Upregulation of Phospholipase Cγ1 Activity during EGF-Induced Proliferation of Corneal Epithelial Cells: Effect of Phosphoinositide-3 Kinase

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PURPOSE. Previously, the authors showed that epidermal growth factor (EGF) stimulates phospholipase Cγ1 (PLCγ1) and phosphoinositide-3 kinase (PI3K) activities in confluent rabbit corneal epithelial cells (RCECs). The purpose of this study was to investigate whether PLCγ1 activity is upregulated during EGF-induced proliferation of RCECs and to determine whether there is any cross-talk between PLCγ1 and PI3K in these cells.

METHODS. Simian virus (SV)-40–immortalized RCECs were cultured in the presence and absence of EGF and other agents. At prescribed time intervals, the cultures were terminated and the cells counted. PLCγ1 activity in intact cells was assessed by measuring the production of [3H]IP3 in [3H]myoinositol-labeled cells. The in vitro enzyme activity was assayed using immunoprecipitated PLCγ1 and [3H]Pi(4,5)P2 as substrate. [3H]IP3, the product of PLCγ1, was analyzed by anion-exchange chromatography. The changes in protein content and level of phosphorylation of PLCγ1 were determined by Western immunoblot analysis, with the appropriate antibodies.

RESULTS. Addition of EGF (50 ng/ml) caused a time-dependent increase in proliferation of RCECs. The effect of EGF peaked at approximately 36 hours. Under the same experimental conditions, EGF-stimulated PLCγ1 activity with a time course similar to that of cell proliferation. Data from Western immunoblot analysis revealed that the EGF-stimulated PLCγ1 activity was due to increased synthesis of the enzyme. Furthermore, during cell proliferation, tyrosine phosphorylation of PLCγ1 increased in a time-dependent manner that corresponded closely with the expression of PLCγ1. EGF exerted its effects both on cell proliferation and PLCγ1 activation in a dose-dependent manner. Treatment of the cells with U-73122, a PLC inhibitor, or myr-GLYRKMRRY, a myristoylated PLCγ1 inhibitor peptide, caused attenuation of both the EGF-stimulated cell proliferation and PLCγ1 activity. Treatment of the cells with the PI3K inhibitors, wortmannin or LY294002, caused inhibition of both EGF-stimulated cell proliferation and PLCγ1 activation. Addition of P(3,4,5)P2 to the in vitro PLCγ1 assay mixture stimulated the enzyme activity in a dose-dependent manner.

CONCLUSIONS. The data suggest a positive correlation between EGF-stimulated PLCγ1 activation and cell proliferation in RCECs. The EGF-stimulated PLCγ1 activity was mirrored by increased synthesis and tyrosine phosphorylation of the enzyme. The data also show that PLCγ1 activation and cell proliferation were inhibited by PI3K inhibitors, suggesting a role for PI3K in EGF-stimulated proliferation of corneal epithelial cells. (Invest Ophthalmol Vis Sci. 2001;42:1472-1478)

Stimulation of a variety of cell surface receptors by the appropriate ligand results in activation of phospholipase C (PLC) and phosphoinositol 3-kinase (PI3K) in several cell types.1–4 PLC catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to generate two intracellular second-messenger molecules, diacylglycerol (DAG) and 1,4,5-trisphosphate (IP3).

ADDENDUM

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of EGF to the injured cornea stimulates cell proliferation and enhances wound repair.9,10 The same outcome results from the addition of EGF to corneal epithelial cells in culture.9,10

The exact biochemical events leading from EGF-receptor interaction and culminating in enhanced cell proliferation are not well understood. Previously, we reported that addition of EGF to quiescent corneal epithelial cells causes activation of PLCγ1 and PI3K.11,12 Recently, we showed that activation of PI3K by EGF, both in vivo and in vitro, correlates positively with cell proliferation and wound repair in rabbit corneal epithelium.6,10 The objective of the present study was to investigate whether there is any correlation between PLCγ1 activation and corneal epithelial cell proliferation induced by EGF. In addition, we examined the effects of PI3K on PLCγ1 activation in the EGF-treated epithelial cells. For this work, we used simian virus (SV)-40–immortalized rabbit corneal epithelial cells (RCECs), which are capable of growing for many passages without any alteration in their morphology or biochemical characteristics.5,14

