Phosphatidylinositol 3-kinase-dependent, MEK-independent proliferation in response to CaR activation

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Bilderback, Tim R., Fred Lee, Nelly Auersperg, and Karin D. Rodland. Phosphatidylinositol 3-kinase-dependent, MEK-independent proliferation in response to CaR activation. Am J Physiol Cell Physiol 283: C282–C288, 2002.—Although ovarian surface epithelial (OSE) cells are responsible for the majority of ovarian tumors, we know relatively little about the pathway(s) that is responsible for regulating their proliferation. We found that phosphatidylinositol 3-kinase (PI3K) is activated in OSE cells in response to elevated extracellular calcium, and the PI3K inhibitors wortmannin and LY-294002 inhibited extracellular signal-regulated kinase (ERK) activation by ∼75%, similar to effects of the mitogen-activated protein kinase/ERK kinase inhibitor PD-98059. However, in assays of proliferation, we found that PD-98059 inhibited proliferation by ∼50%, whereas wortmannin inhibited >90% of the proliferative response to elevated calcium. Expression of a dominant negative PI3K totally inhibited ERK activation in response to calcium. These results demonstrate that ERK activation cannot account for the full proliferative effect of elevated calcium in OSE cells and suggest the presence of an ERK-independent, PI3K-dependent component in the proliferative response.

ovarian surface epithelial cells; signal transduction; extracellular signal-regulated kinase; mitogen-activated protein kinase; Akt; calcium-sensing receptor

OVARIAN CANCER is the most lethal form of female genital cancer, and it is responsible for more than half of the deaths from female genital malignancies. Malignant ovarian tumors are primarily derived from ovarian surface epithelial (OSE) cells (20), whereas tumors from other ovarian tissues are mostly benign (21). OSE cells undergo wounding at ovulation, and this may result in an increase in the formation of OSE tumors, because a positive correlation exists between the number of ovulatory cycles a woman has experienced and her risk of ovarian cancer (21, 23). Because OSE cells are the cell type responsible for ovarian tumors, an understanding of the proliferative pathways used by these cells is of obvious importance. Although OSE cells show a proliferative response to a variety of stimuli, including follicle-stimulating hormone (2), hepatocyte growth factor (13), and elevated calcium (14, 18), the relative importance of distinct signal transduction pathways in mediating the proliferation of OSE cells is not known.

In our previous work, we found that OSE cells respond to elevation of calcium levels with increased proliferation (19). Furthermore, we determined that the calcium-sensing receptor (CaR) is expressed, functional, and essential for calcium-proliferative signaling in human and rat OSE cells (14, 18). The CaR belongs to the C subfamily of seven transmembrane-spanning G protein-coupled receptors (GPCR) (5). In addition to proliferative responses, the CaR is a vital mediator of parathyroid hormone release, with the CaR-inhibiting parathyroid hormone release in the presence of high calcium levels (6). In addition to OSE cells, the CaR is also present in the kidney (22), nerve terminals (25), bone (7), and a number of other tissues.

Activation of the CaR in OSE cells is associated with increased proliferation and increased extracellular signal-regulated kinase (ERK) activity (14, 18). Additional evidence of this association is given by the observation that both the proliferative response to calcium and activation of ERK by calcium are inhibited by a dominant negative (DN) CaR construct (14, 18). The pathways involved in the proliferative response to calcium stimulation were partially elucidated by the observation that the proliferative response of OSE cells to calcium was inhibited by ∼50% in the presence of the mitogen-activated protein kinase/ERK kinase (MEK) inhibitor PD-98059, whereas the general tyrosine kinase inhibitor herbimycin causes a >90% inhibition (14).

