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Inhibition of tyrosine phosphorylation blocks hormone-stimulated calcium influx in an insect steroidogenic gland

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Abstract

In the tobacco hornworm *Manduca sexta* (*M. sexta*) as in other insects, ecdysone synthesis occurs in the prothoracic glands and is stimulated by the brain neuropeptide prothoracicotropic hormone (PTTH). PTTH activates the prothoracic glands through the second messenger cAMP, the synthesis of which is stimulated by calcium. We previously found that the Src kinase inhibitor 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-D]-pyrimidine (PP1) inhibits PTTH-stimulated cAMP synthesis and ecdysone secretion. In the present study, we show that by contrast, PP1 does not block cAMP synthesis stimulated by the calcium ionophore A23187, and that PP1 augments A23187-stimulated ecdysone secretion. Hence, once glandular levels of calcium are elevated, Src family kinase activity is no longer needed for, and may actually inhibit, steroidogenesis. PP1 blocks calcium influx in PTTH-stimulated prothoracic glands, indicating that tyrosine phosphorylation by a member of the Src kinase family is required for calcium influx. These results suggest that prothoracic gland calcium channels are regulated either directly or indirectly by tyrosine phosphorylation.

Keywords: Calcium; Ecdysone; Prothoracicotropic hormone; *Manduca sexta*; Src kinase

1. Introduction

The brain neuropeptide prothoracicotropic hormone (PTTH) is responsible for initiating the production of the insect molting hormones, ecdysteroids, by the prothoracic glands. In the tobacco hornworm, *Manduca sexta* (*M. sexta*), an early event in PTTH action is calcium influx through calcium channels in the plasma membrane (Birkenbeil, 1998; Birkenbeil, 2000). In prothoracic gland membrane preparations, adenylyl cyclase is not stimulated by PTTH but is stimulated by calcium/calmodulin (Meller et al., 1988; Meller and Gilbert, 1990). PTTH increases cAMP formation in prothoracic gland cells, as does the calcium ionophore A23187, with both effects being calcium-dependent (Smith et al., 1985). However, stimulation of ecdysteroidogenesis by cAMP analogs is not calcium-dependent. This indicates that calcium acts in the PTTH-stimulated pathway before cAMP synthesis. Cyclic AMP subsequently activates cAMP-dependent protein kinase, an event accompanied by protein S6 phosphorylation, and by enhanced protein synthesis (Keightley et al., 1990; Kulesza et al., 1994; Rybczynski and Gilbert, 1994; Smith et al., 1986). It is likely that ecdysone synthesis is stimulated by synthesis of an ephemeral protein, such as in vertebrate steroidogenic tissues (Papadopoulos et al., 1997; Stocco, 2001), although this has yet to be

Abbreviations: [Ca^{2+}], calcium ion concentration; cAMP, adenosine 3′,5′-cyclic monophosphate; CYP17, 17-alpha-hydroxylase,17,20-lyase; DHEA(S), dehydroepiandrosterone sulfate; DMSO, dimethylsulfoxide; ERK, extracellular signal-regulated kinase; GDPβS, guanosine-5′-O-(2-thiodiphosphate); kD, kilodalton; MAPK, mitogen-activated protein kinase; PTTH, prothoracicotropic hormone; PP1, 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-D]-pyrimidine; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-D]pyrimidine; PP3, 4-amino-7-phenylpyrazolo[3,4-D]pyrimidine; RIA, radioimmunoassay; Src-family kinase, protein-tyrosine kinase family homologous to the *Rous sarcoma* virus oncogene protein pp60(v-Src); StAR, steroidogenic acute regulatory protein; TRP, transient receptor potential

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shown. In addition, PTTH stimulates rapid phosphorylation of the MAP kinase ERK, a calcium-stimulated event that is dissociable from the action of cAMP (Rybczynski et al., 2001; Rybczynski and Gilbert, 1994; Rybczynski and Gilbert, 2003).

M. sexta prothoracic glands possess voltage-dependent calcium channels (Eusebio and Moody, 1986), though the calcium channels regulated by PTTH are not known. Both L- and T-type channel inhibitors have been found to reduce glandular response (Birkenbeil, 1998; Birkenbeil, 2000). Intracellular addition of the G-protein inhibitor GDPβS does not block PTTH-stimulated calcium influx, suggesting that G proteins are not involved in the control of PTTH-sensitive calcium channels (Birkenbeil, 2000).

