Synergy of Epidermal Growth Factor and 12(S)-Hydroxyeicosatetraenoate on Protein Kinase C Activation in Lens Epithelial Cells*

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Jianzheng Zhou†, Robert N. Fariss‡, and Peggy S. Zelenka†‡

From the †Laboratory of Molecular and Developmental Biology and the ‡Laboratory of Mechanisms of Ocular Diseases, NEI, National Institutes of Health, Bethesda, Maryland 20892

12(S)-Hydroxyeicosatetraenoic acid (12(S)HETE) is a bioactive metabolite of arachidonic acid synthesized by 12-lipoxygenase. The 12-lipoxygenase blocker, baicalein, prevents epidermal growth factor (EGF)-induced activation of protein kinase C (PKC) α and β in lens epithelial cells, whereas supplementation with 12(S)HETE reverses this effect, suggesting that EGF and 12(S)HETE may work together to activate PKC. This study investigates the mechanism of PKCβ activation by EGF and 12(S)HETE. 12(S)HETE alone directed translocation of PKCβ through the C1 rather than the C2 domain, without activating phosphoinositide 3-kinase (PI3K) or MAPK signaling or increasing intracellular calcium concentration. In the presence of baicalein, EGF triggered an asymmetric phosphorylation of the EGF receptor initiating signaling through PI3K and MAPK, but not PLCγ. Together, 12(S)HETE and EGF synergistically increased phosphorylation of PKCβ in the activation loop and C terminus as well as PKCβ-specific activity. PI3K inhibitors blocked phosphorylation, but MEK1 inhibitors did not. Microvesicles containing phosphatidylinositol 3,4,5-trisphosphate mimicked the action of EGF on PKCβ activity in the presence of 12(S)HETE. Kinase-inactive PKCβ mutations in either activation loop or C terminus were effectively translocated by 12(S)HETE, as was PKCβ in the presence of chelerythrine or Gö-6983. These findings indicate that unphosphorylated PKCβ is translocated to the membrane by 12(S)HETE and phosphorylated by EGF-dependent PI3K signaling, to generate catalytically competent PKCβ.

12(S)-Hydroxyeicosatetraenoic acid (12(S)HETE) is a bioactive metabolite of arachidonic acid, which evokes a wide variety of cellular responses, ranging from survival and proliferation to invasion and metastasis (1, 2). Although 12(S)HETE is synthesized primarily in platelets and leukocytes, a number of other cell types have some capacity to make this hydroxylipid, including the epithelial cells of the lens and cornea (3–5). Previous studies from this laboratory found that inhibitors of endogenous 12(S)HETE synthesis prevent EGF-dependent DNA synthesis and c-fos mRNA induction in cultured lens epithelial cells. This effect was specifically reversed by exogenous 12(S)HETE, but not by closely related HETEs, suggesting that 12(S)HETE plays an essential role in regulating lens cell proliferation (6, 7). Further investigation of the role of 12(S)HETE showed that the selective lipoxygenase inhibitor, baicalein, prevented EGF-induced activation of classic PKC isoforms, PKCa and PKCβ (8), raising the possibility that a cooperative effect of EGF and 12(S)HETE is needed for full activation of PKC in these cells. In addition, because inhibition of the classic PKC isoforms was sufficient to block both c-fos mRNA induction and DNA synthesis, these findings pinpointed PKC as an important target of 12(S)HETE action in regulating lens epithelial cell proliferation (8).