MATERIALS AND METHODS

Dulbecco’s modified Eagle’s medium (DMEM), insulin, and gentamicin were purchased from Gibco (Grand Island, NY); human recombinant EGF, leupeptin, aprotinin, protein A (immobilized on Sepharose CL-4B), and phenylmethylsulfonyl fluoride (PMSF) from Sigma (St. Louis, MO); PLCγ1 and β1 polyclonal antibodies from Santa Cruz Biotechnology (Santa Cruz, CA); PI(4,5)P2, PI(3)P, PI(3,4)P2, and PI(3,4,5)P3 from Biomol (Plymouth Meeting, PA); phosphatidyserine (PS) and phosphatidylethanolamine (PE) from Avanti Polar Lipids (Birmingham, AL); and wortmannin, LY294002, U-73122, and U-73343 from Calbiochem (La Jolla, CA). [3H]PIP2 (specific radioactivity 2 Ci/mmol) was purchased from DuPont NEN (Boston, MA) and [3H]thymidine (specific radioactivity 2 Ci/mmol) from ICN Radiochemicals (Irvine, CA).

Cell Culture

The SV-40–transformed RCECs were thawed and suspended in complete medium (DMEM/F-12 containing 40 μg/ml gentamicin, 5 μg/ml insulin, and 10% fetal bovine serum (FBS)) and cultured in a humidified atmosphere of 95% air-5% CO2. The cultures were maintained by addition of EGF to corneal epithelial cells in culture.9,10 The pelleted cells were resuspended in complete medium and then trypsin-0.02% EDTA for 5 minutes at 37°C. Next, complete medium was added and the reaction mixture thoroughly mixed and centrifuged. An aliquot (400 μl) of the upper aqueous phase was removed and counted in a scintillation counter. When the effects of PI(3)P, PI(3,4)P2, and PI(3,4,5)P3 on the activity of PLCγ1 or PLCβ1 were to be determined, the immunoprecipitated enzymes were treated for 15 minutes with different concentrations of these lipids before their addition to the reaction mixture.

Western Immunoblot Analysis

The protein content and level of tyrosine phosphorylation of PLCγ1 were determined by Western immunoblot analysis, as described previously.11 Briefly, RCECs lysates containing equal amounts of protein from EGF-treated and untreated cells were immunoprecipitated using anti-PLCγ1 antibody. The precipitates were boiled in Laemmli’s buffer for 5 minutes, separated by 10% SDS-PAGE, and the proteins transferred to nitrocellulose membranes. To determine total PLCγ1 content, the membranes were successively blotted with anti-PLCγ1 primary antibody and anti-rabbit horseradish peroxidase (HRP)–conjugated goat secondary antibody. To determine the level of PLCγ1 tyrosine phosphorylation, the blots were stripped and reprobed successively with anti-phosphotyrosine primary antibody and anti-mouse HRP-conjugated secondary antibody. The protein bands were visualized using the enhanced chemiluminescence (ECL) Western detection system (Amer sham Pharmacia Biotech, Parsippany, NJ).

Synthesis of Inhibitor Peptides for PLCγ1

The myristoylated PLCγ1 inhibitor peptide, myr-GLYRKMRLRY (myr-PCI(Y)), and its weaker analogue, myr-GLYRKMRLRF (myr-PCI(F)), were synthesized (Pioneer; PerSeptive Biosystems, Framingham, MA) at 0.1 mM, using the 9-fluorenylmethoxycarbonyl (Fmoc) solid phase chemistry. HPLC (Hewlett-Packard, Santa Clara, CA) and mass spectrometry (Finnigan MAT 711, San Jose, CA) were used to confirm peptide purity.

