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Protein kinase A and C are "Gatekeepers" of capacitative Ca^{2+} entry in the prothoracic gland cells of the silkworm, *Bombyx mori*

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ABSTRACT

Application of protein kinases A and C inhibitors to the prothoracic glands cells of the silkworm, *Bombyx mori*, resulted in slow and gradual increases in intracellular Ca^{2+} ($[Ca^{2+}]_i$). Pharmacological manipulation of the Ca^{2+} signalling cascades in the prothoracic gland cells of *B. mori* suggests that these increases of $[Ca^{2+}]_i$ are mediated neither by voltage-gated Ca^{2+} channels nor by intracellular Ca^{2+} stores. Rather they result from slow Ca^{2+} leak from plasma membrane Ca^{2+} channels that are sensitive to agents that inhibit capacitative Ca^{2+} entry and are abolished in the absence of extracellular Ca^{2+}. Okadaic acid, an inhibitor of PP1 and PP2A phosphatases, blocked the increase in $[Ca^{2+}]_i$ produced by the inhibitors of protein kinase A and C. The combined results indicate that the capacitative Ca^{2+} entry channels in prothoracic gland cells of *B. mori* are probably modulated by protein kinases A and C.

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1. Introduction

Decades of research in *Manduca sexta* or *Bombyx mori* prothoracic glands has shown that this ecdysteroidogenic tissue represents a model system for research into steroidogenesis, because it possesses a repertoire of signalling pathways that are developmentally interlinked and dynamically regulated (Rybczynski, 2005). The signalling pathways are so complicatedly linked that, although recent research has shed light in them (Rybczynski, 2005), there are certain aspects that are still regarded as a "black box".

Changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) in prothoracic gland cells are regulated by the prothoracicotropic hormone, a brain neurosecretory protein that stimulates the prothoracic glands to secrete ecdysteroid by stimulating Ca^{2+} influx (Fellner et al., 2005; Priester and Smith, 2005). This protein acts through a transduction cascade requiring extracellular Ca^{2+} (Smith, 1995) and results in activation of a Ca^{2+}/calmodulin-dependent adenylyl cyclase (Meller et al., 1988; Rybczynski, 2005) and increased cAMP levels (Smith, 1995). Although the exact regulatory mechanisms responsible for increases in $[Ca^{2+}]_i$ in prothoracic gland cells are not well understood, pharmacological manipulation of $[Ca^{2+}]_i$ can help identify and distinguish between the various mechanisms underlying the regulation of $[Ca^{2+}]_i$ in these cells (Fellner et al., 2005; Priester and Smith, 2005; Dedos et al., 2005). Such research has identified that activation of Ca^{2+}/calmodulin-dependent adenylyl cyclase(s) in prothoracic gland cells leads to activation of protein kinase A and subsequently increases in ecdysteroid secretion (Rybczynski, 2005). Activation of protein kinase A is therefore regarded as one of the terminal steps in the signalling cascade that leads to ecdysteroid secretion although recent research implicates also protein kinase C as a modulator of ecdysteroidogenesis in the prothoracic gland cells (Rybczynski and Gilbert, 2006). Although the exact role protein kinase A plays in ecdysteroidogenesis is known (Rybczynski, 2005) the exact role of protein kinase C is not yet clarified in detail (Rybczynski and Gilbert, 2006).

In this study we present evidence which suggest that a complex regulatory mechanism exists at the level of activation of both protein kinases A and C. Our results suggest that both of these kinases operate a negative feedback mechanism at the level of extracellular Ca^{2+} entry through capacitative Ca^{2+} entry channels (Putney, 2007). Pharmacological inhibition of either or both of these kinases leads to small but sustained increases in $[Ca^{2+}]_i$ levels in the prothoracic glands cells of the silkworm, *B. mori*.

2. Materials and methods

2.1. Insects

Silkworms, *B. mori* (hybrid J106xDAIZO), were reared on an artificial diet (Nihon Nosan Kogyo Co., Yokohama, Japan) kindly

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provided by Dr. S. Cappellozza (Istituto Sperimentale per la Zoologia Agraria, Sezione Specializzata per la Bachicoltura, Padova, Italy), under a 12:12-L:D photoperiod at 25 ± 1 °C and 60% relative humidity. Larvae were staged after every larval ecdysis, and the day of each ecdysis was designated as day 0. Since the larvae mainly moulted to the final (5th) instar during the scotophase, all the larvae that ecdysed during the scotophase were segregated immediately after the onset of photophase. This time was designated as 0 h of the 5th instar. In this particular hybrid, the 5th instar period lasts about 208 h. The onset of wandering behaviour occurred 144 h (day 6) after the final larval ecdysis.

2.2. Reagents

Forskolin, Adenosine 3′,5′-cyclic Phosphorothioate-Sp (Sp-cAMPS), Adenosine 3′,5′-cyclic Phosphorothioate-Rp (Rp-cAMPS), KT 5720, 12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole (Gö 6976), 2-Aminoethoxydiphenyl borate (2-APB), Fura-2/AM, nitrendipine, ryanodine and okadaic acid sodium salt were purchased from Calbiochem (Bad Soden, Germany). Heparin, gadolinium chloride, lanthanum chloride, dimethyl sulfoxide (DMSO), Ethylene glycol-bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and all other reagents were from Sigma (Deisenhofen, Germany). Stock solutions of forskolin, KT 5720, Gö 6976, and okadaic acid were prepared in DMSO.