In this report, we demonstrate that phosphatidylinositol 3-kinase (PI3K) is a key mediator of the calcium response in OSE cells. Inhibition of PI3K with chemical inhibitors or with a p110α kinase dead DN PI3K construct prevented activation of ERK above the basal level. Additionally, we demonstrate that the PI3K in-
hibitor wortmannin and the ERK inhibitor PD-98059 both inhibit ERK to a nearly equal extent. However, the ERK inhibitor PD-98059 only inhibits \([^{3}H]thymidine\) uptake by \(\sim 50\%\) in response to calcium stimulation, whereas the PI3K inhibitor wortmannin inhibits \([^{3}H]thymidine\) uptake by \(>90\%\). These data strongly suggest that, although PI3K activity is essential for activation of ERK, there are additional proliferative pathways that do not involve ERK yet still require PI3K activity. These pathways appear to be responsible for a significant portion of the proliferative response of OSE cells to calcium.

**MATERIALS AND METHODS**

**Cell culture.** These studies were conducted on immortalized OSE (IOSE)-120 and IOSE-80 cell lines, derived from primary cultures of normal ovarian surface epithelial cells by transfection with SV40 large T antigen to expand the life span of these cells in culture. Although these IOSE cells can no longer be considered normal, they are not immortal (serving as a phosphate donor with equal protein amounts per assay).

**Immunoprecipitation and immunoblotting.** IOSE-120 or IOSE-80 cells were harvested at the indicated times after calcium addition by lysis in M-triton glycerol buffer [1% Triton X-100, 10% glycerol, 20 mM HEPES, pH 8.0, 2 mM Na\(_3\)VO\(_4\), 150 mM NaCl, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% aprotinin, and leupeptin 1%], and lysates were cleared by centrifugation at 6,000 \(\times\) g. Protein concentration was determined by using the Bio-Rad protein assay, and equal amounts of protein were incubated with primary antibody for \(\geq 4\) h, followed by incubation with protein A/G-agarose (Santa Cruz Biotechnology) for \(\geq 1\) h. The immune complex was collected by centrifugation at 2,000 \(\times\) g for 5 min. The pellet was washed extensively with M-TG buffer and boiled for 3 min in 1x Laemml buffer.

Cell lysates containing up to 100 \(\mu\)g of protein or immunoprecipitates from equal amounts of starting protein (up to 500 \(\mu\)g) were size-fractionated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) (Immobilon P, Millipore) by electroblotting. Membranes were blocked in 5% bovine serum albumin, 0.05% Na\(_2\)SO\(_4\) for 1 h at room temperature followed by overnight incubation with the primary antibody indicated at 4°C in TBST (0.05% Tween 20, 20 mM Tris, pH 7.5, 150 mM NaCl). Membranes were washed three times in TBST, incubated with appropriate secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) for \(\geq 2\) h at room temperature and washed extensively in TBST. Bands were visualized by chemiluminescence (Renissance, NEN Life Science Products) and quantified with a Molecular Dynamics PhosphorImager and IP LabGel software.

**PI3K assay.** PI3K was immunoprecipitated from IOSE-120 cells that were made quiescent by overnight culture in serum-free low-calcium medium (0.3 mM Ca\(^{2+}\)) and then placed in 0.05 mM Ca\(^{2+}\) for 4 h to obtain the lowest possible basal activity. Cells were subsequently stimulated with 2.0 mM calcium for the indicated time. Equal amounts of protein were immunoprecipitated with a polyclonal antibody recognizing p110\(\gamma\), \(\beta\), and \(\gamma\) (66-195, Upstate Biotechnology). The pellet was washed in 0.1 mM orthovanadate, 137 mM NaCl, 1 mM CaCl\(_2\), and 1 mM MgCl\(_2\) in 20 mM Tris, pH 7.4. To assay lipid kinase activity, the PI3K bound to protein A/G-agarose beads was resuspended in 0.05 ml of Tris-NaCl-EDTA, phosphatidylinositol was added to a final concentration of 0.27 mg/ml and the reaction was initiated by the addition of 30 \(\mu\)l of [\(\gamma\]^{-32}\]P]ATP. After 10 min at 37°C, the reaction was quenched with 0.02 ml of 6 N HCl. The radio-labeled lipid phosphates were extracted by the addition of 0.16 ml of chloroform:methanol:water:ammonium hydroxide (60:47:11.3:2) as the developing solvent. The products were visualized by autoradiography and identified by comigration with unlabeled PI3P standards that were visualized by iodine staining, and the radioactive bands were quantified by PhosphorImager or by excision and liquid scintillation spectrometry.