In Vitro Assay of PLCγ1 and PLCβ1

Subconfluent (~60%) cultures were serum starved for 24 hours before treatment with EGF or other agents. At appropriate times, the cultures were terminated and the cells scraped and homogenized in 20 mM Tris-HCl buffer (pH 7.0) containing 5 mM MgCl2, 5 mM EDTA, 1 mM EGTA, 1 mM PMSF, 2 mM Na3VO4, 10 μg/ml leupeptin, and 50 μg/ml aprotinin. The homogenate was centrifuged at 600g for 10 minutes, and the resultant supernatant quantified for protein concentration by the method of Lowry et al.15 Supernatants (cell lysates) containing equal amounts of protein were treated with PLCγ1 or PLCβ1 antibody, and the immunoprecipitated protein was used to assay PLC activity as described previously, with minor modifications.16 Briefly, the reaction mixture contained 20 mM Tris-HCl buffer (pH 7.0), 0.1 M NaCl, 2 mM CaCl2, 1 mM EGTA, 1 mM EDTA, 0.1% sodium cholate, 50 μM [3H]PIP2 (30,000 disintegrations per minute [dpm]), and the immunoprecipitated enzyme protein in a final volume of 125 μl. The substrate was prepared by mixing chloroform solutions of [3H]PIP2, unlabeled PIP2, PE, and PS at a molar ratio of 1:2:2, respectively. After evaporation of the solvent under N2, the lipids were suspended by sonication in sodium cholate–containing reaction buffer. The reaction was initiated by addition of the enzyme protein, incubated for 30 minutes at 37°C, and terminated by addition of 0.5 ml chloroform-methanol-concentrated HCl (50:50:1, by volume). Next, 150 μl of 1 M HCl containing 5 mM EGTA was added and the reaction mixture thoroughly mixed and centrifuged. An aliquot (400 μl) of the upper aqueous phase was removed and counted in a scintillation counter. When the effects of PI(3)P, PI(3,4)P2, and PI(3,4,5)P3 on the activity of PLCγ1 or PLCβ1 were to be determined, the immunoprecipitated enzymes were treated for 15 minutes with different concentrations of these lipids before their addition to the reaction mixture.

Statistical Analysis of the Data

Each experiment consisted of incubations that, when pooled, yielded three independent samples for each data point. All experiments were performed at least twice, with the results expressed as mean ± SEM. Statistical analysis was performed with Student’s t-test for nonpaired data. P ≤ 0.05 was considered significant.

RESULTS

Effect of EGF on PLCγ1 Activity and Tyrosine Phosphorylation during Cell Proliferation

To determine whether cell counting can provide an accurate measure for cell proliferation, the data from cell counting were...
The cultures were terminated and PLC\(\gamma\) activity during cell proliferation and cell proliferation. The cells were cultured in the presence of different concentrations of the peptide before counting and determination of PLC\(\gamma\) activity in vitro. myr-PCI(Y) inhibited the EGF-induced cell proliferation in a dose-dependent manner (Fig. 4A). The EGF effect was reduced by approximately 40% in the presence of 10 \(\mu\)M myr-PCI(Y). Unlike U-73122, the peptide did not inhibit the PLC\(\gamma\) activity in the untreated cells. However, it inhibited the EGF-induced activation of PLC\(\gamma\) in a dose-dependent manner (Fig. 4B). At 10 \(\mu\)M myr-PCI(Y), the EGF effect was inhibited by almost 50%. myr-PCI(F), a PCI peptide analogue, was considerably less potent in inhibiting the EGF-induced PLC\(\gamma\) activation and cell proliferation.