2.3. Photomultiplier-based microfluorometry

Dissected *B. mori* prothoracic glands were loaded with 40 μM Fura-2/AM in 1 ml of Ringer's saline (Shirai et al., 1994) for 60 min at 25 °C in the dark. Then glands were rinsed in Ringer's saline for 5 min and placed in 50 μl of the same saline on a cover slip in a tight chamber. In experiments requiring Ca^{2+}-free medium, Ringer's saline was prepared using NaCl (4.5 mM) in place of the standard 4.5 mM $CaCl_2$. HEPES buffer (0.01 M, pH 6.8) and EGTA (0.1 mM) were added prior to use. DMSO, when present, did not interfere with the fluorescence recordings and did not affect intracellular Ca^{2+} levels in the concentrations used (1%). Dual-wavelength measurements were made with a Zeiss (Oberkochen, Germany) microscope-photometer MPM 200 mounted on an inverted Zeiss microscope "Axiovert 135" (with an objective "Fluar" 10x/0.50). Emitted fluorescence was collected through an aperture adjusted to the size of prothoracic gland cells. Background fluorescence was estimated with unloaded cells. The measurements of fluorescence intensity (F) at the two excitation wavelengths were used to calculate $[Ca^{2+}]_i$, assuming a K_d of 224 nM (Grynkiewicz et al., 1985). Free intracellular Ca^{2+} was measured in nine identified cells of each prothoracic gland and was recorded as mean ± S.E.M. Addition of all reagents was made by replacement of the whole bathing medium or by addition of 10 μl saline with a pipette. All reagents added were tested for autofluorescence.

2.4. Statistical analyses

GraphPad Prism™ 4.0 computer software was used for all statistical analyses. Statistical significance of the results was determined by analysis of variance (ANOVA) followed by Tukey multiple comparisons test.

3. Results

3.1. Effects of cAMP signalling pathway modulators on $[Ca^{2+}]_i$ of the prothoracic gland cells on day 6 of the 5th instar of B. mori

Research with prothoracic glands of *B. mori* (Dedos and Birkenbeil, 2003) showed that forskolin and a cAMP analogue, 8-Br-cAMP, increase $[Ca^{2+}]_i$ levels of prothoracic gland cells. We wanted to expand these initial observations and use other similarly acting reagents to test whether these increases in $[Ca^{2+}]_i$ in prothoracic gland cells are mediated by protein kinase A. At a first instance we tested the effects of forskolin in the presence or absence of extracellular Ca^{2+} and observed that forskolin caused an increase of $[Ca^{2+}]_i$ in prothoracic gland cells only when extracellular Ca^{2+} was present. However, unlike 8-Br-cAMP (Dedos and Birkenbeil, 2003), the protein kinase A activator, Sp-cAMPS, did not yield the same results i.e. did not increase $[Ca^{2+}]_i$ in prothoracic gland cells (Fig. 1B). On the contrary, an inhibitor of protein kinase A, Rp-cAMPS, unexpectedly produced an increase in $[Ca^{2+}]_i$ in prothoracic gland cells (Fig. 1B).

3.2. Effects of intracellular Ca^{2+} modulating agents on the ability protein kinases A and C inhibitors to mediate increases of $[Ca^{2+}]_i$ in the prothoracic gland cells of B. mori

The increases of $[Ca^{2+}]_i$ in prothoracic gland cells produced by Rp-cAMPS were modest, never exceeded more than ~50 nM of $\Delta[Ca^{2+}]_i$ during the recording period and could be mimicked by

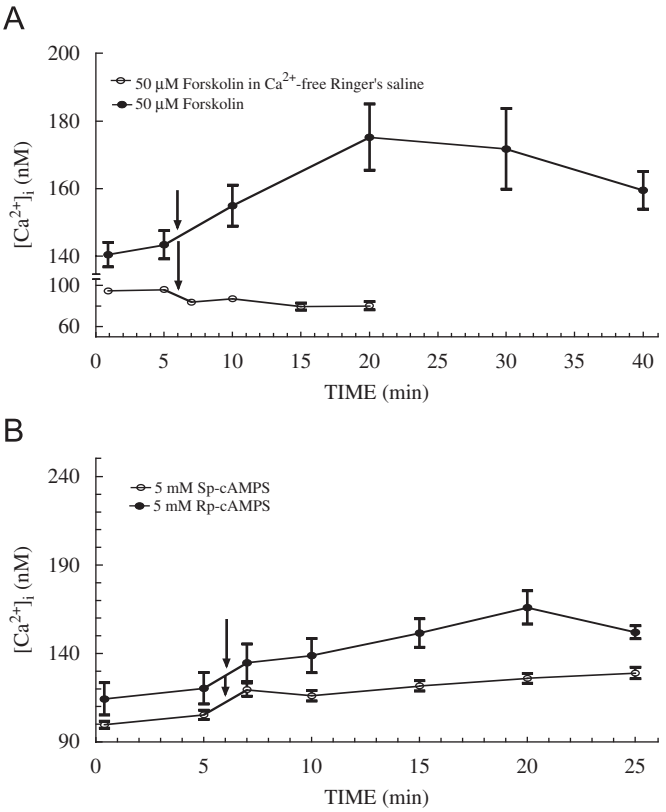

Fig. 1. Effect of forskolin (panel A) and Sp-cAMPS or Rp-cAMPS (panel B) on $[Ca^{2+}]_i$ of *B. mori* prothoracic gland cells. Day 6 prothoracic glands from 5th instar larvae of *B. mori* were loaded with Fura-2/AM and then incubated in Ringer's saline. The arrows indicate the time of addition of each reagent. Results of Tukey multiple comparisons test revealed that foskolin in Ca^{2+}-free Ringer's saline (panel A) and Sp-cAMPS (panel B) did not produce any statistically significant increase of $[Ca^{2+}]_i$ ($P > 0.05$). Each data point is the mean ± SEM of 9–18 independent measurements of prothoracic gland cells.

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