Receptor tyrosine kinases, such as growth factor receptors, and non-receptor tyrosine kinases, such as Src, have been implicated in the regulation of voltage-gated and store-operated calcium channels (Davis et al., 2001; Tsunoda, 1998). Recent work by Smith et al. (2003) shows that PTTH stimulates tyrosine phosphorylation of several prothoracic gland proteins, and that the Src kinase inhibitor PP1 inhibits phosphorylation of these proteins. In addition, PP1 blocks PTTH-stimulated ecdysone and cAMP synthesis (Smith et al., 2003). These findings indicate that a Src kinase is required at an early step in PTTH action. Here, we report that activation of a Src kinase is required prior to calcium influx in PTTH-stimulated ecdysone production. This finding suggests that a Src-family kinase may in some manner regulate prothoracic gland calcium channels.

2. Materials and methods

2.1. Animals

M. sexta eggs were obtained from Carolina Biological Supply (Burlington, NC) or from adults raised from this stock. Larvae were reared on an artificial diet (Bell and Joachim, 1976) at 25 °C under a photoperiod of 16 h-light/8 h-dark cycle. Day 2 fifth larval instar animals were used for experimentation (weight range 4–6 g).

2.2. Reagents

Grace's insect culture medium was obtained from Life Science Technologies (Grand Island, NY). PP1, obtained from BioMol (Plymouth Meeting, PA), was prepared as a stock in DMSO and diluted in Grace's for use in experiments. PP3, obtained from Calbiochem (La Jolla, CA), was prepared similarly. A23187, obtained from ICN Biomedicals (Costa Mesa, CA), was prepared as a 24 mM stock in DMSO and diluted in Grace's to 24 μM for use in experiments. The ecdysone antibody was produced in rabbits against an ecdysone-22-succinyl thyroglobulin synthesized by Dr. D.H.S. Horn (C.S.I.R.O., Canberra, Australia). The calcium indicator Oregon-green dextran BAPTA was obtained from Molecular Probes (Eugene, OR) and prepared as a 75% solution in deionized water for use in experiments. Anti-phosphotyrosine primary antibody and the secondary antibody, anti-mouse IgG, were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

2.3. PTTH

Recombinant PTTH was obtained from H. Kataoka (University of Tokyo) and used at 3 nM in initial experiments. We found the stock to have lost considerable activity at the time of calcium imaging experiments, hence for this portion of the study, brain extracts containing big PTTH were prepared. PTTH extraction was done using frozen day 0 pupal brains homogenized in cold acetone, briefly centrifuged at 1000 × *g* and supernate removed. The remaining sample was then homogenized in cold 2% NaCl and centrifuged for 10 min at 10,000 × *g*. The resulting supernate was heated at 95 °C for 3 min, cooled on ice, and centrifuged for 10 min at 10,000 × *g*. In order to separate big PTTH from other extract materials, the resulting supernate was centrifuged through an Amicon-30 filter, 30 kD MW cut-off (Millipore, Billerica, MA). Mature PTTH is a glycosylated dimmer, which we have found is largely retained in the >30 kD fraction. Retentate was brought up in 2% NaCl, tested for PTTH activity by a standard assay (Bollenbacher et al., 1983) and stored at −80 °C.

2.4. Cyclic AMP and ecdysone radioimmunoassays

Larval prothoracic glands were dissected into lepidopteran saline and maintained in Grace's medium for periods of less than 1 h prior to experimentation. Individual glands were pre-incubated for 20 min in 30 μl PP1, then transferred to fresh droplets containing no PTTH (control), PTTH, A23187, and/or PP1 for designated times. Control glands were placed in medium containing 0.1% DMSO. Experiments were terminated by placing glands directly into ice-cold 0.6 M perchloric acid (for cAMP assays), and medium was removed and stored at −20 °C for ecdysone RIAs. Cyclic AMP radioimmunoassays were conducted using a kit from Biomedical Technologies Inc. (Stoughton, MA), as previously described (Smith and Pasquarello, 1989). Ecdysone RIAs were conducted as previously described (Warren et al., 1984).

2.5. Western blots

Larval prothoracic glands were incubated in test or control solutions for the designated incubation periods, placed into SDS–sample buffer, and boiled for 3–5 min. Details of Western blot procedures are the same as those described previously (Smith et al., 2003). Samples were run on 8% SDS–PAGE gels to separate proteins. The samples were then transferred from gels to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) at 4 °C for 60–75 min. The membranes were incubated with anti-phosphotyrosine antibody (PY99) and secondary anti-mouse antibody, and treated