**Effect of PI3K Inhibitors on PLC\(\gamma\) Activity and Cell Proliferation**

We used wortmannin and LY294002 as inhibitors of PI3K to determine whether PLC\(\gamma\) lies downstream of PI3K in mediating the effect of EGF on proliferation of corneal epithelial cells. Treatment with wortmannin caused a dose-dependent decrease in EGF-stimulated proliferation of RCECs (Fig. 5A). Wortmannin also inhibited the EGF-induced activation of PLC\(\gamma\) in a dose-dependent manner (Fig. 5B). LY294002 also caused a dose-dependent decrease in EGF-stimulated cell proliferation and PLC\(\gamma\) activity in RCECs (Fig. 6).

**Effect of PI3K Products on the Activity of PLC\(\gamma\)**

We used PI(3)P, PI(3,4)P\_2, and PI(3,4,5)P\_3 to investigate whether PLC\(\gamma\) could be a direct target of the PI3K products. PLC\(\gamma\) was immunoprecipitated from cultured RCECs and assayed for its activity in the presence and absence of 3-phosphoinositides. As shown in Figure 7, PI(3,4,5)P\_3 increased the activation of PLC\(\gamma\) in a dose-dependent manner up to 100 \(\mu\)M, followed by a slight tapering off. The maximal stimulation, observed at 100 \(\mu\)M, then decreased at higher concentrations of PI(3,4,5)P\_3. PI(3,4)P\_2 and PI(3,4,5)P\_3 did not have any significant stimulatory effect on PLC\(\gamma\) activity. Similarly, PI(3,4,5)P\_3 had no effect on the activity of PLC\(\beta\)1 immunoprecipitated from RCECs (data not shown).

**DISCUSSION**

Wound repair in corneal epithelium is highly complex and involves cell migration, proliferation and differentiation, and secretion of extracellular matrix proteins. It has been reported that EGF treatment, both in vitro and in vivo, accelerates the wound-healing process by promoting migration and proliferation of the corneal epithelial cells. 7,8 The biochemical events that relay signals from the EGF receptor to the nucleus, resulting in increased DNA synthesis, are not clear. It has been reported that addition of EGF to quiescent corneal epithelial cells causes stimulation of PLC\(\gamma\)1, possibly because of its translocation to, and phosphorylation by, the EGF receptor. 11

The data from the present study show that serum-starved RCECs can proliferate, albeit at a low rate, in the absence of any exogenously added EGF (Fig. 1A). The observed proliferative response is most likely due to autocrine secretion of inhibitory effect on cell proliferation or PLC\(\gamma\) activity. U-73122 exerted no effect on EGF-induced expression or tyrosine phosphorylation of PLC\(\gamma\)1 in RCECs during proliferation (Figs. 3C, 3D).

**Effect of Myristoylated PLC Inhibitor Peptide on IP\_3 Formation and Cell Proliferation**

myr-PCI(Y), the myristoylated PLC\(\gamma\)1 inhibitor peptide, was used to further confirm the involvement of PLC\(\gamma\)1 in EGF-stimulated cell proliferation in RCECs. The cells were cultured in the presence of different concentrations of the peptide before counting and determination of PLC\(\gamma\) activity in vitro. myr-PCI(Y) inhibited the EGF-induced cell proliferation in a dose-dependent manner (Fig. 4A). The EGF effect was reduced by approximately 40% in the presence of 10 \(\mu\)M myr-PCI(Y). Unlike U-73122, the peptide did not inhibit the PLC\(\gamma\)1 activity in the untreated cells. However, it inhibited the EGF-induced activation of PLC\(\gamma\)1 in a dose-dependent manner (Fig. 4B). At 10 \(\mu\)M myr-PCI(Y), the EGF effect was inhibited by almost 50%. myr-PCI(F), a PCI peptide analogue, was considerably less potent in inhibiting the EGF-induced PLC\(\gamma\)1 activation and cell proliferation.
growth-promoting substances by cells in the culture medium. When treated with EGF, the cells exhibited a marked time-dependent increase in cell number. An important finding of the present study is that, under conditions when RCECs were undergoing active proliferation, there was a concomitant increase in PLCγ1 activity both in the EGF-treated and untreated cells (Fig. 1B). Western blot analysis revealed that the increased PLCγ1 activity was due to increased synthesis and tyrosine phosphorylation of the enzyme (Figs. 1C, 1D). The effect of EGF on PLC was found to be specific for the PLCγ1 isoform. Both the activity and expression of PLCβ1 remained unchanged during cell proliferation (data not shown).

