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In vitro and in vivo stimulation of extracellular signal-regulated kinase (ERK) by the prothoracicotropic hormone in prothoracic gland cells and its developmental regulation in the silkworm, *Bombyx mori*

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Abstract

In this study, we investigated activation of the extracellular signal-regulated kinase (ERK) by the prothoracicotropic hormone (PTTH) in prothoracic gland cells of the silkworm, *Bombyx mori*. The results showed that the PTTH stimulated ERK phosphorylation as this depends on time and dose and ecdysteroidogenic activity. The ERK phosphorylation inhibitors, PD 98059 and U0126, blocked both basal and PTTH-stimulated ERK phosphorylation and ecdysteroidogenesis. In addition, activation of glandular ERK phosphorylation by the PTTH appeared to be developmentally regulated with the refractoriness of gland cells to the PTTH occurring during the latter stages of both the fourth and last larval instars. Moreover, in vitro activation of ERK phosphorylation of prothoracic glands by the PTTH was also verified by in vivo experiments: injection of the PTTH into day 6 last instar larvae greatly increased the activity of glandular ERK phosphorylation and ecdysteroidogenesis. These results suggest that development-specific changes in ERK phosphorylation may play a role in PTTH stimulation of ecdysteroidogenesis.
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Keywords: *Bombyx mori*; PTTH; Ecdysteroidogenesis; Prothoracic gland cells; ERK phosphorylation; Signal transduction; In vivo; Development

1. Introduction

Ecdysteroids, synthesized by insect prothoracic glands, are essential for driving the molecular and cellular events that lead to molting and metamorphosis (Gilbert et al., 1996, 2002). Ecdysteroidogenesis in prothoracic glands is under the control of the prothoracicotropic hormone (PTTH), a neuropeptide produced by the brain (Kawakami et al., 1990; Gilbert et al., 1996, 2002). It was previously demonstrated that PTTH stimulation of ecdysteroid synthesis appears to be mediated by cyclic AMP as an intracellular second messenger in both *Manduca sexta* (Smith et al., 1984, 1985, 1993, 1996; Gilbert et al., 1996, 2002) and *Bombyx mori* (Gu et al., 1996, 1997, 2000). The involvement of Ca^{2+} in PTTH stimulation of ecdysteroidogenesis has also been well documented (Smith et al., 1985; Gu et al., 1998; Birkenbeil and Dedos, 2002; Fellner et al., 2005).

By contrast, we previously demonstrated in *B. mori* that PTTH signal transduction pathways undergo specific developmental changes, with a deficiency in transduction in prothoracic gland cells occurring during the early last instar (Gu et al., 1996, 1997; Gu and Chow, 2005). During the first stage of the last larval instar, the PTTH is released from the brain–corpus cardiacum–corpus allatum (BR–CC–CA) complex (Shirai et al., 1993; Dai et al., 1995; Gu et al., 1996), but the prothoracic glands show no increase in either cAMP levels or steroidogenesis in response to stimulation by the PTTH (Gu et al., 1996, 1997; Gu and Chow, 2005). Thus it is clear that both PTTH release and successful PTTH signal transduction are crucial for ecdysteroidogenesis. Development-specific changes in PTTH signal transduction also occur during the penultimate larval instar of *B. mori* (Gu et al., 2000).

Extracellular signal-regulated kinases (ERKs) are members of the mitogen-activated protein kinases (MAPKs).

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Upon extracellular stimulation, ERKs are activated by a network of interacting proteins, which funnel the signals into a multitier kinase cascade (Lewis et al., 1998). Although ERKs were first implicated in the regulation of proliferation and differentiation, it is presently known that these kinases also participate in the control of various cell activities, including cellular morphology, and learning and memory in the central nervous system (Widmann et al., 1999). In *M. sexta*, it was demonstrated that ERKs are involved in PTTH stimulation of ecdysteroidogenesis by the prothoracic glands (Rybczynski et al., 2001; Rybczynski and Gilbert, 2003). However, such research was conducted only on a single insect species. It is not clear whether the results obtained from *M. sexta* apply to other insects. Indeed the available data indicate that some differences exist in cAMP-dependent steroidogenic competence of glands between *M. sexta* and *B. mori* (Smith et al., 1984, 1985; Gilbert et al., 1996, 2002; Gu et al., 1996, 1997; Gu and Chow, 2005). Moreover, although in vitro activation of glandular ERK phosphorylation by the PTTH was observed (Rybczynski et al., 2001; Rybczynski and Gilbert, 2003), it is not clear whether ERK phosphorylation by the PTTH can be demonstrated by in vivo PTTH stimulation.

