A Rapid Increase in cAMP in Response to 20-Hydroxyecdysone in the Anterior Silk Glands of the Silkworm, *Bombyx mori*

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In the anterior silk glands (ASGs) of the silkworm, *Bombyx mori*, intracellular cAMP increases transiently to a very high level shortly after the hemolymph ecdysteroid peak in the prepupal period. In cultured ASGs obtained on the day of gut-purge, cAMP levels were increased by 20-hydroxyecdysone (20E), and this increase was enhanced by an inhibitor of phosphodiesterase, but was not affected by α-amanitin, indicating the 20E action may not be mediated via gene expression. The increase in cAMP occurred within 30 seconds of exposure to a physiological concentration of 20E (1 μM), and also by ponasterone A. Our findings indicate a nongenomic action of ecdysteroids in insects, which may be an additional mechanism by which this steroid hormone induces acute responses in tissues and cells.

Key words: ecdysone, cAMP, silk gland, programmed cell death, *Bombyx mori*

INTRODUCTION

Steroids elicit various physiological responses by modulating gene expression through interaction with their nuclear receptors that serve as ligand-dependent transcription factors (Beato and Klug, 2000; Henrich, 2005). 20-Hydroxyecdysone (20E), the biologically active form of ecdysone, is essential for inducing molecular and cellular events underlying molting and metamorphosis in insects and crustaceans (Gilbert *et al.*, 1996; Henrich *et al.*, 1999). 20E exerts its effects via direct binding to a heterodimeric nuclear receptor, EcR/USP, which modifies the transcriptional activity of target genes (Riddiford *et al.*, 2000).

In addition to the genomic action of steroids, increasing evidence of rapid, non-genomic steroid effects has accumulated for virtually all groups of steroids in vertebrates and plants (Lösel and Wehling, 2003). Some responses to mammalian steroid hormones such as estrogen and aldosterone are known to follow nongenomic pathways, beginning with the elevation of intracellular cAMP (Falkenstein *et al.*, 2000; Lösel and Wehling, 2003). A nongenomic action of 20E has also been postulated to occur in insect cells because of its rapid effects. 20E reduces the amplitude of excitatory potentials at neuromuscular junctions within minutes in the crayfish (Cooper and Ruffner, 1998) and *Drosophila* (Ruffner *et al.*, 1999).

These acute responses to 20E fail to fit the classical

genomic model, and appear instead to rely on mechanisms involving membrane receptors and second messengers. 20E increases cAMP levels in the pupal wing epidermis of *Hyalophora gloveri* (Applebaum and Gilbert, 1972), although the increase is not remarkable (2.5-fold over control levels). In *Mamestra brassicae*, injection of 20E results in an increase in cAMP in the larval fat bodies 3 h after injection (Sass *et al.*, 1983). These earlier studies indicated an involvement of cAMP in the action of 20E, although the evidence was not conclusive.

The anterior silk gland (ASG) of the silkworm degenerates shortly after pupation (Chinzei, 1975; Terashima *et al.*, 2000). After spinning cocoons at the end of the larval stage, the ASGs begin to undergo programmed cell death (PCD) in response to a high hemolymph ecdysteroid concentration, which induces pupal metamorphosis (Terashima *et al.*, 2000). Since 20E acts through binding to a heterodimeric ecdysone receptor, EcR/USP (Yao *et al.*, 1992), which serves as a transcription factor, 20E-induced PCD has been considered to begin with *de novo* gene expression (Huet *et al.*, 1993). However, this was called into question by results obtained with α-amanitin, a potent inhibitor of RNA polymerase II. α-Amanitin prevents PCD induction by 20E when added to a culture of ASGs with 20E from the beginning of the culture, whereas its addition 8 h after the exposure to 20E does not, indicating that the gene transcription needed for the PCD is accomplished by 8 h. Nevertheless, withdrawal of 20E from the culture medium between 8 and 42 h of the culture interferes with the progression of the PCD sequence (Terashima *et al.*, 2000). These previous results suggested a non-genomic action of 20E until 42 h, following its genomic action in the first 8 h.

Here we determined the changes in intracellular cAMP levels in ASGs that were exposed to 20E *in vitro*, as well as in intact ASGs during the prepupal period. Our study shows

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that cAMP levels exhibit rapid changes both *in vivo* and *in vitro*.

