Protein Kinase B/Akt Induces Resumption of Meiosis in Xenopus Oocytes*

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The activation of protein kinase B/Akt is thought to be a critical step in the phosphoinositide 3-kinase pathway that regulates cell growth and differentiation. Because insulin-like growth factor 1 stimulates the resumption of meiosis in Xenopus laevis oocytes via phosphoinositide 3-kinase activation, we investigated the Akt involvement in this process. Injection of mRNA coding for a constitutively active Akt in Xenopus oocytes induced germinal vesicle breakdown (GVBD) to the same extent as progesterone or insulin treatment. Injection of mRNA coding for the wild type Akt kinase was less effective in stimulating GVBD, whereas Akt bearing a lysine mutation in the catalytic domain that abolishes the kinase activity had no effect. A mutant Akt lacking a membrane-targeting sequence did not induce GVBD, despite high levels of expression and activity. As previously reported for insulin, induction of GVBD by Akt was prevented by incubating the oocytes with cisteamide, an inhibitor specific for the type 3 phosphodiesterase (PDE3), suggesting that the activity of a PDE is required for Akt action. That an increase in PDE activity in the oocyte is sufficient to induce meiotic resumption was demonstrated by expression of an active PDE protein. In addition, the constitutively active Akt caused a 2-fold increase in the activity of the endogenous PDE. These data demonstrate that Akt is in the pathway controlling resumption of meiosis in the Xenopus oocyte and that regulation of the activity of a PDE3 is a step distal to the kinase activation.

A critical step in growth factor stimulation of the target cell is the activation of the phosphoinositide 3-kinase (PI3-K)1 pathway. This signaling pathway has been implicated in a wide array of cellular events including mitogenesis, transformation, differentiation, and regulation of metabolism (1). Recently Akt, also known as protein kinase B or related to the A and protein kinase C (2–4), was identified as a kinase distal to PI3-K (1). Three Akt isoforms (α, β, and γ) with closely related properties have been identified. These are proteins of approximately 60 kDa containing a pleckstrin homology (PH) domain (5) and a serine/threonine kinase domain structurally related to the catalytic domains of protein kinases A and C. Phosphatidylinositol 3,4-bisphosphate and 3,4,5-trisphosphate, the products of PI3-K, bind to the PH domain of Akt and serve to anchor the enzyme to the membrane as well as to induce a conformational change in the enzyme (6). The subsequent phosphorylation of Akt, at Thr-308 in the catalytic domain and Ser-473 of the C terminus of Aktα, is crucial for the activation of Akt (7, 8). At least one of the kinases phosphorylating Akt has been identified as the 3-phosphoinositide-dependent kinase-1 (9–11).

Akt is activated by insulin and IGF-1 (12), and this regulation mediates the activation of glucose uptake (13), the phosphorylation and deactivation of GSK3 (14), and activation of p70 S6 kinases (15). Akt also phosphorylates the proapoptotic protein Bad, promoting its interaction with 14-3-3 and thereby preventing apoptosis (16).

In somatic cells including adipocytes, insulin regulates the activation of cGMP-inhibited phosphodiesterase (PDE3) (17). This activation is mediated by PI3-K because wortmannin, a PI3-K inhibitor, blocks the insulin-dependent activation of the PDE (18). The PDE3B isoform expressed in adipocytes is phosphorylated following insulin stimulation, and the phosphorylation is associated with an increase in PDE activity (19). This PDE activation and the consequent decrease in intracellular cAMP are probably responsible for the decrease in the hormone-sensitive lipase activity (17) and for the antilipolytic effects of insulin. The properties of the kinase phosphorylating PDE3B have not been firmly established, although a recent report indicates that Akt coelutes with a kinase activity that phosphorylates PDE3B in a cell-free system (20).

In Xenopus laevis oocytes, IGF-1 induces the dissolution of the nuclear membrane (GVBD) and completion of the first meiotic division (21). Although distinct signaling pathways may be activated, IGF-1, or the physiological stimulus progestrone, activates meiotic resumption by inducing a transient decrease in intracellular cAMP levels, thereby reducing the protein kinase A activity (22, 23). It has been proposed that activation of a PDE and a decrease in cAMP are crucial steps for reentry into the cell cycle. The effects of insulin and IGF-1 on meiosis are blocked by cisteamide, a specific inhibitor of the type 3 family of phosphodiesterases (24). Furthermore, insulin treatment or injection of an activated Ras leads to an increase in the PDE activity in the Xenopus oocyte (25).