Structural studies of the classic PKC isoforms (PKCa, β, and γ) have identified several functional domains (9, 10). These include an autoinhibitory pseudosubstrate domain at the N terminus, the C1 domain, containing a diacylglycerol binding site (11), the C2 domain, containing binding sites for both anionic lipids and calcium (12), an activation loop adjacent to the active site, and the C-terminal domain. The classic PKC isoforms require diacylglycerol and calcium as well as PtdSer for full activity. In contrast, amino acid replacements at certain key residues in the C2 domain of the novel PKC isoforms (PKCδ, ε, η, and θ) have made these isoforms insensitive to calcium signals, whereas changes in both the C1 and C2 domains of the atypical isoforms (PKCγ and ι) have rendered these isoforms insensitive to both calcium and diacylglycerol (9, 10). However, all isoforms require PtdSer or other acidic phospholipids for activity. Activation of PKC involves both phosphorylation of the enzyme at the activation loop and C terminus and translocation to the membrane, where it interacts with its lipid cofactors, PtdSer and diacylglycerol. Phosphorylation at the activation loop seems to be catalyzed by the phospholipid-dependent kinase, PKD1 (13–15), whereas the two phosphorylations in the C terminus appear to be autophosphorylations (16–18). Phosphorylation of PKC is thought to introduce a conformational change, which allows it to respond to lipid second messengers, such as diacylglycerol (18). Upon binding to the membrane, an additional conformational change removes the autoinhibitory substrate domain from the active site and the enzyme becomes catalytically active (9, 10). Because previous studies of lens epithelial cells had suggested that EGF and 12(S)HETE may cooperate in some way to...
activate PKCα and PKCβ, the present study was undertaken to investigate the mechanism of this effect, using a GFP-tagged PKCβ fusion protein to study the respective effects of 12(S)-HETE and EGF on translocation, phosphorylation, and activation. The results indicate that the synergistic effect of EGF and 12(S)-HETE on PKCβ activation is due to 12(S)-HETE-dependent translocation of unphosphorylated PKCβ to the cytoplasmic membrane, where it is phosphorylated by a PI3K-dependent mechanism activated by EGF.

EXPERIMENTAL PROCEDURES

Materials—Wortmannin, LY294002, PD 98059, and the PepTag non-radioactive PKC assay kit were purchased from Promega (Madison, WI). 12-O-Tetradecanoylphorbol 13-acetate (TPA), ionomycin, and chelerythrine chloride were purchased from Sigma Chemical Co. (St. Louis, MO). 12(S)-HETE, baicalein, Go6983, and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3) were from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). EGF was from Invitrogen (Rockville, MD). Polyclonal anti-PKC antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies against phosphorylated sites in PKCβ C terminus, EGF receptor, and phosphorylated sites in EGF receptor as well as PK3 and MAPK signaling sampler kits were all purchased from Cell Signaling Technology Inc. (Beverly, MA). Monoclonal and polyclonal anti-GFP antibodies were from Clontech (Palo Alto, CA). Rabbit polyclonal (P5000) antibody directed against the phosphorylated activation loop of PKCβ was a gift from Dr. Alexandra C. Newton (Department of Pharmacology, University of California, San Diego, CA). Calcium indicator, Fluor-3 ester, was purchased from Molecular Probes, Inc. (Eugene, OR).

Cell Culture—The rabbit lens epithelial cell line, N/N1003A (a gift from Dr. John Reddan, Oakland University, Rochester, MI), was cultured at 35 °C in 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1 mM glutamine, 8% heat-inactivated rabbit serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. For PKC activity assay, cells were plated in 75-mm flasks in an initial density of 2 × 105 cells. At 90–95% confluence, the medium was replaced with serum-free DMEM for 24 h, and the endogenous PKCβ or stably expressed PKCβ-GFP was precipitated with anti-PKCβ or anti-GFP antibodies. For confocal observation of GFP-tagged PKCβ translocation, cells were cultured in a glass-bottomed chamber (LabTek-II Chamber Glass, WVR International, Bridgeport, NJ) and transfected with PKCβ-GFP (Clontech, Palo Alto, CA) using FuGENE transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN). After 48 h, transfected cells were visualized with a Leica TCS SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany). For immunoprecipitation and immunoblotting of PKCβ or PKCβ-GFP, N/N1003A cells were cultured in six-well plates. In all experiments, the cells were incubated for 90 min with 5 μM PKC inhibitors, washed three times with PBS, and lysed on ice and sonicated in a cold-water bath. Lysates were collected at room temperature. Protein concentration was measured by the BCA Protein Assay Reagent kit (Pierce, Rockford, IL). Aliquots of fractions containing 20 μg of protein were mixed with an equal volume of 2× loading buffer, electrophoresed on 4–20% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes (0.45-μm pore size, Novex, San Diego, CA) for 90 min at 400 mA as described previously (20). After transfer, membranes were blocked for 1 h at room temperature in 5% skim milk (Difco, Detroit, MI) in TBST (15 mM Tris-HCl and 150 mM NaCl, pH 7.5, with 0.05% Tween 20). The resultant supernatants were stored at −80 °C for immunoblot analysis. Immunoblot analysis was performed as previously described (19).

