Temporal analysis of ecdysteroidogenic activity of the prothoracic glands during the fourth larval instar of the silkworm, *Bombyx mori*

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Abstract

The cellular mechanism underlying ecdysteroidogenesis during the fourth larval instar of the silkworm, *Bombyx mori*, was analyzed by determining the in vitro ecdysteroid biosynthetic activity of the prothoracic glands, cAMP accumulation of the gland cells, and the in vitro release of prothoracicotropic hormone (PTTH), etc. According to the differential responsiveness of prothoracic glands to PTTH, dibutyryl cAMP (dbcAMP), and 1-methyl-3-isobutylxanthine (MIX), the following different stages were classified and changes in PTTH signal transduction were assumed. During the first stage (between days 0 and 1), the glands showed low basal and PTTH-stimulated activities in both cAMP accumulation and ecdysteroidogenesis, and PTTH release in vitro was maintained at low but detectable levels, implying that a low but sustained PTTH signal may be transduced to prothoracic gland cells. On day 1.5, when low basal ecdysteroid production of the prothoracic glands was being maintained, both the responsiveness of glands to the stimulation of PTTH and PTTH release in vitro dramatically increased, indicating greatly increased PTTH transduction. On day 3 (when the basal ecdysteroidogenesis became maximal) and afterwards, high PTTH release in vitro was maintained, but the gland showed no response to PTTH, implying that the refractoriness of gland cells to PTTH may occur at this stage. We assume that the development-specific changes in PTTH signal transduction during the penultimate larval instar may play a critical role in regulating changes in ecdysteroidogenesis of the prothoracic glands. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Ecdysteroids, synthesized by insect prothoracic glands, play important roles in directing insect growth and development, with the major ecdysteroid peaks eliciting molting and metamorphosis (Gilbert et al. 1988, 1996; Sehnal, 1989; Smith, 1985). In addition, subtle fluctuations exist in basal ecdysteroid levels during larval stages of several insect species (Sehnal, 1989). However, their developmental significance has been little examined. Recently, the physiological significance of differential basal ecdysteroid levels between the early penultimate and last larval instars has been demonstrated (Gu et al. 1992, 1995; Gu and Chow 1993, 1996). It was found that the relatively high ecdysteroid levels during the early stages of the penultimate instar are important for the corpora allata (CA) to continue producing juvenile hormone (JH) (Gu and Chow, 1996). When these ecdysteroid levels are artificially reduced to very low levels, the CA stop producing JH, and therefore precocious metamorphosis is induced at the end of the penultimate larval instar. In light of this, the low but continued ecdysteroid production by the prothoracic glands (which is responsible for the relatively high ecdysteroid levels) during the early penultimate larval instar is critical for ensuring that the next molting is an additional larval-larval molting. Unfortunately, very few studies have been conducted on the cellular mechanism underlying sustained ecdysteroid biosynthesis by the prothoracic glands during the fourth larval instar.

On the other hand, PTTH is the primary regulator of
ecdysteroidogenesis of prothoracic glands (Bollenbacher and Granger, 1985; Gilbert et al. 1988, 1996; Ishizaki and Suzuki, 1994; Kawakami et al., 1990; Smith, 1993). The cellular mechanism of PTTH action has been well documented in prothoracic gland cells of the last instar larvae of both *M. sexta* (Gilbert et al. 1988, 1996; Henrich et al., 1999; Smith et al., 1984; Smith 1993, 1995) and *B. mori* (Gu et al. 1996, 1997). PTTH appears to stimulate ecdysteroid secretion through cell surface receptors, resulting in the stimulation of a calcium/calmodulin-sensitive adenylate cyclase, increases in both cellular Ca\(^{2+}\) and cAMP levels, and enhanced protein phosphorylation and synthesis (Gilbert et al. 1988, 1996; Gu et al. 1996, 1997; Smith et al., 1984; Smith 1993, 1995). However, the developmental changes in PTTH signal transduction during a larval-larval molting cycle, have not been extensively studied.

The purpose of the present study is to clarify changes in PTTH signal transduction during the fourth (penultimate) larval instar of the silkworm, *B. mori*. We looked at the developmental changes of the steroidogenic capability of the prothoracic glands and their responsiveness to PTTH, dibutyryl cAMP (dbcAMP), as well as 1-methyl-3-isobutylxanthine (MIX) during the penultimate larval instar. Changes in cAMP accumulation by prothoracic gland cells in vitro after PTTH or MIX stimulation and in vitro PTTH release from brain-corpus cardiacum-corpus allatum complexes (BR-CC-CA) were also examined. The role of PTTH release in regulating changes in responsiveness of prothoracic glands to PTTH is discussed.