MATERIALS AND METHODS

Animals

Larvae of the silkworm, *Bombyx mori*, were reared on an artificial diet at 25°C under a photoperiod cycle of 12 h-light/12 h-dark, as described previously (Terashima *et al.*, 2000). Day 0 was designated as the day consisting of a scotophase during which larvae underwent gut purge, and the following photophase; ASGs were dissected during the day 0 photophase, unless otherwise stated.

Hormones and chemicals

20E (Sigma, St. Louis, MO) and ponasterone A (PonA, 25-deoxy-20-hydroxyecdysone; Sigma) were dissolved in ethanol (2 mM) and stored at −20°C until use. α-Amanitin (Sigma) was dissolved in water (1 mg/ml) and diluted to 10 μg/ml with Grace's medium. The phosphodiesterase inhibitor 1-methyl-3-isobutylxanthine (IBMX, Sigma) was dissolved in dimethylsulfoxide (DMSO). When added to culture media, the inhibitor concentration in DMSO was adjusted so that the final DMSO concentration was less than 5%.

Cultures

ASGs were rinsed with Grace's medium and cultured separately in 0.3 ml medium at 25°C (Terashima *et al.*, 2000). For *in-vitro* experiments, one of a pair of ASGs was used as a control gland and the contralateral gland as a test gland.

Measurement of intracellular cAMP

ASGs were kept at −80°C until cAMP extraction. Frozen ASGs were homogenized in cold 6% (W/V) trichloroacetic acid and centrifuged at 2,000×g for 15 min at 4°C. The resulting supernatant was washed four times with 5 vols of water-saturated diethyl ether, and the upper ether layer was discarded after each wash. The aqueous extracts and pellets were lyophilized separately and stored at −20°C until cAMP quantification. Amounts of cAMP in extracts were determined with a cAMP Enzyme Immunoassay Kit (Amersham Pharmacia, Piscataway, NJ) according to the manufacturer's instructions and were expressed in pmol/mg protein on the basis of the amount of protein in pellets. Four ASGs were used for one measurement.

RESULTS

Changes in intracellular cAMP levels during the prepupal period

Intracellular cAMP levels were determined from the day of gut purge (day 0) until pupation (Fig. 1A). Shortly after gut purge, cAMP increased transiently to 1.16 pmol/mg protein, and then decreased to a low level 24 h thereafter. After remaining at this level for two days, the concentration abruptly increased to a high of 4.08 pmol/mg protein at the beginning of day 3 photophase, and decreased to a low level 6 h thereafter. The peak on day 3 appeared approximately 12 h after the peak titer of ecdysteroid in the hemolymph.

Changes in cAMP levels during culture with 20E

To examine whether the increase in cAMP was brought about by 20E *in vitro*, day 0 ASGs were cultured with or without 20E (1 μM) (Fig. 1B). In the absence of 20E, basal cAMP levels remained low, ranging from 0.88–1.34 pmol/mg protein. In the contralateral ASGs that were exposed to continuous 20E, the cAMP level began to increase at 18 h
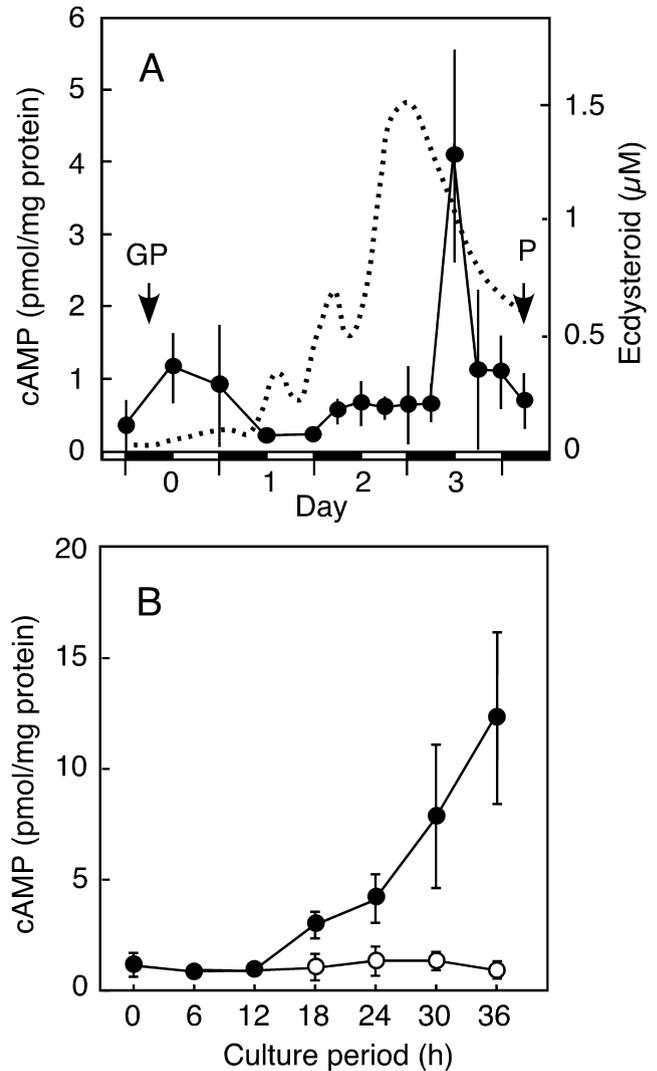