Translocation of Wild-type and Mutated PKCβ-GFP—PKCβ-GFP–transfected cells were spread onto the glass-bottomed chamber (LabTek-II, Ashland, MA) and cultured for at least 36 h. Serum-containing medium was replaced with serum-free DMEM 24 h before experiments. PKCβ-GFP fluorescence was measured by confocal laser scanning microscopy (Leica TCS SP2, Leica Microsystems) using 488-nm argon laser excitation, a 500-nm RSP dichroic filter, and a 500- to 550-nm emission spectrum. Reagents were diluted directly into the DMEM medium to obtain appropriate concentrations. Real-time images were collected at room temperature.

Immunoblot Analysis—Protein concentration was measured by the bicinchoninic acid method (BCA Protein Assay Reagent kit, Pierce, Rockford, IL). Aliquots of fractions containing 20 μg of protein were mixed with an equal volume of 2× loading buffer, electrophoresed on 4–20% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes (0.45-μm pore size, Novex, San Diego, CA) for 90 min at 400 mA as described previously (20). After transfer, membranes were blocked for 1 h at room temperature in 5% skim milk (Difco, Detroit, MI) in TBST (15 mM Tris-HCl and 150 mM NaCl, pH 7.5, with 0.05% Tween 20). The resultant supernatants were stored at −80 °C for immunoblot analysis. Antibodies used were as follows: PKCβ (1:500) mouse monoclonal (Transduction Laboratories, Lexington, KY), rabbit polyclonal antibody to Erk1/2 and phospho-Erk1/2 (T202/Y204) (1:1000), and rabbit polyclonal antibody to Akt (Akt) or phospho-Akt (New England BioLabs, Beverly, MA). The immunoblots were incubated with primary antibodies for 1 h at room temperature on a shaking platform, washed three times with TBST, and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody, either anti-rabbit or anti-mouse IgG (1:2500, New England BioLabs) for 30 min at room temperature. Specific immunoactive bands were detected by enhanced chemiluminescence (ECL Plus, Amersham Biosciences, Buckinghamshire, UK). Chemiluminescence was quantified by densitometric scanning of x-ray films with image analysis software (ImageQuaNT Scientific Software, version 5.0, Amersham Biosciences, Piscataway, NJ).

PepTag Assay for Nonradioactive Detection of PKC Activity—The PepTag assay utilizes a brightly colored, fluorescent peptide substrate that is highly specific to PKCα (Promega). Fluorescently labeled PKC changes the net charge of the substrate from +1 to −1, thereby allowing the phosphorylated and nonphosphorylated versions of the substrate to be separated on an agarose (0.8%) gel. The phosphorylated species migrates toward the positive electrode, whereas the nonphosphorylated substrate migrates toward the negative electrode. The phosphorylated substrate (after visualization under UV light) is used to immunoprecipitate PKC incubated with PKC reaction mixture (25 μl) according to the manufacturer’s protocol (Promega) at 30 °C for 30 min. The reactions were stopped by placing the tubes in a boiling water bath for 10 min. After adding 80% glycerol (1 μl), the samples were loaded
onto an agarose gel (0.8% agarose in 50 mM Tris-HCl, pH 8.0). The samples were separated on the agarose gel in the same buffer at 75 V for 25 min, and the bands were visualized under UV light and quantified by ImageQuaNT Scientific Software (version 5.0, Amersham Biosciences).

Confocal Ca$^{2+}$ Imaging—Intracellular Ca$^{2+}$ was monitored with Fluo-3 AM, a membrane-permeable long wavelength fluorescence indicator. N/N1003 cells were plated in glass-bottomed chambers (LabTek II Chamber Coverglass, VWR International, Bridgewater, NJ) and cultured in the standard medium for 24 h. The cells were loaded with acetoxymethyl ester of Fluo-3 (5 μM) for 30 min; after loading, a period of at least 30 min elapsed before experimentation to allow for deesterification of the intracellularly accumulated Fluo-3 AM. A Leica TCS-SP2 laser scanning confocal microscope (Leica Microsystems, Germany) was used to visualize Ca$^{2+}$-mediated fluorescence in the cells. Fluo-3 was excited with the 488-nm line of an argon laser, and Fluo-3 fluorescence was collected between 524 and 540 nm. Scanning was performed every 10 s for a total of 20 min after treatment with 12(S)HETE (800 nM) or EGF (15 ng/ml). For quantitative measurements, scanning was performed every 30 s for 40 min, and the ratio of fluorescence intensity to initial fluorescence intensity (F/F₀) was calculated at each point. Data were collected on 14 cells, and the results were averaged.

