. In Manduca, the glandular phosphodiesterase activity is high in the early fifth instar when dbcAMP does not effectively stimulate ecdysteroidogenesis, but IBMX does (Smith and Pasquarello, 1989). Moreover, the developmental profile of glandular phosphodiesterase activity (Smith and Pasquarello, 1989) is consistent with that of the hemolymph JH titer in the last instar of Manduca (Baker et al., 1987) and Bombyx (Niimi and Sakurai, 1997). Taken together, it is possible that JH is involved in upregulation of phosphodiesterase activity, and an increase of enzyme activity bestows, at least in part, the fifth instar-specific characteristics on the prothoracic glands.

The most pronounced finding of the present study is that sensitivity of the glands to 20E, or efficacy of feedback inhibition, changes from the early to late fourth instar but not from the third to early fourth instar. When feedback inhibition was examined in the glands 24 h after 20E injections, a dose of 0.4 µg was shown to effectively inhibit the prothoracic glands in the late fourth instar, while the same dose did not significantly inhibit the glands in the third and early fourth instars.
When 20E effects were examined 48 h after injection, 1 µg 20E had completely inhibited the glands so that they did not produce any ecdysone or respond to PTTH. Those glands did not respond to either dbcAMP or IBMX. These responses resemble those of the glands in the early fifth instar.

Feedback inhibition was reduced in larvae from which the brain and corpora allata had been removed prior to 20E injection. When assayed 48 h after 1 µg 20E injection at 18 h of the fourth instar, the glands retained ecldysteroidogenic capacity in contrast to the intact larval glands that were completely inhibited by the same dose. A similar result was obtained when the glands were examined 48 h after 4 µg 20E injection. The time of examination corresponds to 6 h of the fifth instar in intact larvae when the glands do not exhibit secretory activity nor respond to PTTH. When CA are removed at ecldysis, the prothoracic glands recover their responsiveness to PTTH in 6 h and recover their basal secretory activity in 9 h (Sakurai et al., 1989). In other words, the glands recover competence to respond to PTTH before exhibiting basal secretory activity. Thus, JH in the early fifth instar appears to suppress the PTTH transductory cascade as well as the ecdysone biosynthetic pathway, thereby maintaining the inhibited state of the glands. Taken together, we suggest that inactivity of the glands in the early fifth instar is caused by feedback inhibition in the late fourth instar, and JH functions to maintain the inactive state. Strong feedback inhibition may be provided by an increased sensitivity to 20E, because sensitivity is much lower in late third instar than in late fourth instar. Accordingly, the fourth instar larvae are committed to undergo a stationary molt in late third instar because the glands are not completely inhibited due to a rather low sensitivity to 20E, while the fifth instar is destined to pupal metamorphosis before the last larval ecldysis because of the high sensitivity to 20E in late fourth instar.

The postulated regulation of prothoracic gland activity in the fourth through middle fifth instar is summarized in Fig. 9. Until the middle of the fourth instar, gland sensitivity to feedback inhibition is weak, and therefore gland activity is not suppressed through negative feedback. In the late fourth instar, the glands become sensitive to 20E so that they are strongly inhibited by 20E. The inhibited state is maintained by hemolymph JH, which peaks at the time of ecldysis (Niimi and Sakurai, 1997). Since ecldysteroid serves to maintain CA activity (Whisenton et al., 1987a, b; Gu et al., 1996), JH secretory activity of the CA declines in the early fifth instar due to the very low ecldysteroid titer. As the JH titer declines to a sub-threshold level, which is a consequence of an elevation of JH-specific esterase activity in hemolymph (Baker et al., 1987) in addition to a decline in CA secretory activity, the prothoracic glands are released gradually from feedback inhibition and recover ecldysteroidogenic capacity, although still low, and competence to respond to PTTH as well (Sakurai et al., 1989). The glands also become activated through a positive feedback that is suppressed by JH (Sakurai and Imokawa, 1988; Sakurai et al., 1989). The secretory program of the prothoracic glands is thus switched from the young larval one to the last instar program that leads to pupal metamorphosis.

Although the molecular mechanism of feedback inhibition remain to be seen, it may be mediated by the heterodimeric nuclear receptor composed of the ecdysone receptor (EcR) and Ultraspiracle (USP) proteins (Gilbert et al., 2002). In the fifth instar of Manduca sexta, two USP isoforms are identified, and the translational expression of the smaller isoform is selectively upregulated by 20E, resulting in the down regulation of ecldysteroidogenesis, during a period in the prepupal stage when hemolymph ecldysteroids are at their highest level (Song and Gilbert, 1998). A similar molecular mechanism is likely involved in the control of sensitivity to 20E that increases from the early to late fourth instar, and this issue is under study.
Acknowledgements
The present study was supported partially by a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science to S.S.

References


