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Intracellular mobilization of Ca^{2+} by the insect steroid hormone 20-hydroxyecdysone during programmed cell death in silkworm anterior silk glands

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ABSTRACT

20-Hydroxyecdysone (20E) triggers programmed cell death (PCD) and regulates *de novo* gene expression in the anterior silk glands (ASGs) of the silkworm *Bombyx mori*. PCD is mediated via a nongenomic pathway that includes Ca^{2+} as a second messenger and the activation of protein kinase C/caspase-3-like protease; however, the steps leading to a concomitant buildup of intracellular Ca^{2+} are unknown. We employed pharmacological tools to identify the components of this pathway. ASGs were cultured in the presence of 1 μM 20E and one of the following inhibitors: a G-protein-coupled receptor (GPCR) inhibitor, a phospholipase C (PLC) inhibitor, an inositol 1,4,5-trisphosphate receptor (IP_3R) antagonist, and an L- or T-type Ca^{2+} channel blocker. The T-type Ca^{2+} channel blocker inhibited 20E-induced nuclear and DNA fragmentation; in contrast, PCD was induced by 20E in Ca^{2+}-free medium, indicating that the source of Ca^{2+} is an intracellular reservoir. The IP_3R antagonist inhibited nuclear and DNA fragmentation, suggesting that the endoplasmic reticulum may be the Ca^{2+} source. Finally, the GPCR and PLC inhibitors effectively blocked nuclear and DNA fragmentation. Our results indicate that 20E increases the intracellular level of Ca^{2+} by activating IP_3R, and that this effect may be brought about by the serial activation of GPCR, PLC, and IP_3.

1. Introduction

20-Hydroxyecdysone (20E) is an insect steroid hormone that plays important roles in larval growth and metamorphosis. During metamorphosis, 20E triggers programmed cell death (PCD) in various tissues, including larval-specific muscles (Schwartz, 1992), the motoneurons innervating those muscles (Streichert et al., 1997), and anterior silk glands (ASGs) (Terashima et al., 2000; Kakei et al., 2005). In the silkworm *Bombyx mori*, ASGs undergo degeneration through PCD in response to a metamorphic increase in hemolymphatic ecdysteroid at the end of the last (fifth) larval instar (Chinzei, 1975; Terashima et al., 2000). 20E-induced PCD is characterized by a series of cellular changes, including cell shrinkage, nuclear condensation, DNA fragmentation, nuclear fragmentation, and apoptotic body formation (Terashima et al., 2000). Cycloheximide (CHX), a protein synthesis inhibitor, inhibited induction of cell shrinkage and apoptotic body formation induced by 20E. Such indicates that *de novo* gene expression is required for these cellular responses, and therefore, they are under the control of the genomic pathway. By contrast, 20E induces nuclear condensation, DNA fragmentation, and nuclear fragmentation in the presence of CHX (Iga et al., 2007), indicating that these events are mediated through a nongenomic pathway. Thus, 20E functions via both genomic and nongenomic pathways, and both are required to initiate PCD. In the nongenomic pathway, Ca^{2+} acts as a second messenger to activate protein kinase C (PKC) and caspase-3-like protease, which leads to DNA and nuclear fragmentation (Iga et al., 2007), but the events leading to an increase in the intracellular Ca^{2+} concentration are unknown.

In vertebrates, a rapid increase in the intracellular Ca^{2+} level in response to steroid hormones has been widely demonstrated. For example, estrogen increases the intracellular Ca^{2+} concentration in maturing chicken oocytes (Morley et al., 1992). Other examples include progesterone in human spermatozoa (Baldi et al., 1991; Blackmore et al., 1991; Falkenstein et al., 1999) and pig ovarian granulosa cells (Machelon et al., 1996), aldosterone in cultured rat skeletal muscle cells and mouse cortical collecting duct cells (Estrada et al., 2000; Harvey and Higgins, 2000), and vitamin D in

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mouse osteoblasts and osteoblast-like cells (Civitelli et al., 1990; Lieberherr, 1987). Such increases in the cytosolic Ca^{2+} level are brought about by an influx of extracellular Ca^{2+} through calcium channels and/or an efflux of Ca^{2+} from an intracellular reservoir in response to steroid binding to a G-protein-coupled receptor (GPCR) (Marinissen and Gutkind, 2001). The increase in cytosolic Ca^{2+} by GPCR activation is mediated through a steroid signaling pathway that begins with the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C (PLC), resulting in the production of the intracellular second messenger inositol 1,4,5-triphosphate (IP_3) (Rhee, 2001). The binding of IP_3 to its receptor IP_3R on the endoplasmic reticulum (ER) causes the rapid release of Ca^{2+} from the ER into the cytosol (Berridge, 1993; Szlufcik et al., 2006).

B. mori ASG cells are believed to carry a putative membrane ecdysone receptor (mEcR) on the plasma membrane (Elmogy et al., 2004, 2006), and 20E-induced PCD has been shown to be mediated by an increase in cytosolic Ca^{2+} (Iga et al., 2007). Thus, the steps leading to an increased intracellular Ca^{2+} concentration during 20E-induced PCD may be similar to the nongenomic mechanism demonstrated for vertebrate steroids. In this study, using pharmacological tools, we identified a pathway beginning at the plasma membrane that leads to the activation of a $PLC/IP_3/IP_3R$ pathway.