RESULTS

12(S)HETE and EGF Act in Synergy to Increase PKCβ-specific Enzymatic Activity—To examine the effect of 12(S)HETE and EGF on PKCβ activation, we immunoprecipitated both endogenous PKCβ and exogenous GFP-tagged PKCβ from the membrane fraction of N/N1003 cells and measured its activity using the PKC peptide substrate, PepTag. We also calculated the specific enzymatic activity of PKCβ by normalizing the relative level of phosphorylated PepTag to the relative amount of PKCβ in the immunoprecipitate, as determined by immunoblotting. The results showed that EGF (15 ng/ml) in the presence of baicalein (30 μM) did not significantly increase either the phosphorylation of the PepTag substrate (Fig. 1A, upper panel) or the amount of PKCβ in the membrane fraction (Fig. 1A, lower panel). Treatment with 12(S)HETE (300 nM) increased the amount of PKCβ in the membrane fraction and increased the phosphorylation of the PepTag substrate somewhat but produced no increase in the specific enzymatic activity of PKCβ (Fig. 1, A and B). However, combining 12(S)HETE (300 nM) with EGF (15 ng/ml) significantly enhanced PepTag phosphorylation and produced an ~2-fold increase in the specific enzymatic activity of PKCβ (Fig. 1, A and B). These results confirm that 12(S)HETE and EGF have a synergistic effect on PKCβ activation. For further studies of this effect, we established a cell line that stably expresses GFP-tagged PKCβ. The GFP tag was fused with the C terminus of PKCβ (Fig. 2A), because this fusion protein has previously been shown to retain full catalytic competence (21). Immunoprecipitation of PKCβ-GFP with monoclonal anti-GFP antibody confirmed that its behavior was indistinguishable from that of endogenous PKCβ with respect to activation in the presence and absence of 12(S)HETE (Fig. 1B, lower panel).

12(S)HETE Promotes Translocation of PKCβ-GFP—Because translocation of PKC to the membrane is often considered sufficient for its activation, we next examined the effect of 12(S)HETE and EGF on PKCβ translocation, using real-time imaging to follow the movement of PKCβ-GFP in living cells. As in the above experiments, the cells were incubated with the selective 12-lipoxygenase inhibitor, baicalein (30 μM), to block the endogenous generation of 12(S)HETE (8). Under these conditions, exogenous application of 12(S)HETE (300 nM) rap-
idly delivered GFP-tagged PKCβ to the plasma membrane (Fig. 2B). PKCβ-GFP fluorescence began to localize at the plasma membrane about 10 min after 12(S)HETE addition and reached a plateau after about 15 min. In contrast, in the presence of baicalein, EGF (15 ng/ml) had no effect on PKCβ-GFP translocation, whereas 12(S)HETE plus EGF showed a time course of PKCβ translocation very similar to that seen with 12(S)HETE alone (Fig. 2B). To quantify the time course of PKCβ-GFP redistribution, a translocation factor was calculated by comparing changes in the fluorescence intensity in two pre-assigned areas, one in the membrane and another in the cytosol. 12(S)HETE and EGF in combination showed no synergistic effect on PKCβ-GFP membrane translocation within 20 min (lower panel). D, time series images showing C1-GFP (upper panel, scale bar, 25 μm) or C2-GFP (middle panel, scale bar, 50 μm) translocation and Fluo-3 fluorescence changes after treatment with 12(S)HETE (800 nM). This concentration of 12(S)HETE had no effect on C2-GFP translocation or Fluo-3 fluorescence (lower panel, scale bar, 45 μm), although both parameters responded rapidly to ionomycin (0.5 μM) after 12(S)HETE was washed out.