Fig. 1. Changes in intracellular cAMP levels in ASGs *in vivo* and *in vitro*. **(A)** Changes in cAMP during the prepupal period. GP, gut purge; P, pupation. The dotted line indicates the changes in ecdysteroids in hemolymph (Terashima *et al.*, 2000). Open and filled bars indicate day and night, respectively. **(B)** *In vitro* increase in cAMP by 20E. Day 0 ASGs were cultured with continuous 20E (1 μM) (closed circles) or in medium alone (open circles), and cAMP levels were determined every 6 h. Each datum point is a mean ± SD (n=3).

of culture, and at 24 h attained a value similar to the highest recorded *in vivo*, after which it continuously increased to 12.2 pmol/mg protein at 36 h.

Effects of 20E on cAMP levels

The above results showed that it takes more than 12 h for cAMP to increase in response to 20E. Our previous results (Terashima *et al.*, 2000) indicated that a nongenomic action of 20E could be involved after 12–18 h of the culture with 20E. This suggested that pre-culture of the ASGs with 20E might be necessary to examine the 20E effects on cAMP. In addition, we inserted a culture period in a hormone-free medium so as to clarify the 20E effects (Fig. 2). Day 0 ASGs were cultured with 20E (1 μM) for 6, 12, 18, or 24 h (pre-culture), followed by culture in a hormone-free
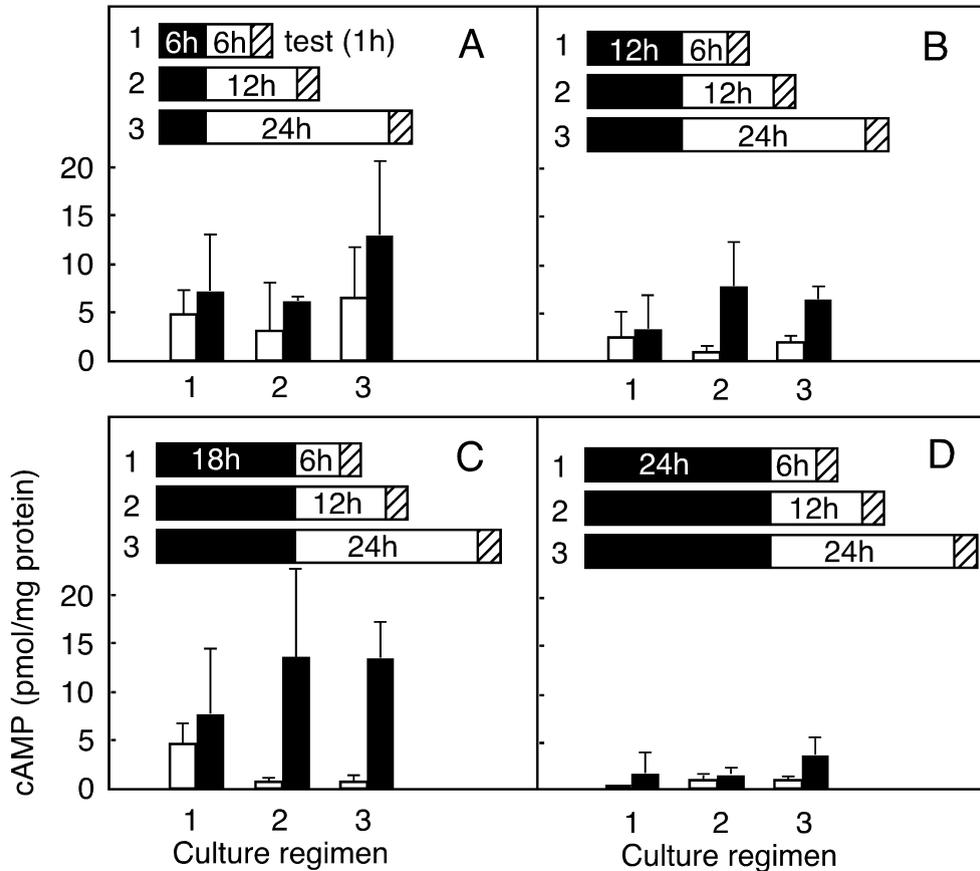
Fig. 2. Effects of insertion of a 20E-free period on the increase in cAMP levels. **(A–D)** Optimum conditions for examining the 20E effects on the increase in cAMP levels were determined by changing the periods of pre-culture with 20E and the second culture without 20E. Day 0 glands were cultured with 1 μM 20E for **(A)** 6 h, **(B)** 12 h, **(C)** 18h, or **(D)** 24 h, and were further cultured in the absence of 20E for 6, 12 or 24 h (indicated in each panel). The ASGs were then again exposed to 20E (1 μM) for 1 h (closed bars) or incubated in medium alone for 1 h (open bars), and cAMP was measured. Each datum point is a mean ± SD (n=3).