**ERK activity assays.** In vitro kinase assays were conducted on immunoprecipitates as previously described (24). IOSE-80 cells at 80% confluence were transfected with hemagglutinin (HA)-tagged ERK in 10-cm plates or with empty vector as described in Transfections. The cells were cultured in serum-free Ham’s F-12 (0.3 mM Ca\(^{2+}\)) for 16 h, followed by 4 h in 0.05 mM Ca\(^{2+}\). The cells were then stimulated with 2.0 mM Ca\(^{2+}\) for 10 min before lysis in 300 \(\mu\)l M-TG lysis buffer (20 mM HEPES, pH 8.0, 1% Triton X-100, 10% glycerol, 2 mM Na\(_3\)VO\(_4\), 150 mM NaCl, 1 mM NaF, 1 mM PMSF, 1% aprotinin, and 1% leupeptin). Lysates were cleared by addition of 20 \(\mu\)g protein A/G-agarose and then underwent rotation at 4°C for 30 min and subsequent centrifugation at 12,000 \(\times\) g for 10 min. Aliquots of cleared lysates normalized for protein content were subjected to immunoprecipitation overnight at 4°C using 12CA5 anti-HA (a gift from B. Druker, Oregon Health Sciences University), followed by addition of protein A/G-agarose and an additional 2-h incubation at 4°C. Immunoprecipitates were recovered by centrifugation and washed once in M-TG lysis buffer, once in LiCl buffer [500 mM LiCl, 100 mM Tris-HCl, pH 7.6, 0.1% Triton X-100, 1 mM dithiothreitol (DTT), 1 mM vanadate, and 0.4% M-PMSF], and once in MOPS assay buffer (20 mM MOPS, pH 7.2, 20 mM MgCl\(_2\), 2 mM EGTA, 2 mM DTT, and 0.2% Triton X-100). The pellets were resuspended in 20 \(\mu\)l of kinase assay buffer (10 mM MOPS, pH 7.2, 20 mM MgCl\(_2\), 1 mM EGTA, 1 mM DTT, 0.1% Triton X-100, and 1 \(\mu\)l of [\(\gamma\]^{-32}\]P]ATP and incubated at 30°C for 30 min. Glutathione-S-transferase (GST)-Elk1 (3 \(\mu\)g per reaction) was added as a substrate for phosphorylation. Phosphorylated proteins were resolved by SDS-PAGE in 12% acrylamide gels. Proteins were electrophoretically transferred to PVDF membranes (Millipore), and radioactivity was visualized and quantified with a Molecular Dynamics PhosphorImager and IP LabGel software. The membranes were then stripped and immunoblotted with anti-ERK1/2 antibody (Santa Cruz Biotechnology) to verify equal loading of protein.

**Raf assays.** For Raf assays, IOSE-80 cells were incubated with DMSO, PD-98059, or wortmannin for 1 h. Unstimulated control cells and cells treated with 2.0 mM calcium for 15 min were lysed in ice-cold 1% Nonidet P-40 buffer containing 10 mM Tris, pH 7.4, 5 mM EDTA, 50 mM NaCl, and 1 mM PMSF. Immune complex kinase assays were performed as described (26) by using MEK1 as a substrate and [\(\gamma\]^{-32}\]P]ATP as a phosphate donor with equal protein amounts per assay. The reaction products of all kinase assays were resolved by
Cells were then stimulated with calcium for the indicated times and harvested. Parallel dishes of cells were transfected with pEGFP (enhanced green fluorescent protein plasmid), and transfection efficiency was quantified by determining the percentage of cells that expressed green fluorescent protein.