A time series of confocal images was taken, and the value of R was calculated automatically by comparing the changes of fluorescence intensity in two pre-assigned areas: one in the membrane (representing IM) and another in the cytosol (representing IC). Thus,

\[ R = \frac{I_M - I_C}{I_C} \]  

(Eq. 1)

To investigate the mechanism underlying the 12(S)HETE-dependent translocation of PKCβ, we tested the effect of 12(S)HETE on GFP-tagged constructs of the truncated C1 and C2 domains of PKCβ. Previous studies have identified these domains as membrane targeting modules that mediate PKC translocation by binding with diacylglycerol and calcium, respectively (11, 12, 22, 23). In control experiments, TPA (1.5 μg/ml) induced a translocation of the C1-GFP domain to the membrane, where it remained for more than 20 min (not shown). Similarly, control experiments with ionomycin (0.5 μM) induced translocation of GFP-C2 (Fig. 2D). In this case, however, the time course of translocation was very rapid, with membrane-associated fluorescence reaching a peak and returning to baseline within 60 s in most cells (Fig. 2D). Interestingly, ionomycin (0.5 μM) also caused translocation of GFP-C1 to the membrane (data not shown). Because previous studies of the isolated C1 domain indicate that it binds to the membranes in a calcium-independent manner (12), the response to ionomycin may reflect a calcium-dependent change in membrane lipids.
Incubating cells in 300 nM 12(S)HETE, a concentration that promoted translocation of full-length PKC/H9252-GFP (30/34 cells imaged) within about 14 min, had a weak effect on C1-GFP membrane translocation (2/15 cells imaged) and no effect on C2-GFP (data not shown). Increasing the concentration of 12(S)HETE to 800 nM strongly directed C1-GFP to the membrane (14/16 cells imaged) but had no effect on C2-GFP translocation (Fig. 2D). These findings suggest that 12(S)HETE promotes the translocation of PKCβ by affecting either the lipid composition of the membrane or the lipid binding capacity of PKCβ, rather than by mobilizing Ca$^{2+}$. In addition, the observation that higher concentrations of 12(S)HETE are needed for translocation of the isolated C1 domain than for the full-length protein suggests that interactions between C1 and other regions of PKCβ, such as the recently reported interaction between C1 and C2 (24), may facilitate membrane binding.

In view of the inability of 12(S)HETE to direct the translocation of the calcium-responsive C2 domain, we next tested whether 12(S)HETE has any effect on cytoplasmic Ca$^{2+}$ levels. To measure changes in cytoplasmic calcium, we loaded N/NI1003A cells with the membrane-permeable calcium indicator, Fluo-3 ester, and monitored the Ca$^{2+}$-dependent fluorescence by real-time imaging, sampling every 10 s for 20 min. Application of 12(S)HETE (800 nM) had no effect on intracellular Ca$^{2+}$ levels (Fig. 2D). Thus, 12(S)HETE seems to mediate translocation of PKCβ through a preferential effect on the C1 domain, which is not accompanied by changes in intracellular calcium.

**EGF Induces PI3K and MAPK Signaling Cascades But Not the PLC-γ Pathway**—The EGF receptor undergoes tyrosine autophosphorylation in its intracellular domain after binding with EGF (25). The phosphorylated tyrosine residues then serve as docking sites for recruitment of different signaling molecules, leading to the activation of signaling through PI3K, MAPK, and PLC-γ (25). Phosphorylation of tyrosine 1068 leads to activation of PI3K and MAPK through the adaptor proteins.
Grb2 or Gab1 (26, 27), whereas phosphorylation at tyrosine 992 or 1173 activates PLC-γ (28, 29) (Fig. 3A, upper panel). In addition, EGF receptor phosphorylation by c-Src at tyrosine 845 increases the receptor kinase activity (30). To investigate the signaling pathways activated by EGF in N/N1003A cells, we examined the phosphorylation state of each of these tyrosines using phospho-specific antibodies. The results confirmed that the EGF receptor is expressed (Fig. 3A) and undergoes phosphorylation at tyrosine 845 and 1068 in response to EGF (15 ng/ml). However, no phosphorylation of tyrosine 992 was observed (Fig. 3A). Consistent with these findings, EGF treatment triggered strong phosphorylation of Akt, a kinase downstream of PI3K, and of Erk1/2 (Fig. 3B), indicating that both the PI3K and MAPK signaling cascades are activated. The lack of autophosphorylation at tyrosine 992 suggested that EGF may fail to activate signaling through PLC-γ in N/N1003 cells under the present conditions. In this case, EGF would also not produce the increase in cytosolic calcium that is the expected consequence of PLC-γ activation. To test this possibility, we examined the ability of EGF (15 ng/ml) to deliver