medium for 6, 12, or 24 h (second culture), and then the glands were incubated with or without 20E for 1 h. Under the culture regimen with a 6 h pre-culture (Fig. 2A), cAMP levels after culture without 20E were quite high, which obscured the 20E effects. In the culture regimen with a 12 h pre-culture, followed by a second culture for 12 or 24 h, 20E significantly increased cAMP levels, but the stimulation was not very prominent (Fig. 2B). In contrast, a culture regimen consisting of an 18 h pre-culture with 20E and a second culture for 12 h, cAMP levels increased by 20-fold (Fig. 2C). Similarly, an 18-fold increase was recorded after the second culture for 24 h. Although the high stimulation was partly due to the low basal levels, the absolute values of cAMP were also the highest among the experimental regimens. Pre-culture for 24 h was far less effective than that for 18h (Fig. 2D). Accordingly, a pre-culture with 20E for 18 h, and a second culture in a hormone-free medium for 12 h, was considered to be the most suitable culture regimen.

Effects of α-amanitin and IBMX on the cAMP response to 20E

The effects of 20E on the increase in cAMP could be nongenomic, similar to mammalian steroid hormones (Falkenstein *et al.*, 2000), although 20E has been believed to

exert its effects via a genomic pathway (Gilbert *et al.*, 1996; Henrich *et al*, 1999). To address this issue, α-amanitin was added to the 1-h incubation with 20E after the culture regimen that gave the maximal increase in cAMP (Fig. 3A). α-Amanitin did not affect the cAMP increase in response to 20E. We next examined the effects of IBMX on the 20E effects by adding IBMX with 20E after the second culture and incubating the ASGs for 1–3 h (Fig. 3B). IBMX did not elicit any effect in 1 h, but cAMP levels in the ASGs incubated in the presence of IBMX were significantly higher than in the absence of IBMX at 2 and 3 h. These results indicate that 20E may increase cAMP production.