Here we have tested the hypothesis that Akt is involved in the IGF-1-induced resumption of meiosis by activating a PDE present in the Xenopus oocyte. Our data demonstrate that Akt is a signal for resumption of meiosis in the Xenopus oocyte and that activation of a PDE3 is a step distal to the Akt activation.

MATERIALS AND METHODS

Construction of mRNA Expression Vectors—The constructs encoding the wild type (WT)-Akt, myristoylated (myr)-Akt, K179M-Akt, and A2myr-Akt have been described (12, 13). These constructs were transferred from the pECE vector to the XbaI-SalI sites of the mRNA expression vector pSP64(poly(A)) (Promega, Madison WI). The pCMV-5 iSH2-p110 (a gift from M. Birnbaum) was subcloned into pSP64(poly(A)) using XbaI and BamHI for the p110 subunit and XbaI

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The abbreviations used are: PI3-K, phosphatidylinositol 3-kinase; PDE, phosphodiesterase; IGF-1, insulin-like growth factor 1; HA, hemagglutinin; myr, myristoylation; PH, pleckstrin homology; GVBD, germinal vesicle breakdown; WT, wild type.
for the inter-SH-2 domain (iSH2-I) of p85. The pCMV-5 rat PDE4D3 construct has been described previously (26). The complete open reading frame of PDE4D3 was subcloned from pCMV-5 into EcoRI of pBluescript KSII and then into pSP64(poly(A)) using AvaI-HindIII. All Akt constructs had the influenza virus HA epitope tag fused in-frame to the C terminus to monitor protein expression.

In Vitro mRNA Synthesis—To express Akt, PI3-K, and PDE4D3 mRNAs, the pSP64(poly(A)) constructs were transcribed using the SP6 polymerase according to the procedure supplied by the manufacturer (Message Machine Kit, Promega). The transcribed mRNA was purified by phenol/chloroform extraction, precipitated at −20 °C with 1 volume of isopropanol alcohol, and resuspended in diethylpyrocarbonate water. The mRNA concentration was measured by A260, and transcript size was determined by formaldehyde gel electrophoresis. The mRNA was diluted to 1 ng/μl in diethylpyrocarbonate water and stored at −70 °C.

Injection into Xenopus Oocytes—Ovary fragments were surgically removed from pregnant mare serum gonadotropin-primed X. laevis, and the oocytes were isolated manually. Stage VI oocytes were selected for all the experiments. Oocyte storage and experiments were carried out in OR2 solution (82.5 mM NaCl, 2.5 mM KCl, 1.0 mM CaCl2, 1.0 mM MgCl2, 1.0 mM Na2HPO4, 5.0 mM HEPES, pH 7.8). Oocytes were routinely tested for their insulin responsiveness by incubation of 10 oocytes in 1 μl of OR2 solution overnight at room temperature. Oocytes that exhibited an insulin-stimulated GVBD of 80–100% were used. The mRNAs (0.5 μl) (vehicle) was injected using a micromanipulator (Drummond) into defolliculated Xenopus oocytes. Resumption of meiosis was scored by the appearance of a white spot on the animal pole of the oocyte.

Western Blot Analysis—Expression of HA-tagged Akt was analyzed after lysing injected oocytes in 10 μl of lysis buffer (250 mM sucrose, 1 mM KCl, 1 mM MgCl2, 0.2 mM phenylmethylsulfonflouride) per oocyte. Oocyte extracts were isolated by centrifugation at 15,000 × g for 10 min at 4 °C. The lipid supernatant was removed, and the clarified supernatants were transferred to Eppendorf tubes. Oocyte extract were analyzed by gel electrophoresis on 8% SDS-polyacrylamide gel electrophoresis (1:30 bis-acrylamide). After transfer, nitrocellulose membranes were blocked overnight at 4 °C in 5% bovine serum albumin in Tris-buffered saline with 0.02% Tween 20. Immunoassaying to detect Akt-HA expression was performed by incubating for 1 h with a 1:1,000 dilution of an anti-rabbit Ig conjugated to horseradish peroxidase (Amersham Pharmacia Biotech). Blots were visualized by the ECL procedure (Amersham Pharmacia Biotech). Western blot for endogenous ERK2 phosphorylation was performed as described previously (27).