2. Materials and methods

2.1. Experimental animals

Tetramolter silkworm larvae were reared on fresh mulberry leaves at 25°C under a 12 L:12 D photoperiod. Newly-ecdysoned fourth instar larvae were collected and used for each experiment.

2.2. Reagents

[23, 24-\(^{3}\)H] Ecdysone was purchased from New England Nuclear (Boston, MA, USA), and unlabeled 20-hydroxyecdysone, MIX, and dbcAMP were from Sigma Chemical Company (St. Louis, MO, USA). Grace’s insect cell culture medium was purchased from Gibco (Grand Island, NY, USA).

2.3. PTTH preparation

To study changes in the responsiveness of the prothoracic glands at different developmental stages to stimulation by PTTH, we prepared PTTH sources as described previously (Gu et al. 1996, 1997). We used pooled medium that was pre-incubated with BR-CC-CA from day 0 last instar tetramolter larvae as a PTTH source. Although previous studies indicated that BR-CC-CA from silkworm larvae may contain prothoracostatic and other unknown factors that may affect the response of the prothoracic glands (Hua et al., 1999; Dedos et al., 1999; Gu, unpublished), we defined our pre-incubated medium as the PTTH source because of the following reasons: 1) A basically similar activation pattern in ecdysteroidogenesis was observed when glands were treated with recombinant PTTH (Gu, unpublished); and 2) It has been reported that the in vitro release of PTTH from BR-CC-CA is compatible with the timing of in vivo release of PTTH in both *B. mori* and *Rhodnius prolixus* (Shirai et al., 1993; Vafopoulou and Steel, 1996).

2.4. In vitro incubation of prothoracic glands and radioimmunoassay (RIA) of ecdysteroid

Prothoracic glands from precisely timed larvae were dissected under lepidopteran saline (Carrow et al., 1981) and then incubated for 2 h with gentle shaking in 50 μl Grace’s medium containing either dbcAMP, MIX, PTTH, or control medium. After incubation, the released ecdysteroid was determined by RIA according to procedures described in previous studies (Takeda et al., 1986; Gu et al., 1995; Gu and Chow, 1996). The assay was calibrated with 20-hydroxyecdysone as the standard. The antiserum has an approximate binding ratio of 2.5:1 for 20-hydroxyecdysone to ecdysone (Takeda et al., 1986). The detection limit of this RIA is 0.03 ng/50 μl medium.

2.5. In vitro assay of PTTH release from BR-CC-CA

To determine the PTTH release of BR-CC-CA at different developmental stages, BR-CC-CA were isolated at specific developmental stages of fourth instar larvae, then incubated in Grace’s medium for 4 h (each complex was incubated in 100 μl medium). The complexes were then removed and the medium obtained was used for determining in vitro PTTH release. For each prothoracic gland incubation, we used 50 μl of pre-incubated medium. The prothoracic glands from day 1.5 fourth instar larvae were isolated and maintained in Grace’s medium on ice prior to use. After dissection, glands were pre-incubated in Grace’s medium for 30 min. Subsequently, glands were transferred to pre-incubated medium containing PTTH or to control medium. After incubation for 2 h, the amount of released ecdysteroid in the medium was determined by RIA. From a comparison of different responses of prothoracic glands to pre-incubated medium containing PTTH and to control medium, the PTTH release in vitro was assessed. We selected glands from day 1.5 fourth instar tetramolters.
for determining in vitro PTTH release, because we found that these glands showed constant low basal activity (0.1–0.2 ng per 2 h incubation) but the most sensitive response to PTTH (see results, Fig. 1).

Fig. 1. Effects of PTTH, dbcAMP, and MIX on ecdysteroid release by prothoracic glands during the fourth larval instar. Glands were isolated every 12 h throughout the fourth larval instar and then incubated in Grace’s medium containing 10 mM dbcAMP (d), 300 μM MIX (M), PTTH (P) or in normal medium (controls, C) for 2 h, and the released ecdysteroids were quantified by RIA. PTTH sources were prepared as follows: BR-CC-CA from newly-ecdysed last instar larvae were isolated and incubated in 100 μl Grace’s medium for 4 h. The complexes were then removed, and the medium obtained was used as PTTH sources. For each incubation of a prothoracic gland, we used 50 μl of pre-incubated medium (0.5 brain equivalents). Each point represents the mean ± SEM of 8 separate assays.