Time-course and ecdysteroid-concentration responses for cAMP

The time course of the changes in cAMP showed that cAMP increased within 30 seconds after the 20E challenge, and attained a plateau level at 10 min, after which it did not decline (Fig. 4A). Although cAMP levels appeared to increase until 60 min, there was no significant difference between the levels at 10 and 60 min. Concentration-responses were examined for 20E and PonA (Fig. 4B). Both ecdysteroids elicited an increase in cAMP levels in a concentration-dependent manner, and the effect of PonA was
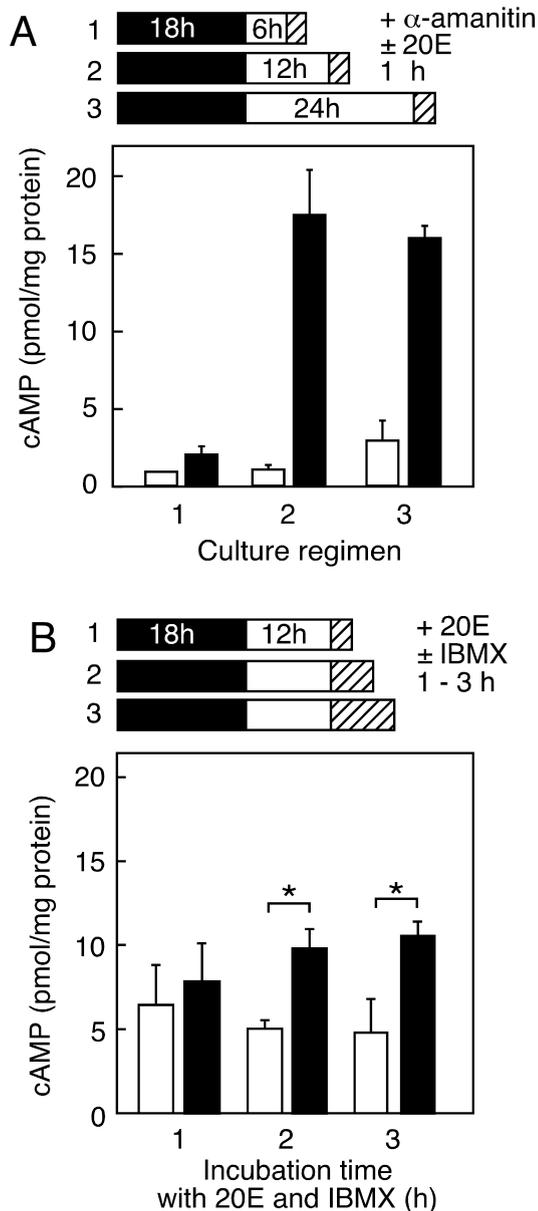
Fig. 3. Effect of 20E on cAMP is nongenomic. **(A)** Day 0 ASGs were cultured with 20E (1μM) for 18 h followed by a second culture in a hormone-free medium for 6 h, 12 h, or 24 h. The ASGs were then incubated for 1 h in the presence of α-amanitin alone (10 μg/ml; open bars) or α-amanitin and 20E (closed bars). **(B)** Effects of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) on cAMP levels. Day 0 ASGs were cultured with 20E (1 μM) for 18 h, followed by a second culture for 12 h. The ASGs were then incubated in the presence of 20E alone (open bars) or 20E and 0.1 mM IBMX (filled bars) for 1 h, 2 h, or 3 h. Asterisk indicates a significant difference in cAMP level at $P<0.05$ (ANOVA). Individual culture programs are indicated above the graphs. Each datum point is a mean ± SD (n=3).

slightly higher than that of 20E.