In the present study, we investigated activation of ERK phosphorylation by the PTTH in prothoracic gland cells of the silkworm, *B. mori*. Our results showed that in vitro activation of glandular ERK phosphorylation by PTTH appears to be developmentally regulated with refractoriness of gland cells to PTTH occurring during the latter stages of both the fourth and last larval instars. Moreover, in vitro activation of ERK phosphorylation of the prothoracic glands by the PTTH was also verified by in vivo experiments: injection of the PTTH into day 6 last instar larvae greatly increased the activities of glandular ERK phosphorylation and ecdysteroidogenesis. These results suggest that development-specific changes in ERK phosphorylation may play a role in PTTH stimulation of ecdysteroidogenesis.

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 ecdysed fourth and last instar larvae were respectively collected and used for each experiment. Larvae began wandering on day 7 of the last larval instar, and pupation occurred on day 11.

2.2. Reagents

20-Hydroxyecdysone, forskolin, and dibutyryl cAMP (dbcAMP) were from Sigma (St. Louis, MO, USA). PD 98059 and U0126 were from Calbiochem (La Jolla, CA, USA). Grace's insect cell culture medium was purchased from Gibco (Invitrogen, Carlsbad, CA, USA). [23,24-^{3}H] Ecdysone was purchased from New England Nuclear (Boston, MA, USA). Recombinant *B. mori* PTTH (PTTH) was produced by infection of *Spodoptera frugiperda*-SF21 cells with the vWTPTTHM baculovirus as described previously (O'Reilly et al., 1995). The same PTTH as that previously reported (O'Reilly et al., 1995; Gu et al., 1998; Chen and Gu, 2006; Gu, 2007) was used in the present study. Although much time has passed since we obtained it, our previous studies showed that the PTTH is still stable (Chen and Gu, 2006; Gu, 2007). In the present study, the extracellular fluid from cells infected with vWTPTTHM was used as the PTTH source and diluted it 200 times with medium except for the experiments on the dose-dependent effects of PTTH and with the in vivo injection.

2.3. In vitro incubation of the prothoracic glands and radioimmunoassay (RIA) of ecdysteroids

Prothoracic glands from precisely timed larvae were dissected in lepidopteran saline (Gu et al., 1995a, b; Gu and Chow, 1996) and then incubated for 2 h with gentle shaking in 50 μl of Grace's medium. After incubation, the released ecdysteroids were determined by RIA according to procedures described in previous studies (Takeda et al., 1986; Gu et al., 1995a, b; Gu and Chow, 1996). The assay was calibrated with 20-hydroxyecdysone as the standard. The antiserum has an approximate binding ratio of 3:1 for 20-hydroxyecdysone to ecdysone (Takeda et al., 1986). The detection limit of the RIA was 0.03 ng. To determine changes in hemolymph ecdysteroids, hemolymph (50 μl from each animal) from precisely timed larvae was collected and stored at −20 °C before use. Ecdysteroids were extracted from the hemolymph using methanol; after centrifugation, the supernatants were evaporated to dryness. Ecdysteroid activity was measured by RIA as described above. To study the effects of PTTH injection on hemolymph ecdysteroid levels and ecdysteroidogenic activity of the prothoracic glands, last instar larvae from days 1 and 6 were injected with 10 μl saline containing 1 μl of the original PTTH solution. Larvae that were only injected with 10 μl saline were used as the controls. Before and 2 h after the injection, hemolymph from each larva was collected. The prothoracic glands were dissected 2 h after the injection and then incubated in Grace's medium for 2 h, and their ecdysteroidogenic activity was determined.

2.4. Antibodies

Anti-phospho-ERK and anti-total-ERK antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). A horseradish peroxidase (HRP)-linked goat anti-rabbit second antibody was purchased from PerkinElmer Life Sciences, Inc. (Boston, MA, USA).

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