2.6. Cyclic AMP determination

The content of cAMP in the prothoracic glands (five glands per point, repeated five times) was quantified by an enzyme immunoassay (EIA) using a kit and protocol available from Cayman Chemical Company (Ann Arbor, MI 48108, USA) as described previously (Gu et al. 1996, 1997).

3. Results

3.1. Changes in the responsiveness of glands to PTTH, dbcAMP, and MIX

Fig. 1 shows a developmental change in the responsiveness of glands to either PTTH, dbcAMP, or MIX throughout the fourth larval instar. On day 0, basal ecdysteroid production was low (0.05 ng/gland). However, glands showed an activation response to stimulation by either dbcAMP, MIX, or PTTH, leading to increases in ecdysteroid production (0.50, 0.20, and 0.14 ng/gland for dbcAMP-, MIX- and PTTH-stimulated activities, respectively). Glands also showed moderately increased ecdysteroid secretion to PTTH, implying that functional PTTH receptors may be maintained at low but detectable levels. On days 0.5 and 1, the stimulatory patterns of dbcAMP, MIX, and PTTH on ecdysteroid production were basically similar to those on day 0. Among the treatments of dbcAMP, MIX and PTTH on day 1.5, PTTH showed the most potent stimulatory effect on ecdysteroid production, leading to greatly increased ecdysteroid production (1.10, 0.68, 1.22, and 0.19 ng/gland for dbcAMP-, MIX-, and PTTH stimulated and basal ecdysteroid production, respectively). The basically similar stimulatory patterns of responses to either PTTH, dbcAMP, or MIX were also observed on days 2 and 2.5, indicating that PTTH signal transduction may be maintained at a high level. However, on day 2.5, basal ecdyssteroidogenic activity was greatly enhanced. On day 3, basal ecdysteroid production was 5.21 ng/gland, reaching maximal activity in the penultimate larval instar. Glands at this stage showed no response to the stimulation by PTTH. However, glands exhibited greatly increased ecdysteroid production upon stimulation by either dbcAMP or MIX. Between days 3.5 and 4.5, gland activity dramatically decreased, leading to no ecdysteroid production on day 5 when larvae ecdysed to the fifth instar. Similar to those on day 3, the glands during these stages showed no response to the stimulation by PTTH, but dbcAMP and MIX could stimulate ecdysteroid production during these stages, although the stimulatory effects of dbcAMP and MIX were steeply decreased.
3.2. Changes in in vitro PTTH release from BR-CC-CA

For determining changes in PTTH release in vitro, BR-CC-CA were isolated from larvae every 12 h or 24 h throughout the fourth instar, then incubated in Grace’s medium for 4 h. After incubation, the medium obtained was used to incubate the prothoracic glands from day 1.5 fourth instar for assaying PTTH activity. Glands from day 1.5 fourth instar were used, because of their sensitive responsiveness to stimulation by PTTH (Fig. 1). As shown in Fig. 2, ecdysteroid production was enhanced in medium pre-incubated with BR-CC-CA from day 0 fourth instar (0.22 and 0.11 ng/gland for pre-incubated and control medium, respectively), indicating that BR-CC-CA from day 0 fourth instar release low but detectable PTTH, leading to enhanced ecdysteroid secretion. PTTH release in vitro slightly increased on days 0.5 and 1 as compared with that on day 0. Greatly increased ecdysteroid production was observed in medium pre-incubated with BR-CC-CA from day 1.5 fourth instar larvae, implying that PTTH release in vitro became high at this stage (0.96 and 0.11 ng/gland for pre-incubated and control medium, respectively). The high PTTH release in vitro was maintained throughout the later stages of the fourth instar.

3.3. Changes in cAMP accumulation by gland cells and their stimulation by PTTH and MIX

Finally, we examined developmental changes in cAMP accumulation of gland cells and their stimulation by either PTTH or MIX at specific stages during the fourth larval instar. As shown in Fig. 3, cAMP levels on day 1 were 21.25, 26.90, and 70.65 fmol/gland for control, PTTH-, and MIX-stimulated glands, respectively. Similar to ecdysteroid production, PTTH slightly increased cAMP accumulation at this stage. On day 2, cAMP levels were 20.05, 81.90, and 81.90 fmol/gland for control, PTTH-, and MIX-stimulated glands, respectively. The glands showed greatly increased cAMP accumulation upon stimulation by PTTH, as compared with those from day 1 larvae. However, on day 3, cAMP levels were 73.15, 72.20, and 612.5 fmol/gland for control, PTTH-, and MIX-stimulated glands, respectively. Glands showed no increase in cAMP accumulation in response to the stimulation by PTTH, but MIX dramatically increased cAMP accumulation, implying the desensitization of PTTH-responsive adenylate cyclase and high phosphodiesterase activity. The glands on day 4 also showed a similar pattern of responses to the stimulation by either PTTH or MIX, as those from day 3, but the MIX-stimulated effect was much smaller.