DISCUSSION

A rapid increase in intracellular cAMP levels in response to 20E and the inability of α-amanitin to suppress the increase suggest that 20E may act through mechanisms
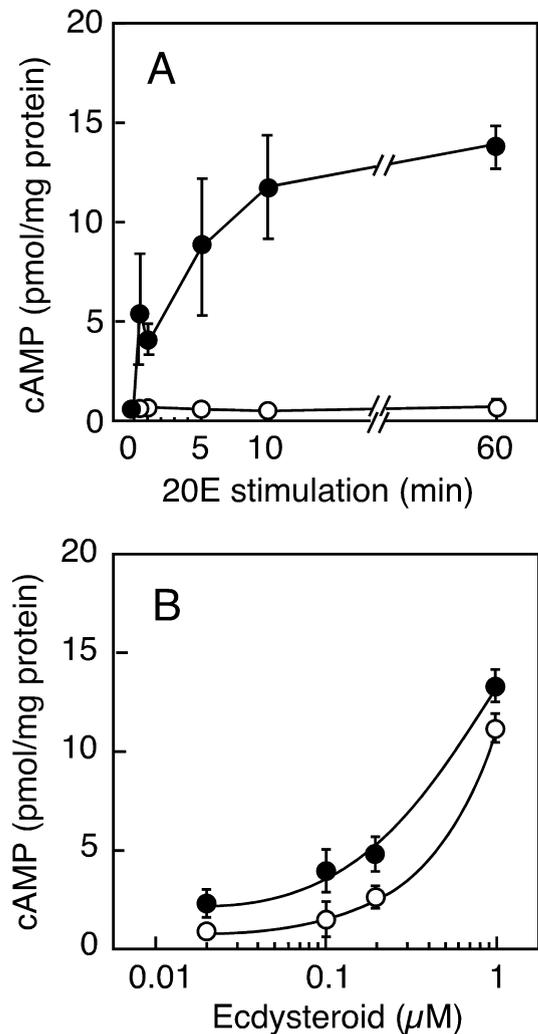

Fig. 4. Quick response of ASGs to 20E. **(A)** Time course for the increase in cAMP in response to 20E. Day 0 ASGs were cultured with 20E (1 μM) for 18 h, followed by a second culture in a hormone-free medium for 12 h. The glands were then challenged by 1 μM 20E (closed circles) or incubated in medium alone (open circles) for various time-periods. **(B)** Ecdysteroid concentration dependency. ASGs were cultured with 20E (1 μM) for 18 h followed by a second culture in a hormone-free medium for 12 h, and then exposed to various concentrations of 20E (open circles) or PonA (closed circles) for 1 h. Each datum point is a mean ± SD (n=3).

that are unrelated to its genomic action. Effects of steroid hormones on intracellular cAMP and adenylate cyclase activity have been described in vertebrate tissues (Falkenstein *et al.*, 2000), but there is only one report based on *in vitro* experimental results using insect tissues. In the moth *Hyalophora glover,* 20E enhances adenylate cyclase activity in the pupal wing epidermis within 10 min after exposure *in vitro* (Applebaum and Gilbert, 1972). 20E thus provokes an increase in cAMP in two different tissues, the wing epidermis and the ASGs, indicating that 20E-cAMP signaling could occur in various tissues in insects.

Several steroid hormones have been shown to exert rapid effects on cells by interacting with specific G-protein-coupled receptors (GPCRs) present on the cell surface (Lösel and Wehling, 2003; Simoncini and Genazzani, 2003;

Filardo *et al.*, 2002; Zhu *et al.*, 2003; Revankar *et al.*, 2005). In *Drosophila*, the dopamine/ecdysteroid receptor functions as a cell-surface GPCR, although 20E and PonA suppress the increase in cAMP by dopamine (Srivastava *et al.*, 2005). In mammalian cells, estrogen increases the intracellular cAMP level via a specific GPCR (Filardo *et al.*, 2002). A membrane ecdysteroid receptor could similarly mediate the rapid increase in cAMP levels in ASG cells, although further studies are necessary to determine whether a membrane receptor is involved in the activation of adenylate cyclase.

The correlation between increases in cAMP and the progression of PCD seems to be negative. A sharp increase in cAMP occurred at the time when the ASGs were capable of undergoing PCD without further 20E stimulation *in vivo*. Execution of PCD in ASGs begins in response to a high hemolymph ecdysteroid concentration in day-2 photophase, which induces pupation (Terashima *et al.*, 2000). In addition, the increase in cAMP occurred approximately 24 h or less after the large increase in hemolymph ecdysteroids. In an *in-vitro* culture system, cAMP began to increase after 18 h of culture with 20E. Although the increases in cAMP levels, both *in vivo* and *in vitro*, concomitant with the progression of PCD implies an involvement of cAMP in this process, this hypothesis is tenuous, since neither the membrane-permeable cAMP analog dibutyryladenosine 3',5'-cyclic monophosphate (dbcAMP) nor cAMP-dependent protein kinase peptide inhibitor (PKA inhibitor) affected PCD at all (Iga and Sakurai, unpublished data). Consequently, cAMP may not be involved in modulation of the rate of PCD, and its role in this process remains obscure.

ACKNOWLEDGMENTS

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