**Fig. 4.** Co-application of 12(S)HETE (300 nM) with EGF (15 ng/ml) has no synergistic effect on PI3K or MAPK signaling. Akt and Erk1/2 phosphorylation were determined as indicators for PI3K and MAPK signaling cascades activation, respectively. LY294002 (10 μM) or wortmannin (100 nM) was utilized to inhibit PI3K activity, whereas PD98059 (10 μM) was used for MEK inhibition. 12(S)HETE, alone or in combination with EGF, had no effect on Akt phosphorylation (left panel) or Erk1/2 phosphorylation (right panel). Phosphorylation of Akt was abolished by Ly294002 (10 μM) or wortmannin (100 nM) and phosphorylation of Erk1/2 by PD98059 (10 μM) confirming the involvement of PI3K and MEK, respectively.

**Fig. 5.** Co-application of 12(S)HETE (300 nM) with EGF (15 ng/ml) synergistically affects PI3K-dependent phosphorylation of endogenous PKCβ in the activation loop and C terminus. Endogenously expressed PKCβ from either membrane-associated fraction (A) or whole cell lysate (B) were immunoprecipitated with rabbit anti-PKCβ. Total PKCβ was immunoblotted with mouse monoclonal anti-PKCβ. PKCβ phosphorylation in the activation loop and C terminus was determined with phosphospecific antibodies. Immunoblots were quantified by densitometry and results were expressed as percentage of control. Five separate experiments were performed (mean ± S.E.). A, representative immunoblots showing total PKCβ and phosphorylation in either activation loop or C terminus from the membrane-associated fractions. Bar graphs of quantitative results show a PI3K-dependent synergistic effect of 12(S)HETE and EGF on PKCβ phosphorylation in activation loop and C terminus. B, representative immunoblots showing total PKCβ and phosphorylation in either activation loop or C terminus from whole cell lysate. Bar graphs of quantitative results show a PI3K-dependent synergistic effect of 12(S)HETE and EGF on PKCβ phosphorylation in activation loop and C terminus.
the calcium-responsive GFP-tagged C2 domain of PKCβ to the membrane. EGF failed to cause translocation of the GFP-tagged C2 domain (Fig. 3, upper panel), although ionomycin (0.5 μM) produced translocation within 40 s after EGF was washed out (not shown).

To further define the effect of EGF on the PLCγ signaling pathway, we monitored changes in free cytosolic Ca2+ level using the membrane-permeable calcium indicator, Fluo-3 ester. Cells were treated with EGF (15 ng/ml), and fluorescence was monitored every 10 s for 20 min. We observed no increase in cytosolic Ca2+ level within this time period (Fig. 3, lower panel). In contrast, when EGF was washed out and replaced by ionomycin (0.5 μM), Ca2+ levels were elevated within 20 s (Fig. 3C, lower panel). For a more quantitative measure of Ca2+ levels, the ratio of fluorescence intensity to initial fluorescence intensity (FF/FF₀) was measured every 30 s for 40 min. Data were collected from fourteen individual cells, and the results were averaged (Fig. 3D). We detected no increase in FF/FF₀, although this ratio increased sharply when EGF was washed out and replaced by ionomycin. Thus, under these experimental conditions, the PLC-γ pathway appears to be silent after treatment with EGF (15 ng/ml). This may explain the unusual finding that EGF (15 ng/ml) alone is unable to induce PKCβ translocation (Fig. 2A).

12(S)HETE Does Not Activate PI3K and MAPK Signaling Cascades—To test the effect of 12(S)HETE and EGF on the PI3K and MAPK signaling cascades, N/N1003 cells were treated with baicalein, followed by exposure to 12(S)HETE, EGF, or both, for 20 min, and specific antibodies were used to detect the activated forms of Akt and Erk1/2. As expected, EGF treatment led to phosphorylation of both Thr-308 and Ser-473 of Akt, as well as phosphorylation of Erk1/2 (Fig. 4). In contrast, treatment with 12(S)HETE did not lead to phosphorylation of either Akt or Erk1/2. Adding both agents in combination did not increase the extent of phosphorylation above the levels produced by EGF alone. Phosphorylation of Akt and Erk were blocked by the PI3K inhibitor, Ly294002, and the MEK1 inhibitor, PD98059, respectively, confirming that the observed phosphorylations resulted from signaling through PI3K and MEK1. These results imply that EGF is able to initiate signaling through these pathways, whereas 12(S)HETE is not.

