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Protein kinase C modulates ecdysteroidogenesis in the prothoracic gland of the tobacco hornworm, *Manduca sexta*

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Abstract

The prothoracic gland is the primary source of ecdysteroid hormones in the immature insect. Ecdysteroids coordinate gene expression necessary for growth, molting and metamorphosis. Prothoracicotropic hormone (PTTH), a brain neuropeptide, regulates ecdysteroid synthesis in the prothoracic gland. PTTH stimulates ecdysteroid synthesis through a signal transduction cascade that involves at least four protein kinases: protein kinase A (PKA), p70 S6 kinase, an unidentified tyrosine kinase, and the extracellular signal-regulated kinase (ERK). In this report, the participation of protein kinase C (PKC) in PTTH signalling is demonstrated and characterized. PTTH stimulates PKC activity through a PLC and Ca^{2+}-dependent pathway that is not cAMP regulated. Inhibition of PKC inhibits PTTH-stimulated ecdysteroidogenesis as well as PTTH-stimulated phosphorylation of ERK and its upstream regulator, MAP/ERK kinase (MEK). These observations reveal that the acute regulation of prothoracic gland steroidogenesis is dependent on a web of interacting kinase pathways, which probably converge on factors that regulate translation.

Keywords: Signal transduction; Phosphorylation; Phospholipase C; Insect molting; Insect steroids; Extracellular signal-regulated kinase (ERK)

1. Introduction

The control of gene expression by ecdysteroids, primarily 20-hydroxyecdysone (20E) is critical for coordinating gene expression in insects, especially during molting and metamorphosis (see Henrich et al., 1999). Periodic increases in ecdysteroid levels result from stimulation of the prothoracic glands by the brain neuropeptide prothoracicotropic hormone (PTTH) and/or by direct neuronal input (see Rybczynski, 2005). Ecdysteroids are synthesized from cholesterol, and the transduction pathway by which PTTH up-regulates ecdysteroid synthesis was first believed to be relatively simple, involving cAMP generation and protein kinase A activation (PKA) (see Rybczynski, 2005). Recent studies have progressively revealed a more complicated PTTH signal transduction cascade, dependent on increases in internal Ca^{2+} and involving additional kinases (see Gilbert et al., 2002; Rybczynski, 2005). It is now known that the 70 kDa S6 kinase (p70S6K), is also activated by PTTH (Song and Gilbert, 1994), resulting in the phosphorylation of ribosomal protein S6 with a concomitant and necessary increase in protein translation (Song and Gilbert, 1997; see Palen and Traugh, 1987). Additionally, a member of the extracellular signal-activated regulated kinase (ERK) subfamily of mitogen-activated protein kinases (MAPKs) is also activated rapidly by PTTH, and inhibitors of ERK phosphorylation can block PTTH-stimulated ecdysteroidogenesis (Rybczynski et al., 2001). Finally, recent data indicate that PTTH also activates one or more tyrosine kinases and that inhibiting tyrosine kinase activity can inhibit PTTH-stimulated ecdysteroid synthesis (Smith et al., 2003).

Phospholipase Cβ (PLCβ) metabolizes phosphatidylinositol-4,5-bisphosphate (PIP_2), yielding inositol trisphosphate (IP_3) and diacylglycerol (DAG) (see Rhee, 2001). In a recent study of the sources and mechanisms of PTTH-stimulated increases of cytoplasmic free Ca^{2+} ($[Ca^{2+}]_i$) in the prothoracic gland of the tobacco hornworm *Manduca sexta*, Fellner et al. (2005) found that an inhibitor of PLCβ blocked such $[Ca^{2+}]_i$ increases. Furthermore, PTTH stimulated Ca^{2+} release from the IP_3 receptor in the endoplasmic reticulum (ER) and inhibitors of the IP_3 receptor blocked PTTH-stimulated ecdysteroidogenesis (Fellner et al., 2005). These observations suggested that PTTH stimulates PIP_2 metabolism, even though two studies have failed to detect such metabolites in either *Bombyx mori* or *Manduca* prothoracic glands (Girgenrath and Smith, 1996; Dedos and Fugo, 2001). These data suggested

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also that PTTH might stimulate protein kinase C (PKC) in the prothoracic gland since DAG, a product of PIP_2 metabolism, in concert with Ca^{2+}, activates several isoforms of PKC (see Mellor and Parker, 1998). In addition, the same study that found no PIP_2 metabolism in PTTH-stimulated *Bombyx* prothoracic glands, did find that a PKC inhibitor blocked PTTH-stimulated ecdysteroidogenesis (Dedos and Fugo, 2001). The present study was undertaken to determine specifically if PTTH stimulates PKC activity in the *Manduca* prothoracic gland and, if so, to begin the characterization of the upstream regulators and targets of PKC activity in the gland.

2. Methods and materials

2.1. Animal rearing and dissections

Manduca larvae were group-reared on an artificial diet, under high humidity, at 26°C, with a 16:8 light:dark cycle (Rybczynski and Gilbert, 1994). Under these conditions, the fifth (final) larval instar stage last ≈10 days. Animals were immobilized rapidly in an ice-water slurry, their prothoracic glands extirpated under insect saline and transferred to wells of a spot test plate containing 50 μl Grace's medium (Rybczynski and Gilbert, 1994). Each animal possesses a pair of prothoracic glands and one gland served as a matched control for the gland that received an experimental treatment. Following the dissection of six pairs of prothoracic glands, the medium was replaced with fresh medium (±any inhibitors) and a 30 min preincubation period was initiated. At the end of the preincubation, the medium was replaced rapidly with 25 μl of fresh Grace's medium ± experimental materials (inhibitors, PTTH) and a 20 min to 4 h incubation period commenced. DMSO was used to dissolve all hydrophobic compounds, which were prepared as 100× stocks. When such compounds were used, the control glands also received DMSO (1%). After the incubation the glands were flash frozen at −80°C for future SDS PAGE analyses, while the media samples were frozen at −20°C with the addition of 100 μl of PBS, for later radioimmunoassay of ecdysteroids.