In Vivo PDE Assay—PDE activity was measured in Xenopus oocytes by the method described by Sadler and Muller (23). In brief, oocytes were first stimulated with insulin or injected with either PI3-K, K179M-Akt, or myr-Akt mRNA. Three hours later, they were reinfected with 50 nl of 2 mM H[3]HcAMP to a final concentration of 200 μM (600–700 cpm/μl). The reaction products were separated and quantified as previously described (25, 28).

Akt Assay—Oocytes were lysed as described above, and the expressed Akt was immunoprecipitated and analyzed for activity as described (12). GSK peptide GRPRTSSFAEG was used as substrate (14). Akt activity was determined by both densitometry and scintillation counting.

RESULTS

Akt Stimulation of Meiotic Resumption in Xenopus Oocytes—To test whether Akt is involved in the resumption of meiosis, an mRNA encoding a constitutively active Akt (7) was microinjected in Xenopus oocytes; in this construct, the Akt coding region with a deletion in the PH domain (Δ4–129) is fused to a 14-amino acid leader containing the myr sequence of src (myr-Akt). This engineered protein is constitutively active in ST3L cells by being targeted to the membrane (7). The expression of this constitutively active Akt caused resumption of meiosis. Western blot analyses of Xenopus extracts with mitogen-activated protein kinase antibodies verified that injection of the constitutively active Akt, but not the kinase-dead mutant, induces phosphorylation of ERK2 together with the induction of GVBD (data not shown).

Activation of a PDE3 Is Distal to Akt in the Maturation Pathway—Previous work by Sadler (24) had shown that the maturation induced by insulin and IGF-1 is blocked by type 3 PDE inhibitors. To investigate whether a PDE is distal to Akt, different concentrations (0.2, 0.5, and 1.0 mg/ml) of constitutively active Akt (myr-Akt) mRNA were injected into Xenopus oocytes preincubated with inhibitors of PDE3. A 1-h preincubation with 10 μM cilostamide effectively prevented the myr-Akt-induced meiotic resumption (Fig. 3). Under the same conditions, 10 μM cilostamide also blocked insulin-induced...
maturation (data not shown). Whereas preincubation of oocytes in 10 μM cilostamide reduced the effect of microinjected myr-Akt no inhibition was observed by preincubating oocytes with a type 4 PDE inhibitor, rolipram (data not shown). This suggests that Akt-stimulated resumption of meiosis requires the activity of a PDE3, as does IGF-1-stimulated oocyte maturation (24).

To determine whether PDE activation per se is sufficient to induce resumption of meiosis, a PDE mRNA was injected into the oocytes. The mRNA coding for a PDE4 (PDE4D3) rather than PDE3 was used for these experiments to distinguish the expressed PDE from the endogenous PDE activity. Injection of the PDE4D3 mRNA induced GVBD to the same extent as progesterone or insulin treatment (Fig. 4). This effect was blocked by inhibitors specific for type 4 PDE (10 μM rolipram) but not by inhibitors specific for type 3 PDE (cilostamide) (Fig. 4). This finding demonstrates that the activity of the expressed PDE4 is required for induction of meiosis. It is also important to note that cilostamide could not block the meiotic resumption induced by the PDE4D3 injection, thus ruling out the possibility that the effect of this inhibitor on the Akt-stimulated maturation is because of effects other than PDE3 inhibition.

To test whether Akt activates the endogenous PDE, we next measured cAMP hydrolysis. Oocytes were injected with 50 ng of myr-Akt mRNA 3 h prior to injection of [3H]cAMP (23). Oocytes injected with K179M-Akt were used as a control. In parallel, a group of oocytes was stimulated with insulin 2 h before [3H]cAMP injection. Injection of the constitutively active Akt caused a 2-fold increase in cAMP hydrolysis over the control (Table 1). As previously reported (23), insulin treatment also stimulated cAMP hydrolysis (data not shown).

**DISCUSSION**

Akt is a serine-threonine kinase in the PI3-K signaling pathway that mediates growth factor regulation of cell differentiation and survival (1). Here we provide evidence for an additional role of this Akt kinase in the control of the cell cycle and meiosis. Akt expression in the *Xenopus* oocyte causes germinal vesicle breakdown and mitogen-activated protein kinase phosphorylation, hallmarks of the resumption of meiosis. Furthermore, our findings indicate that a PDE3 is distal to Akt activation. This conclusion is supported by both the pharmacological manipulation of the oocyte PDE and by the observation that an increase in PDE activity follows expression of the Akt kinase in oocyte. Finally, the finding that expression of a PDE in the oocyte per se is sufficient to promote a meiotic resumption is consistent with the above conclusions.