**RESULTS**

**PI3K plays an important role in the proliferative response of OSE cells to elevated Ca\(^{2+}\).** Among the possible mechanisms linking activation of GPCRs such as the CaR to the Ras-mitogen-activated protein (MAP) kinase cascade is the G\(_{i/o}\) activation of the p110\(\gamma\) catalytic subunit of PI3K (12). If PI3K is involved in signal transduction downstream of the CaR, we would expect to see increased phosphorylation of Akt, a major PI3K target, in response to activation of the CaR. As shown in Fig. 1, IOSE cells responded to 2 mM Ca\(^{2+}\) with an increase in both Akt phosphorylation (Fig. 1A) and ERK phosphorylation (Fig. 1B). The Akt response to calcium stimulation peaked at 10 min, but peak ERK phosphorylation was not observed until 15 min after stimulation. ERK1 responded more robustly to calcium stimulation than did ERK2. Because Akt is activated downstream of PI3K, we used two chemically dissimilar inhibitors of PI3K, wortmannin and LY-294002, to test whether activation of PI3K was an essential intermediary between CaR activation and ERK activation in IOSE cells. As shown in Fig. 2, the increase in ERK phosphorylation was inhibited in both Akt and ERK phosphorylation in the presence of wortmannin. In contrast, LY-294002 inhibited ERK phosphorylation but not Akt phosphorylation. These results suggest that PI3K plays a critical role in the proliferative response of OSE cells to elevated Ca\(^{2+}\).
activation normally seen when IOSE cells are shifted from 0.05 to 2.0 mM Ca\(^{2+}\) was completely inhibited in the presence of either wortmannin or LY-294002. Neither wortmannin nor LY-294002 had a statistically significant effect on ERK kinase activity under low-calcium conditions. In addition to blocking any increase in ERK kinase activity upon shifting to 2 mM Ca\(^{2+}\), wortmannin produced a variable inhibition of ERK kinase activity to levels slightly below those measured at low calcium concentrations. This inhibition sometimes approached statistical significance (0.01 < \(P < 0.05\); Fig. 2).

Activation of PI3K by extracellular calcium. The efficacy of wortmannin and LY-294002 as inhibitors of Akt and ERK phosphorylation in response to 2 mM Ca\(^{2+}\) suggests that PI3K is a downstream effector of the CaR. This possibility was tested directly by measurement of PI3K enzymatic activity in response to extracellular calcium (Fig. 3). Switching the IOSE cells from 0.05 to 2.0 mM Ca\(^{2+}\) produced a 1.5-fold increase in PI3P formation at 5 min, as measured by incorporation of \(^{32}\)P into PI3P (Fig. 3), as well as a threefold increase in Akt phosphorylation, as detected by anti-phospho-Akt antibodies (Fig. 4). This increase in Akt phosphorylation is consistent with activation of PI3K (and, by inference, the PI3K target Akt), we measured Akt kinase activity (11).

To determine whether activation of the Raf-MEK-ERK pathway was required for activation of PI3K (and, by inference, the PI3K target Akt), we measured Akt phosphorylation and ERK phosphorylation in parallel, in the presence of the PI3K inhibitor wortmannin and the MEK inhibitor PD-98059 (Figs. 4 and 5). Both the MEK inhibitor PD-98059 and the PI3K inhibitor wortmannin were effective inhibitors of basal and stimulated ERK phosphorylation (Fig. 5). In contrast, Akt phosphorylation was inhibited by wortmannin but not by PD-98059 (Fig. 4), suggesting that MEK activation was not required for increased Akt phosphorylation but that PI3K enzymatic activity, however, was required.