2.2. Reagents

Grace's medium was obtained from GibcoBRL (Grand Island, NY). Partially purified PTTH was extracted from brains of day 1 pupae as described by Rybczynski and Gilbert (1995); this material has the same effects on prothoracic gland ecdysteroidogenesis and signal transduction as does pure, recombinant *Manduca* PTTH (Gilbert et al., 2000; Rybczynski et al., 2001). Recombinant PTTH was a generous gift of Dr. H. Kataoka (University of Tokyo, Japan). A monoclonal antibody against the dually phosphorylated *Drosophila melanogaster* ERK was obtained from Sigma–Aldrich (St. Louis, MO), the antibodies against PKC substrate proteins and against phosphorylated PKC and MEK were from Cell Signalling Technology (Beverly, MA). The antibody against phosphorylated PKC recognizes several vertebrate PKCs (α, β_I, β_{II}, δ, ε, and η; Zhang et al., 2002), if they are phosphorylated on a serine located in a conserved hydrophobic region of the C-terminal. Calphostin C and U73122 were from Calbiochem; U73443, chelerythrine C, 2-nitro-4-carboxyphenyl-*N*,*N*-diphenylcarbamate, suramin, and mastoparan were from Sigma–Aldrich

2.3. Immunoblots

SDS-PAGE and immunoblotting were performed as described previously (Rybczynski and Gilbert, 2003). Briefly, prothoracic glands were boiled directly in sample buffer. Following centrifugation (15,800 × *g*) for 3 min to remove particulate matter, aliquots of the supernatants were loaded onto 10% SDS gels and proteins were transferred to polyvinylidene difluoride membranes, using a Bio-Rad (Hercules, CA) semi-dry apparatus. Membranes were blocked at 37°C for 30 min in phosphate-buffered saline with 0.1% Tween 20 (PBST) and 2% bovine serum albumin (BSA) and 2% non-fat powdered dry milk (PBST/block) and then incubated overnight at 4 °C with the primary antibody (1:3000) in PBST/block, followed by three 5 min washes in PBST (room temperature) and then incubated with a horseradish peroxidase-linked second antibody (1:3000) in PBST/block. After three washes, immunoreactivity was visualized with chemiluminescence, using the SuperSignal® West Femto Maximum Sensitivity Substrate reagents from Pierce Biotechnology (Rockford, IL). Chemiluminescence was measured directly with an Alpha Innotech FluroChem 8800 Imaging System and quantified using AlfaEaseFC software (San Leandro, CA). Results are presented as means ± S.E.M.

2.4. Radioimmuoassay of ecdysteroids

Thawed samples were vortexed, centrifuged at 15,000 × *g* for 5 min and 40 μl aliquots of the supernatant/PBS mix were assayed in duplicate for ecdysteroid content as described previously, using the H-22 antibody (Warren and Gilbert, 1986, 1988; Kiriishi et al., 1990). The results of the RIA experiments are expressed as percent of control, i.e., the ratio of the synthetic activity of each treated gland to its contralateral control multiplied by 100%. This ratio corrects for inter-animal variation and allows the comparison of results obtained from animals with varying basal ecdysteroid production. RIA results are presented as the mean ± S.E.M.

3. Results

3.1. PTTH stimulates multiple PKC-dependent phosphorylations

The subfamily of PKCs comprising the conventional PKCs (cPKCs) phosphorylates target proteins on serines or threonines with arginine or lysine at the −3, −2 and +2 positions, and an uncharged hydrophobic amino acid at position +1 (see Hofmann, 1997). A polyclonal antibody against the serine cPKC motif (Zhang et al., 2002) was used to assess the possibility that PTTH stimulates PKC activity in the prothoracic gland. The data indicate that PTTH stimulates multiple phosphorylations in both the larval and pupal prothoracic gland (Fig. 1), as recognized by the PKC substrate antibody. Only those proteins that are consistently phosphorylated in response to both recombinant PTTH and partially purified PTTH are labeled by molecular weight in Fig. 1. Unfortunately our supply and the activity of recombinant PTTH is currently too low to use in all experiments and, thus, we are unable to determine whether several additional phosphoproteins detected occasionally were phosphorylated in response to PTTH or in response to some other factor present in the partially purified preparation. Additionally, some proteins were inconsistently phosphorylated in response to both rPTTH and partially purified PTTH, e.g., the 66 kDa protein in Fig. 1, and it is unclear whether this variation indicates a handling artifact or a subtle developmentally-specific response for which the animal staging criteria are too broad to include consistently.

At least two PTTH-related phosphorylations were detected only after 4 h of PTTH stimulation and the phosphorylation of the 79 kDa protein was much higher in pupal than larval glands. Several PKC inhibitors, e.g., chelerythrine C and calphostin C, were tested for their effect on the phosphorylation of these proteins, as detected by the PKC substrate antibody (Fig. 2A). The results reveal that the PTTH-stimulated protein phosphorylations are inhibited by the PKC inhibitors and that both compounds affected the phosphorylation of the same proteins. These two inhibitors were used at the lowest concentrations that yielded