Numerous observations have underscored the importance of the lipid PI3-K in insulin and growth factor signaling in somatic cells (1) as well as in *Xenopus* oocytes. Because insulin and IGF-1 effects on meiosis are inhibited by wortmannin (29), PI3-K has been implicated in oocyte maturation. Injection of a constitutively active PI3-K mRNA causes resumption of meiosis (30), whereas injection of the lipid phosphatase SIP/SHP blocks the insulin effects (31). Our data extend these observations by identifying downstream steps in the pathway by which IGF-1 induces resumption of meiosis. As for somatic cells where the PI3-K effects are mediated by activation of Akt, we have shown that Akt activates resumption of meiosis in a manner similar to insulin/IGF-1.
Although the myr-Akt efficiently promoted oocyte maturation, a wild type Akt was only partially effective in the oocyte model, consistent with observations in mammalian cells showing that wild type Akt has low basal activity in the absence of insulin or growth factor signals (7, 8, 12, 13). Unlike the myr-Akt, the A2myr-Akt lacking the PH domain and with a mutation in the myristoylation signal was ineffective in inducing resumption of meiosis. This protein was efficiently expressed, and the overall Akt activity recovered in oocyte extracts was comparable with that obtained by expression of myr-Akt. When the activity of A2myr-Akt was corrected for the amount of protein expressed, this mutant kinase had one-fourth the specific activity of the myr-Akt. Because the only difference between A2myr-Akt and myr-Akt is the ability to interact with the lipid bilayer, we can conclude that Akt interaction with lipids is essential for the signaling meiotic resumption.

Our findings that PDE3 inhibitors block the oocyte maturation induced by insulin and Akt, but not progesterone, indicate that a PDE3 is involved in the PI3-K signal transduction pathway activated by IGF-1/insulin. In agreement with this hypothesis, we have shown that insulin, PI3-K, and Akt stimulate the activity of PDE3 endogenous to the oocyte. That Akt activation of PDE3 may be a sufficient signal for meiosis resumption is strongly suggested by the observation that expression of a PDE causes GVBD. Our conclusion is consistent with the finding in adipocytes, where it has been shown that insulin through the PI3-K pathway activates PDE3B (32).

Our data do not exclude the possibility that Akt is at a branch point in the signaling pathway controlling maturation. Upon activation, this kinase may phosphorylate several substrates in addition to a PDE3, and the activation of a PDE3 has only a permissive role on meiotic maturation. According to this model, both the release of a cAMP-dependent blockade and the activation of a distinct signaling cascade may be required for meiotic resumption. For instance, Akt activates the p70 ribosomal S6 kinase, which may be involved in the regulation of mos translation, a crucial step in oocyte meiosis (33).

Regardless of the mechanism of Akt action, our findings indicate a role for this enzyme in oocyte maturation. Interestingly, the cloning of 3-phosphoinositide-dependent kinase-1, the kinase-phosphorylating Thr-308 of Akt, has uncovered a structural and function homology with the Drosophila DSTRPK61 kinase. Although little is known about the function of the PI3-K and Akt pathway in Drosophila, DSTRPK61 kinase may play an important role in the differentiation of the female and male germ cells (9). Our data in the Xenopus oocyte are in line with the view that this pathway is involved in oocyte maturation. The physiological signals activating the PI3-K and the Akt pathway are unknown at present but may be a means by which somatic cells control the function of germ cells. It is worth noting that the PDE3A mRNA (34) and protein are expressed in mammalian oocytes. Consistent with that shown in Xenopus, PDE3 inhibitors block the spontaneous resumption of meiosis in rat and mouse oocytes as well as the maturation induced by the luteinizing hormone in the intact follicle (34, 35). Recent in vivo observations have further confirmed the crucial role of PDE3 for resumption of meiosis in mammals (35). Thus, our data on the Xenopus oocyte open the possibility that, in the mammalian follicle, physiological signals promoting resumption of meiosis regulate the inractioo cAMP levels via the PI3-K/Akt signaling pathway.

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