To further define the relationship between PI3K activation and the Raf-MEK-ERK pathway, we compared the effects of wortmannin and PD-98059 on Raf activation in response to 2 mM Ca\(^{2+}\). Raf activation, measured as the ability of immunoprecipitated Raf to phosphorylate recombinant MEK in vitro, was significantly inhibited in the presence of wortmannin but not PD-98059 (Fig. 6). Equivalent results were obtained when U-0126 was used to inhibit MEK and LY-294002...
was used to inhibit PI3K (data not shown). These results suggest that PI3K activation is upstream of the Ras-Raf-MEK pathway. Effects of DN and CA PI3K constructs on ERK activation. Although pharmacological inhibitors such as wortmannin are very useful tools in understanding signaling pathways, there is always doubt as to the specificity of the effects of an inhibitor on other pathways. For this reason, we chose to transiently transfet IOSE cells with a DN mutant of PI3K that consists of the p110 subunit of PI3K mutated to lack kinase ability (DN PI3K). The expression of the DN PI3K construct has previously been demonstrated to be an effective inhibitor of PI3K activity (3). Transient transfection of IOSE-80 cells with the DN PI3K inhibited ERK activation in low-calcium medium to 5% of control basal levels (Fig. 7). When IOSE cells were shifted from 0.05 to 2.0 mM Ca\textsuperscript{2+}, there was a 2.5-fold increase in ERK activity in the mock-transfected control. However, when DN PI3K transfected cells were shifted from 0.05 to 2.0 mM Ca\textsuperscript{2+}, there was a total block of ERK activity in response to elevated calcium. Importantly, cells transfected with the DN PI3K had no apparent change in viability or morphology for the 48-h period of the experiment. The dramatic block of ERK activity in response to increased calcium levels gives strong supporting evidence that PI3K activation is specifically required for activation of ERK in response to elevated calcium levels. In contrast, transfection with CA PI3K, a mutated version of p110\textalpha containing the inter-SH2 domain of the p85\textbeta subunit that confers constitutive kinase activity in a variety of systems (15), had relatively little effect on ERK activity levels in IOSE cells (Fig. 7). Expression of CA PI3K failed to significantly increase either basal or calcium-stimulated ERK kinase activity compared with vector-transfected IOSE cells. Although there was a trend toward increased ERK activity in response to 2 mM Ca\textsuperscript{2+} in the presence of CA PI3K, the difference was not statistically significant.

Relative sensitivity of OSE proliferation to MEK vs. PI3K inhibitors. To determine the relative contribution of MEK-dependent and PI3K-dependent pathways to the proliferative response induced by 2 mM Ca\textsuperscript{2+}, we compared the effects of relevant chemical inhibitors on both ERK activity and thymidine incorporation. As shown in Fig. 8A, the MEK inhibitor PD-98059 and the PI3K inhibitor wortmannin were equally effective at preventing any increase in ERK activation in response to 2 mM Ca\textsuperscript{2+}. However, a 1.8-fold increase in thymidine incorporation was still evident in PD-98059 cells stimulated with 2 mM Ca\textsuperscript{2+}, whereas wortmannin treatment inhibited thymidine incorporation to 20\% of basal control levels (Fig. 8B). In a replicate experiment comparing two MEK inhibitors, PD-98059 and U-0126, with two PI3K inhibitors, wortmannin and LY-294002, a residual proliferative response to 2 mM Ca\textsuperscript{2+} was observed in the presence of either MEK inhibitor, whereas both PI3K inhibitors prevented any increase in thymidine incorporation between 0.05 and 2.0 mM Ca\textsuperscript{2+} (Fig. 8C). The doses of PD-98059, wortmannin, and LY-294002 were determined from previous experiments in which inhibitor concentrations had been titrated against ERK activation (data not shown). These data suggest that PI3K is involved in the inhibition of other proliferative pathways in OSE cells that respond to elevated calcium, independent of ERK.
DISCUSSION

In this report, we present evidence that PI3K plays an important role in signal transduction from the CaR to calcium-sensitive proliferative pathways including but not necessarily limited to ERK. This study was performed using human OSE cell lines to expand our knowledge of the regulation of a proliferative response in the cell type that forms the most aggressive and lethal ovarian tumors (20, 21). Work in this report and others (14) indicates that normal OSE cells modulate their rate of proliferation in response to the concentration of extracellular calcium. Therefore, understanding the proliferative pathways activated by the CaR in normal OSE cells is a prerequisite to identifying possible disruptions of these pathways in ovarian adenocarcinomas.