2. Materials and methods

2.1. Animals

B. mori larvae (Kinshu × Showa) were reared on an artificial diet (Silkmate II; Nihon Nosan Kogyo, Yokohama, Japan) at 25 °C under a 12-h light:12-h dark cycle and staged as previously described (Sakurai et al., 1998). Only those fifth instar larvae that exhibited gut purge in the scotophase on day 6 or 7 of the instar were used (Iga et al., 2007).

2.2. Reagents

20E, suramin sodium salt (a GPCR inhibitor), verapamil hydrochloride (an L-type Ca^{2+} channel blocker), flunarizine dihydrochloride (a T-type Ca^{2+} channel blocker), 2-APB (an IP_3R antagonist), and U73122 (a PLC inhibitor) were obtained from Sigma (St. Louis, MO). 20E was dissolved in water (1 mg/ml) and stored at −20 °C. All other chemicals were dissolved individually in dimethylsulfoxide (DMSO) or sterilized water at the desired concentration and stored at −20 °C for up to 3 months according to the manufacturer's recommendations. The concentration of each chemical in DMSO was adjusted to give a final concentration of less than 5% DMSO in the culture medium; at this concentration, DMSO had no effect on the response of the ASGs to 20E.

2.3. Tissue culture

ASGs were dissected under dry conditions, then rinsed with Grace's insect cell culture medium (Gibco BRL, Rockville, MD) or Ca^{2+}-free Grace's medium (Grace, 1962) and cultured individually in 0.3 ml of medium using 24-well plates (Greiner Bio-One, Frickenhausen, Germany) at 25 °C for 120 h. Cellular morphology was recorded daily according to the PCD scoring system described by Terashima et al. (2000).

2.4. 4′,6-Diamidino-2-phenylindole (DAPI) staining

ASGs were fixed in 4% formaldehyde for 30 min followed by washing with phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.47 mM KH_2PO_4, pH 7.4), and then incubated in PBS containing 0.1 μg/ml DAPI (Sigma) at 25 °C in the dark for 10 min. The ASGs were then washed with PBS and observed under a fluorescence microscope using a UV excitation filter (BX-50; Olympus, Tokyo, Japan). All images were processed with Photoshop® (Adobe Systems Inc., San Jose, CA).

2.5. Detection of DNA fragmentation

ASGs were homogenized and mixed with DNA extraction buffer (10 mM Tris–HCl, 150 mM NaCl, 10 mM EDTA–NaOH, and 0.1% SDS, pH 8.0) on ice, and then the homogenates were treated with RNase (20 μg/ml, 37 °C for 30 min) and proteinase K (100 μg/ml, 50 °C for 60 min). DNA was extracted by standard procedures using phenol–chloroform and chloroform, and 1 μg of each sample was loaded onto a 2% agarose-S gel for electrophoresis. The gels were stained with ethidium bromide (EtBr) and observed under a UV transilluminator connected to a CCD gel scanning system (Bioinstrument; Atto Corp., Tokyo, Japan).

3. Results

3.1. Effects of Ca^{2+} channel blockers on 20E-induced PCD

Ca^{2+} serves as a second messenger in the nongenomic pathway of 20E (Iga et al., 2007). We examined an involvement of an extracellular Ca^{2+} influx by inhibiting a possible Ca^{2+} influx using an L-type Ca^{2+} channel blocker, verapamil, and a T-type Ca^{2+} channel blocker, flunarizine (Fig. 1). When the blockers were added at the start of the culture period with 1 μM 20E, 0.1 mM flunarizine inhibited most of the changes in cellular morphology associated with PCD progression (Fig. 1A). When the glands were cultured at lower concentrations, no difference was noted in the progression of PCD between the glands cultured with 20E and the inhibitors and those cultured with 20E only (Fig. 1B); moreover, it completely blocked nuclear fragmentation and DNA fragmentation (Fig. 1C) at a concentration of 0.1 mM, whereas verapamil exerted no inhibitory effect on either event at the highest concentration examined (0.1 mM; data not shown). Nuclear condensation did, however, occur in the presence of both blockers at the same time as in ASGs cultured with 20E alone (data not shown), indicating that the pathway through which nuclear condensation is induced differs from that leading to DNA and nuclear fragmentation. Flunarizine did not exert these effects when added at 18 h of culture or later (Fig. 1D), indicating that the portion of the pathway affected by flunarizine may be activated by 20E within 18 h of a 20E challenge.

3.2. Induction of PCD by 20E in Ca^{2+}-free medium

The inhibitory effects of flunarizine suggested the involvement of a T-type channel at the plasma membrane in the influx of Ca^{2+}. If this were the case, the influx of Ca^{2+} would depend on the amount of extracellular Ca^{2+} (i.e., the amount of Ca^{2+} in Grace's medium); therefore, PCD in those glands cultured with 20E in Ca^{2+}-free medium should be incomplete. To test this postulate, we cultured ASGs with 20E in Ca^{2+}-free medium. Unexpectedly, the rate of PCD in the Ca^{2+}-free medium was exactly the same as that in normal Grace's medium (Fig. 2). To ensure the removal of Ca^{2+} from the medium, we added EGTA, a divalent cation chelator, to the Ca^{2+}-free medium and cultured ASGs with 20E in the medium for 144 h. Under these conditions, the rate of PCD again matched the rate in normal Grace's medium (Fig. 2A); in addition, DNA fragmentation occurred at the same time in both types of media (Fig. 2B). These findings indicate that the rise in cytosolic Ca^{2+} in response to 20E