Synergistic Effect of EGF and 12(S)HETE on PKCβ Phosphorylation—Because PKC phosphorylation is regarded as an important factor in regulating PKC activity, we next tested the respective roles of EGF and 12(S)HETE in phosphorylating the activation loop and C terminus of PKCβ, using specific antibodies against the phosphorylated motifs in the activation loop DGVT Kendrick and C terminus NFDKFFTRGQPVLpTFCGTPD and C terminus NFDFKKFTGQPVLpTFCGTPD. PKCβ was immunoprecipitated from both membrane fraction and whole cell lysate then analyzed by immunoblotting to determine its phosphorylation state. Unstimulated N/N1003A cells contained very low levels of phosphorylation in the activation loop (T500) and C terminus (T641), whereas 12(S)HETE is not. PKCβ phosphorylation at both sites, whereas 12(S)HETE (300 nM) alone had no significant effect on PKCβ phosphorylation in the activation loop and C terminus (T500) and C terminus (T641) in either the membrane fraction or whole cell lysate as determined by immunoblotting with phospho-specific antibodies (Fig. 5, A and B). Incubating baicalein-treated cells with EGF (15 ng/ml) alone for 20 min produced approximately a 70% increase in PKCβ phosphorylation at both sites, whereas 12(S)HETE (300 nM) alone had no significant effect on PKCβ phosphorylation under the same conditions (Fig. 5, A and B). In contrast, the combination of EGF and 12(S)HETE had a strong synergistic effect on PKCβ phosphorylation in both activation loop and C terminus with approximately a 4- to 5-fold increase of PKCβ phosphorylation (Fig. 5, A and B). To investigate the signaling cascades required for this synergistic effect on PKC phosphorylation, cells were pretreated with PI3K inhibitor...
LY294002 (10 μM) or MEK inhibitor PD98059 (10 μM) for 30 min. Pretreatment with LY294002 (10 μM) abolished the phosphorylation of PKC produced by combined treatment with EGF and 12(S)HETE, whereas the MEK inhibitor, PD98059, had no effect (Fig. 5, A and B), suggesting that signaling through PI3K is required.

To confirm that EGF and 12(S)HETE have a synergistic effect on phosphorylation of the PKCβ-GFP fusion protein, as well as the endogenous enzyme, PKCβ-GFP was immunoprecipitated from stably transfected cells using anti-GFP antibody. The phosphorylation state of its activation loop and C terminus were then examined by immunoblotting with phospho-specific antibodies. Once again, the combination of 12(S)HETE and EGF had a synergistic effect on phosphorylation of the activation loop and C terminus (Fig. 6, A and B), which was prevented by pretreatment with PI3K blocker, LY294002 (10 μM), but not by the MEK inhibitor, PD98059 (Fig. 6, A and B).

**Synergy of 12(S)HETE and EGF on PKCβ Activation Is PI3K-dependent**—The above results imply that the combined effect of EGF and 12(S)HETE on PKCβ activation may be due to EGF-dependent phosphorylation of PKCβ following its translocation to the membrane by 12(S)HETE. As a direct test of this possibility, we measured the effect of PI3K inhibitors on the specific enzymatic activity of PKCβ after stimulating with 12(S)HETE, EGF, or both agents combined. If phosphorylation in either the activation loop or C terminus is essential to the increase in specific enzymatic activity of PKCβ produced by these agents in combination, co-treatment with these inhibitors should block this effect. The results indicate that this is the case (Fig. 7). The synergistic effect of 12(S)HETE and EGF on the specific enzymatic activity of PKCβ was prevented by pretreatment with PI3K blockers, wortmannin (100 nM) or LY294002 (10 μM), but not by the MEK inhibitor, PD98059 (10 μM) (Fig. 7A). The same result was obtained using PKCβ-GFP immunoprecipitated from stably transfected N/N1003 cells with monoclonal anti-GFP antibody (data not shown). Because 12(S)HETE had no effect on PI3K signaling (Fig. 4), these results imply that the synergistic effect of EGF and 12(S)HETE on PKCβ activation requires EGF-dependent signaling through PI3K.