The effective inhibition of ERK kinase activity by chemical inhibitors of PI3K has been observed in murine interleukin (IL)-3-BaF/3 cells in which LY-294002 inhibited IL-3 activation of MEK, ERK1, and ERK2 (10). LY-294002 has also been shown to inhibit insulin-like growth factor-I-induced activation of both MEK and ERK in MCF-7 cells (27) as well as staurosporine-induced phosphorylation of ERK1/ERK2 in peritoneal macrophages (28). In contrast, wortmannin had no effect on ERK activation in response to the ligand-stimulated chemokine receptor CXCR3 in hepatic stellate cells (4). LY-294002 had no effect on ERK activation in erythropoietin-stimulated erythroid cells (8), and LY-294002 and wortmannin had no effect on activation of ERK in response to epidermal growth factor in glioblastoma cells (16). These mixed observations led us to examine whether PI3K may be activated in response to stimulation of the CaR. We saw a substantial increase in phosphatidylinositol 3-phosphate levels 5 min after shifting calcium from 0.05 to 2.0 mM, indicating that activation of the CaR is coupled to activation of PI3K.

The selective ERK inhibitor PD-98059 has previously been shown to inhibit phosphorylation of ERK in rat OSE cells and inhibit proliferation (14). We have expanded on this observation in dissecting the respective roles of ERK and PI3K in the proliferative response to elevated calcium concentration. Our observations demonstrate that PD-98059 does not affect signaling through PI3K, i.e., it does not prevent phosphorylation of Akt (Fig. 4) and does not interfere with Raf activation (Fig. 6). This clearly shows that PI3K is active upstream of MEK. In contrast, the PI3K inhibitor wortmannin prevents the stimulation of Akt, Raf, and ERK. Furthermore, we strengthened the evidence for inhibition of ERK by wortmannin and PD-98059 by measuring ERK kinase activity as well as phosphorylation and obtaining similar results in terms of the level of ERK phosphorylation. However, when we examined the effects of MEK inhibitors and PI3K inhibitors on [3H]thymidine uptake in response to elevated calcium, the PI3K inhibitors completely inhibited any increase in thymidine incorporation in response to 2 mM calcium, whereas the MEK inhibitors appeared to act predominantly on basal (low calcium) levels of thymidine incorporation, with a 50–70% increase in thymidine incorporation still evident upon switching to 2 mM calcium. The observation of a residual Ca$_{2+}$-induced increase in thymidine incorporation when ERK activation was completely suppressed suggests the presence of an ERK-independent proliferative pathway in IOSE cells. The complete inhibition of thymidine incorporation observed in the presence of wortmannin and LY-294002 suggests that this ERK-independent pathway is downstream of and dependent on PI3K.

Although wortmannin is known to inhibit PI3K without interfering with upstream events, pharmacological inhibitors always raise concerns about their specificity. To address this concern, we transiently transfected...
IOSE-80 with either a DN or a CA PI3K. The DN PI3K totally inhibited ERK activation in response to elevated calcium (Fig. 7). This result convincingly demonstrates that PI3K is required for activation of ERK through the CaR in OSE cells. However, when the same cells were transfected with the CA PI3K, we saw no significant change in the levels of ERK activation. The CA PI 3-kinase construct did not activate ERK above basal levels at low calcium levels, and it did not significantly increase ERK activation at high calcium levels. Together, these results demonstrate that, although PI3K is necessary for ERK activation in response to elevated calcium signaling through the CaR, increased p110 activity alone may not be sufficient for ERK activation. One possible explanation for this discrepancy is the possibility that ERK activation may be dependent on activation of p110γ, such that the DN PI3K interferes with signaling from any catalytic PI3K subunit, whereas the CA PI3K can only mimic the effects of p110 activity.

In summary, we have demonstrated that PI3K is involved in signal transduction from the CaR to ERK. Our data suggest that there may be other calcium-sensitive proliferative pathways that are independent of ERK but require the activation of PI3K. Further investigation is needed to fully understand the pathway by which the CaR signals through PI3K to ERK. There is also a need to define the pathways involved in the proliferative response to calcium mediated through PI3K but independent of ERK.

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