4. Discussion

As the first step to understanding the cellular mechanism of ecdysteroidogenesis in prothoracic glands during the fourth larval instar, we examined the changes in both basal ecdysteroidogenesis and the responsiveness of glands to either PTTH, dbcAMP, or MIX. We used PTTH, dbcAMP, and MIX as stimulators of in vitro ecdysteroidogenesis for the following reasons: 1) PTTH is the primary stimulator of prothoracic glands in insects (Bollenbacher and Granger 1985), with a sensitive
response to PTTH indicating higher potency of glands in PTTH signal transduction; 2) Cyclic AMP appears to be involved in PTTH signal transduction (Gilbert et al., 1988, 1996; Smith, 1993, 1995; Gu et al., 1996, 1997). Dibutyryl cAMP is a cAMP analog that enters cells more rapidly than cAMP. A sensitive response to dbcAMP implies that the potency of glands for ecdysteroidogenesis is high, if the cAMP levels in gland cells are artificially elevated; and, 3) A stage-specific difference in phosphodiesterase activity in Manduca prothoracic glands has been reported (Smith and Pasquarello, 1989). MIX is a potent phosphodiesterase inhibitor, and if both endogenous cAMP levels and phosphodiesterase activity are high, glands may show a sensitive response to stimulation by MIX.

The present study clearly shows that the responsiveness of prothoracic glands in ecdysteroidogenesis to dbcAMP, PTTH, and MIX undergoes development-specific changes during the fourth larval instar of B. mori (Fig. 1). During the first stage (between days 0 and 1), basal gland activity was maintained at low but detectable levels. Among the effects of dbcAMP, PTTH, and MIX, dbcAMP showed the highest stimulation of ecdysteroidogenesis of the glands, indicating that the glands have the ability to increase the synthesis of ecdysteroids, provided cAMP levels are artificially elevated in gland cells. Thus the low cAMP-generating machinery of prothoracic gland cells may account, at least in part, for the low basal ecdysteroid production at this stage. By contrast, the in vitro PTTH release from BR-CC-CA during this stage was maintained at low but demonstrable levels (Fig. 2). Low but detectable levels of hemolymph PTTH were also observed at this stage (Dai et al., 1995). PTTH slightly stimulated both ecdysteroid secretion during this stage and cAMP accumulation on day 1 (Fig. 3), suggesting that some functional PTTH receptors may exist. This developmental state of glands differs from that of early last instar silkworm larvae: glands from early last instar cannot synthesize ecdysteroid and show no response in either cAMP accumulation or ecdysteroidogenesis to stimulation by PTTH, implying the absence of functional PTTH receptors in gland cells (Okuda et al., 1985; Gu et al., 1996, 1997). We suppose that PTTH, which exerts its action in a cAMP-dependent signal cascade, may be transduced to prothoracic gland cells in a low but sustained manner during this stage for maintaining low but continued ecdysteroid production in vivo. To our knowledge, this is the first study to demonstrate the cellular mechanism of sustained ecdysteroidogenesis during the early penultimate larval instar in an insect system.

However, the above assumption contrasts with our current knowledge on PTTH release and its action. It is generally accepted that PTTH is a trigger in the hormone cascade relating to molting and metamorphosis, and its release from BR-CC-CA activates the prothoracic glands, resulting in a major increase in ecdysteroid secretion (Bollenbacher and Granger, 1985; Gilbert et al., 1988, 1996; Smith, 1993). In several insect species, it has been well demonstrated that PTTH release occurs in a pulsatile fashion (Bollenbacher and Granger, 1985; Shirai et al., 1993; Vafopoulou and Steel, 1996). During the penultimate larval instar of M. sexta, PTTH is released in a single burst over a few hours to elicit the major increase in ecdysteoid production (Bollenbacher, 1988). However, previous research on M. sexta usually used the neck ligation method, and the head critical period was assumed to be the PTTH release period (Bollenbacher and Granger, 1985). Characterization of Manduca PTTH remains to be carried out. The determination of hemolymph PTTH levels in B. mori larvae clearly indicated that although some fluctuations in PTTH levels were observed, throughout the penultimate and last larval instars some detectable PTTH levels always exist (Dai et al., 1995). This result strongly supports our present assumption.