**FIGURE 1.** Time-course effect of EGF on PLCγ1 activity during RCEC proliferation. Serum-starved cells were cultured for different time intervals in the absence and presence of EGF (50 ng/ml). (A) Cells were trypsinized and counted. (B) Cells were lysed and the lysate immunoprecipitated with PLCγ1 antibody. The immunoprecipitate was collected and assayed for PLC activity using [3H]PIP2 as substrate. Data are mean ± SEM of three independent experiments with triplicate cultures for each data point. *Statistically significant (P < 0.05) increase in cell number, compared with the control.

**FIGURE 2.** Dose-response effect of EGF on cell proliferation and PLCγ1 activity in RCECs. Serum-starved cells were cultured for 36 hours in the absence and presence of different concentrations of EGF. (A) Cells were trypsinized and counted. (B) Cells were lysed and the lysate immunoprecipitated with PLCγ1 antibody. The immunoprecipitate was collected and assayed for PLC activity using [3H]PIP2 as substrate. Data are mean ± SEM of three independent experiments with triplicate cultures for each data point. *Statistically significant (P < 0.05) increase in cell number, compared with the control.
These data provide evidence that PLCγ1 is involved in a signaling pathway that mediates the EGF’s effect on DNA synthesis in RCECs. The findings are in accord with previously published reports in which microinjection of anti-PLCγ1 antibody blocked serum- and Ras-stimulated DNA synthesis.17 In other studies, microinjection of PLCγ1 SH2 domains into Madin-Darby canine kidney (MDCK) epithelial cells or fibroblasts blocked the platelet-derived growth factor (PDGF)-induced DNA synthesis.18,19

Additional evidence to support the suggestion that PLCγ1 is involved in corneal epithelial cell proliferation comes from the use of the PLC inhibitor U-73122. This inhibitor has been widely used in a number of studies examining the role of PLC in intracellular signaling mechanisms.20,21 In the present study, by using U-73122, we were able to significantly inhibit the EGF-stimulated PLC-γ1 activation, which resulted in a corresponding decrease in EGF-induced proliferation in RCECs (Fig. 3). The inhibitory effect of U-73122 was not due to the inhibition of PLCγ1 synthesis or its phosphorylation. It has been reported that PLCγ1 possesses, adjacent to its SH2 and SH3 motifs, a PLC inhibitor (PCI) region that strongly suppresses its catalytic activity.22 It has been suggested that stimulation of the cells with growth factors probably dissociates the PCI region of PLCγ1 from the catalytic region, resulting in the activation of the enzyme. Myristoylation of PCI facilitates its entry into Swiss 3T3 cells causing inhibition of cell growth and phosphoinositide hydrolysis.23 We used myr-PCI(Y) to further examine the involvement of PLCγ1 in RCEC proliferation. When added to RCECs, myr-PCI(Y) suppressed the EGF-induced PLCγ1 activation and cell proliferation, even when used at high concentrations. Taken together, these data provide convincing evidence that PLCγ1 is upregulated during EGF-induced proliferation of RCECs.

