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PTTH-stimulated ERK phosphorylation in prothoracic glands of the silkworm, *Bombyx mori*: Role of Ca^{2+}/calmodulin and receptor tyrosine kinase

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ABSTRACT

Our previous studies showed that the prothoracicotropic hormone (PTTH) stimulated extracellular signal-regulated kinase (ERK) phosphorylation in prothoracic glands of *Bombyx mori* both in vitro and in vivo. In the present study, the signaling pathway by which PTTH activates ERK phosphorylation was further investigated using PTTH, second messenger analogs, and various inhibitors. ERK phosphorylation induced by PTTH was partially reduced in Ca^{2+}-free medium. The calmodulin antagonist, calmidazolium, partially inhibited both PTTH-stimulated ERK phosphorylation and ecdysteroidogenesis, indicating the involvement of calmodulin. When the prothoracic glands were treated with agents that directly elevate the intracellular Ca^{2+} concentration [either A23187, thapsigargin, or the protein kinase C (PKC) activator, phorbol 12-myristate acetate (PMA)], a great increase in ERK phosphorylation was observed. In addition, it was found that PTTH-stimulated ecdysteroidogenesis was greatly attenuated by treatment with PKC inhibitors (either calphostin C or chelerythrine C). However, PTTH-stimulated ERK phosphorylation was not attenuated by the above PKC inhibitors, indicating that PKC is not involved in PTTH-stimulated ERK phosphorylation. A potent and specific inhibitor of insulin receptor tyrosine kinase, HNMPA-$(AM)_3$, greatly inhibited the ability of PTTH to activate ERK phosphorylation and stimulate ecdysteroidogenesis. However, genistein, another tyrosine kinase inhibitor, did not inhibit PTTH-stimulated ERK phosphorylation, although it did markedly attenuate the ability of A23187 to activate ERK phosphorylation. From these results, it is suggested that PTTH-stimulated ERK phosphorylation is only partially Ca^{2+}- and calmodulin-dependent and that HNMPA-$(AM)_3$-sensitive receptor tyrosine kinase is involved in activation of ERK phosphorylation by PTTH.

1. Introduction

The complex physiological processes of insect molting and metamorphosis are regulated by ecdysteroids, synthesized by the prothoracic glands (Gilbert et al., 1996, 2002). Ecdysteroidogenesis in the prothoracic glands is under the control of the prothoracicotropic hormone (PTTH), a neuropeptide produced by the brain (Kawakami et al., 1990; Ishizaki and Suzuki, 1994; Gilbert et al., 1996, 2002; Rybczynski, 2005). Previous studies demonstrated that PTTH stimulation of ecdysteroid synthesis appears to be mediated by cAMP and Ca^{2+} as intracellular second messengers in both *Manduca sexta* (Smith et al., 1984, 1985; Gilbert et al., 1996, 2002; Fellner et al., 2005; Rybczynski, 2005) and *Bombyx mori* (Gu et al., 1996, 1997, 1998, 2000; Birkenbeil and Dedos, 2002). In addition, it was well demonstrated that p70 S6 kinase and ribosomal protein S6 are involved in PTTH-stimulated ecdysteroidogenesis in *M. sexta* (Song and Gilbert, 1994, 1995, 1997). Recently, a quantitative phosphoproteomics was also used to investigate PTTH signal transduction events (Rewitz et al., 2009).

Extracellular signal-regulated kinases (ERKs) are members of the mitogen-activated protein kinase (MAPK) family. Upon activation, ERKs regulate the activities of a variety of key signal transduction enzymes and transcription factors, which ultimately affect gene expression and mitogenesis (Lewis et al., 1998; Kolch, 2005). An initial study showed that ERKs are involved in regulating proliferation and differentiation. Presently, it is known that these kinases participate in controlling various cell activities, including cellular morphology, learning, and memory in the central nervous system (Widmann et al., 1999; Kolch, 2005). In both *M. sexta* and *B. mori*, it was demonstrated that ERKs are involved in PTTH stimulation of ecdysteroidogenesis by prothoracic glands (Rybczynski et al., 2001; Lin and Gu, 2007). Moreover, the involvement of

Abbreviations: PTTH, prothoracicotropic hormone; ERK, extracellular signal-regulated kinase; PKC, protein kinase C; PMA, phorbol 12-myristate acetate; MAPK, mitogen-activated protein kinase; TBS, Tris-buffered saline; MW, molecular weight; TOR, target of rapamycin; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; RIA, radioimmunoassay; BSA, bovine serum albumin.

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Ca^{2+} and protein kinase C (PKC) in PTTH-stimulated ERK phosphorylation was demonstrated in *Manduca* prothoracic glands (Rybczynski and Gilbert, 2003, 2006).

In the present study, we used *Bombyx* PTTH, second messenger analogs, and various inhibitors to investigate the signaling pathway by which PTTH activates ERK phosphorylation. We demonstrated that HNMPA-$(AM)_3$-sensitive receptor tyrosine kinase is involved in activation of ERK phosphorylation by PTTH, that PTTH-stimulated ERK phosphorylation is partially dependent on Ca^{2+}/calmodulin, and that PKC is involved in PTTH-stimulated ecdysteroidogenesis by *Bombyx* prothoracic glands in an ERK-independent manner.