On day 1.5, basal ecdysteroid production of glands remained at low levels (Fig. 1). Among the effects of
dbcAMP, PTTH, and MIX, PTTH showed the highest stimulation of ecdysteroidogenesis. In mammalian ovary, it is now well established that the response of ovarian cells to gonadotropins in steroidogenesis is dependent not only on the concentration of these hormones in serum, but also on the content of their receptors in target cells and on the adenylate cyclase system (Richards, 1980; Richards and Hedin, 1988). Moreover, gonadotropins can up- or down-regulate the contents of their own receptors (homologous regulation) (Richards, 1980; Richards and Hedin, 1988). In the present study, in vitro PTTH release from BR-CC-CA on day 1.5 was also greatly increased as compared to those during the first stage, indicating that increased PTTH release may play a role in the enhanced responsiveness to PTTH. The glands also showed greatly increased responsiveness in the accumulation of cAMP to stimulation by PTTH (Fig. 3), suggesting enhancement in the activity of receptor-coupled adenylate cyclase. The greatly increased responsiveness of glands to PTTH may be due to increases in PTTH receptor levels in gland cells, the activity in receptor-coupled adenylate cyclase, as well as other factors. We suppose that the increases in both PTTH release and the responsiveness of glands to PTTH imply the possibility that the released PTTH may play a role in the rapid induction of unoccupied PTTH receptors, in a way similar to that observed in gonadotropin receptors of mammalian ovarian cells (Richards, 1980; Richards and Hedin, 1988), thus leading to a dramatic increase in ecdysteroidogenesis of gland cells during the later stage.

Prothoracic glands on days 2 and 2.5 showed similar activation patterns upon stimulation by PTTH, dbcAMP, and MIX, as those from day 1.5 larvae, but the basal gland activity on day 2.5 was greatly enhanced. Glands on day 3 showed a maximal basal ecdysteroid production of 5.21 ng/gland. Glands from this stage showed no response to PTTH in ecdysteroid secretion, but dbcAMP and MIX could further stimulate ecdysteroidogenesis, leading to greatly increased activities (10.58 and 13.50 ng/gland for dbcAMP- and MIX-stimulated activities, respectively). This result indicates that refractoriness of gland cells to PTTH may occur at this stage, but that the ecdysteroidogenic competency of glands can be further enhanced by agonists that bypass the receptor-adenylate cyclase, and act intracellularly. Prothoracic gland cells on day 3 also showed no response in cAMP accumulation to stimulation by PTTH, but MIX could further greatly increase cAMP accumulation as compared to that in the controls (Fig. 3). This result implies that a loss in the ability of gland cells to elevate adenylate cyclase activity and cAMP production in response to stimulation by PTTH may be responsible, at least in part, for the occurrence of the refractory state in gland cells.

Glands on day 4 showed decreased ecdysteroidogenesis and no response to PTTH in both cAMP accumulation and ecdysteroidogenesis. Dilbutryl cAMP and MIX still further stimulated ecdysteroid production, but these stimulatory effects had also greatly declined, implying that the artificial elevation of cAMP levels in gland cells by agonists (dbcAMP and MIX) that bypass the receptor-adenylate cyclase failed to prevent the decrease in ecdysteroid production. In mammalian ovary, it has been well documented that the reduced steroidogenic capacity of granulosa cells during follicular maturation is not only a consequence of the decrease in both the number of functional gonadotropin receptors and the receptor-coupled adenylate cyclase activity, but also that it is linked to the decline in post-cyclic AMP activities involving possible key steroidogenic enzymes (Hertelendy and Asem, 1984). We suppose that a similar mechanism may exist in the decrease in ecdysteroidogenesis of prothoracic gland cells; steps beyond the generation of cAMP also seem to be involved in decreased ecdysteroidogenesis, even though, to our knowledge, no such measurements have so far been reported. The occurrence of a refractory state of the prothoracic gland cells to stimulation by PTTH and the continued decrease in ecdysteroidogenesis during the last stage of the fourth larval instar appear to be important for larvae to terminate the fourth larval instar and to begin a new larval stage (fifth instar), thus leading to a disruptive, non-continuous pattern of insect development. The possible mechanism underlying the occurrence of this refractory state is currently being investigated.

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