Activation of EGF receptor is followed by autophosphorylation of its tyrosine residues, which serve as high-affinity

FIGURE 3. Effect of U-73122 on EGF-stimulated cell proliferation and PLCγ1 activity in RCECs. Serum-starved cells were cultured for 36 hours in the presence and absence of EGF (50 ng/ml) and different concentrations of U-73122. The inactive analogue U-73343 was used as a negative control. (A) Cells were trypsinized and counted. (B) Cells were lysed and immunoprecipitated using PLCγ1 antibody. The resultant immunoprecipitate was assayed for PLC activity using [3H]PIP2 as the substrate. (C) Western blot of the immunoprecipitated PLCγ1. (D) PLCγ1 immunoblot stripped and reprobed with anti-phosphotyrosine antibody. Data are mean ± SEM from three independent experiments with three to four cultures for each data point. *Statistically significant (P < 0.05) decrease in cell number or PLCγ1 activity, compared with the corresponding cultures not treated with U-73122.

FIGURE 4. Effect of myristoylated PCI peptides on EGF-stimulated cell proliferation and PLCγ1 activity in RCECs. Serum-starved cells were cultured for 36 hours in the absence and presence of EGF (50 ng/ml) and different concentrations of myr-PCI(Y). myr-PCI(F) was used as a negative control. (A) Cells were trypsinized and counted. (B) Cells were lysed and immunoprecipitated using PLCγ1 antibody. The resultant immunoprecipitate was assayed for PLC activity using [3H]PIP2 as substrate. Data represent mean ± SEM of three independent experiments with at least duplicate cultures for each data point. *Statistically significant (P < 0.05) decrease in cell number or [3H]IP3, compared with the corresponding cultures not treated with myr-PCI(Y).
binding sites for SH2-containing proteins, including PLCγ1. Recruitment of PLCγ1 to the receptor results in tyrosine phosphorylation and stimulation of its enzyme activity. Tyrosine phosphorylation of PLCγ1 has been reported to be critical in transducing mitogenic signals from growth factor receptors to the interior of the cell. In particular, substitution of Phe for Tyr783 completely blocks the activation of PLCγ1 by PDGF in NIH 3T3 cells. Furthermore, overexpression of PI3K in COS-7 cells causes increased activation of PLCγ1 that is reduced to the normal level when the cells are treated with PI3K inhibitors. In other studies, the SH2 and PH domains of PLCγ1 have been found to potently bind PIPS generated by PI3K.

To investigate whether EGF-induced PLCγ1 activation in RCECs is dependent on concomitant activation of PI3K, PLCγ1 activity was assayed in cells treated with the PI3K inhibitors wortmannin and LY294002. The results showed that both wortmannin and LY294002 dose-dependently inhibited EGF-stimulated PLCγ1 activation and cell proliferation (Figs. 5, 6).

Because the cell number remained essentially unchanged with increasing concentrations of wortmannin or LY294002, it was unlikely that the inhibitory effects of these compounds on EGF-induced responses was due to increased cell death. When wortmannin or LY294002 was added directly to the PLCγ1 assay mixture, there was no inhibition of the enzyme activity (data not shown), which suggests that these compounds inhibit PLCγ1 by selectively blocking PI3K activity. Further evidence that PLCγ1 lies downstream of PI3K is provided by the experiments in which PLCγ1 was assayed in the presence of 3-phosphoinositides. The enzyme activity was markedly increased by PI(3,4,5)P3, PI(3,4)P2 and PI(3)P was without effect (Fig. 7).

Taken together, the data provide support for the hypothesis that EGF-induced proliferation of RCECs involves both increased PLCγ1 synthesis and enzyme activation by PI(3,4,5)P3, a product of PI3K. One likely mechanism for PLCγ1 activation by PI(3,4,5)P3 may involve recruitment of the enzyme by PI(3,4,5)P3 to the plasma membrane adjacent to its substrate, PIP2. The SH2, SH3, and PH domains of PLCγ1 can specifically interact with inositol phospholipids. After PLCγ1 recruitment to the plasma membrane, the enzyme could be activated directly by PI(3,4,5)P3.

In conclusion, the current data demonstrate a close correlation between PLCγ1 activation and cell proliferation in EGF-
tracellular signal-regulated (ERK) pathway. Therefore, it is 
is DAG, which is a natural activator of PKC. There are several 
results of the manuscript.

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Table of references