2. Materials and methods

2.1. Experimental animals

Larvae of an F1 racial hybrid, Guofu × Nongfong of *B. mori*, were reared on fresh mulberry leaves at 25 °C under a 12-light:12-dark photoperiod. Newly-ecdysed last instar larvae were collected and used for each experiment.

2.2. Reagents

20-Hydroxyecdysone, A23187, thapsigargin, the PKC activator (phorbol 12-myristate acetate, PMA), calmidazolium, and genistein were from Sigma (St. Louis, MO, USA). HNMPA-$(AM)_3$, calphostin C, and chelerythrine C were from Calbiochem (San Diego, CA, USA). Grace's insect cell culture medium was purchased from Invitrogen (Carlsbad, CA, USA). [23,24-^{3}H] Ecdysone was obtained 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. In the present study, the extracellular fluid from cells infected with vWTPTTHM was used as the PTTH source, and it was diluted 500 times with medium.

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

Prothoracic glands from day 7 last instar larvae were dissected in lepidopteran saline (Gu, 2006, 2007; Gu and Chow, 1996). Following dissection, the medium was replaced with fresh medium (± any inhibitors) or saline (see Section 3), and a 30-min preincubation period was initiated. After preincubation, glands were rapidly transferred to fresh medium (± experimental materials, such as inhibitors or PTTH) and then incubated with gentle shaking. Incubation lasted 1 h (for most experiments) or 30 min (for Western blotting experiments of A23187, thapsigargin, and PMA). DMSO was used to dissolve all hydrophobic chemicals, which were prepared as 100× or 200× stocks. When these chemicals were used, the control glands also received DMSO (less than 1%). After incubation, glands were flash frozen at −70 °C for future sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, while samples of media for ecdysteroid determination were frozen at −20 °C. The released ecdysteroids were determined by an RIA according to procedures described in previous studies (Takeda et al., 1986; Gu and Chow, 1996). The assay was calibrated with 20-hydroxyecdysone as the standard. The antiserum has an approximate binding ratio of 3:1 of 20-hydroxyecdysone to ecdysone (Takeda et al., 1986). The detection limit of the RIA was 0.03 ng. Each ecdysteroid value is an average ± SEM (n = 8).

2.4. Antibodies

Anti-phospho-ERK and anti-total-ERK antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA) (antibody catalog numbers 9101, 9102, respectively). Horseradish peroxidase (HRP)-labeled anti-rabbit immunoglobulin G (IgG) was purchased from PerkinElmer Life Sciences (Boston, MA, USA).

2.5. Western blot analysis

SDS-PAGE and immunoblotting were performed as described previously (Lin and Gu, 2007; Gu et al., 2009). Briefly, the treated or control prothoracic glands from day 7 last instar larvae were homogenized in lysis buffer (10 mM Tris and 0.1% Triton x-100) at 4 °C, then boiled in an equal volume of SDS sample buffer for 4 min followed by centrifugation at 15,800 × g for 3 min to remove any particulate matter. Aliquots of the supernatants were loaded onto 12% SDS gels. Following electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes using an Owl (Portsmouth, NH, USA) Bandit™ Tank Electroblotting System, and then washed with Tris-buffered saline (TBS) for 5 min at room temperature. Blots were blocked at room temperature for 1 h in TBS containing 0.1% Tween 20 (TBST) and 5% (w/v) nonfat powdered dry milk, followed by washing three times for 5 min each with TBST. Blots were incubated overnight at 4 °C with the primary antibody in TBST with 5% bovine serum albumin (BSA). Blots were then washed three times in TBST for 10 min each and further incubated with the HRP-linked second antibody in TBST with 1% BSA. Following three additional washes, the immunoreactivity was visualized by chemiluminescence using Western Lightning Chemiluminescence Reagent *Plus* from PerkinElmer Life Sciences. Films exposed to the chemiluminescent reaction were scanned and quantified using an AlphaImager Imaging System and AlphaEaseFC software (Alpha Innotech, San Leandro, CA, USA).

To measure the effect a treatment had on ERK phosphorylation, fold changes in ERK phosphorylation were calculated as the ratio of the phosphorylation signal detected in experimentally treated glands divided by the phosphorylation signal detected in the control glands after being standardized against total ERK levels per sample. A pair of prothoracic glands was found in each insect, and one served as a matched control for each gland receiving experimental treatment in the experiments on the last instar larvae.

2.6. Data analysis

Results are expressed as the mean ± S.E.M. Data were compared by analysis of variance (ANOVA) followed by Tukey's multiple comparison test. $p < 0.05$ was considered significant.

3. Results

3.1. Specificity of the antibodies and involvement of Ca^{2+} in PTTH-stimulated ERK phosphorylation

Activation of ERK phosphorylation by PTTH was previously demonstrated in prothoracic glands of both *M. sexta* and *B. mori* (Rybczynski et al., 2001; Lin and Gu, 2007). In addition, PTTH-stimulated ERK phosphorylation in *Manduca* prothoracic glands appears to be Ca^{2+}-dependent (Rybczynski and Gilbert, 2003). In the present study, we first investigated the specificity of anti-phospho-ERK and anti-total-ERK antibodies. Fig. 1 shows whole blots of lysates of prothoracic glands. Using an anti-phospho-ERK antibody, which recognizes only the phosphorylated form, a strongly immunoreactive protein with a molecular weight (MW) of about 40,000 was readily detectable in lysate of one prothoracic