One of the principal products of phosphoinositide phosphorylation by PI3K is PtdIns(3,4,5)P₃ (31). This lipid generates a membrane docking site for a variety of pleckstrin homology domain proteins, including PDK1, the kinase implicated in PKC phosphorylation (13–15). To confirm that the EGF-dependent activation of PI3K is responsible for the synergistic effect of EGF and 12(S)HETE on PKCβ activation, we loaded N/N1003A cells with PtdIns(3,4,5)P₃ microvesicles and measured the specific enzymatic activity of PKCβ in the presence or absence of 12(S)HETE. PtdIns(3,4,5)P₃ had no effect on the specific enzymatic activity of PKCβ when added alone (Fig. 7B). However, in combination with 12(S)HETE, PtdIns(3,4,5)P₃ increased the specific enzymatic activity by about 2-fold (Fig. 7B). Thus, when combined with 12(S)HETE, PtdIns(3,4,5)P₃ is able to enhance the specific activity of PKCβ to about the same extent as EGF. These findings strengthen the view that, when 12(S)HETE and EGF act together to increase the specific enzymatic activity of PKCβ, the role of EGF is to increase PKCβ phosphorylation via PI3K signaling.

**12(S)HETE Delivers Unphosphorylated PKCβ to the Membrane**—These findings implied that the contribution of 12(S)HETE to the synergistic effect with EGF lies in its ability to deliver unphosphorylated PKCβ to the plasma membrane, where it can be phosphorylated by EGF-dependent activation of the PI3K cascade. To explore this idea in greater detail, we generated stably transfected N/N1003A cell lines expressing PKCβ-GFP containing specific mutations at phosphorylation sites in the activation loop (T500A or T641A/S660A) (Fig. 8A). As expected, neither the T500A mu-
Although both inhibitors effectively blocked PKC, we tested whether abolishing PKC activity with the inhibitors chelerythrine or Go 6983 interfered with the ability of 12(S)HETE or TPA in the presence of two PKC inhibitors, chelerythrine (10 \( \mu \)M) or Go 6983 (1 \( \mu \)M). Together these findings confirm that 12(S)HETE is able to direct membrane translocation of unphosphorylated, inactive forms of PKC and demonstrate that translocation and phosphorylation are separable events.

**DISCUSSION**

The present findings indicate that submicromolar concentrations of 12(S)HETE are sufficient to cause translocation of PKC to the plasma membrane. Moreover, 12(S)HETE also directs translocation of the kinase inactive construct PKC(T500A), which can not be phosphorylated in either the transactivation loop or C-terminal domain. Because this construct would be expected to retain the conformation of the unphosphorylated enzyme, as previously shown for PKC(T500V) (17, 34), conversion to the mature, phosphorylated conformation of the enzyme is apparently not required for 12(S)HETE-dependent translocation. These findings contrast with previous findings indicating that PKC must be fully phosphorylated to respond to lipid cofactors such as diacylglycerol or TPA (18) and suggest that certain lipids can recruit the unphosphorylated enzyme to the membrane for further processing by phosphorylation.

Although the mechanism responsible for 12(S)HETE-dependent translocation is unclear, it does not seem to involve
esterified diacylglycerols that specifically activate PKC. The hydroxylipid, 15(S)HETE, has been shown to form 15(S)HETE-esterified diacylglycerol is involved. Nevertheless, the related hydroxy acid, 12(S)HETE, may participate in PKC activation in lens epithelial cells, if endogenous 12(S)HETE synthesis is blocked and suggest that endogenously synthesized 12(S)HETE may participate in PKC activation in lens epithelial cells by promoting translocation. Interestingly, this may provide a mechanism for activating cPKC isoforms that does not require calcium mobilization. Exactly how endogenously synthesized 12(S)HETE might participate in this process is not yet clear, however. One possibility is that 12(S)HETE may be esterified to phospholipids, then released in response to EGF by the action of cPLA2, which is activated by MAPK (35, 36). Alternatively, arachidonic acid might be released by cPLA2 then converted to 12(S)HETE by 12-lipoxygenase. Because our results suggest that EGF does not activate PLCγ in this cell type, it seems unlikely that formation of a 12(S)HETE-esterified diacylglycerol is involved. Nevertheless, the related hydroxy acid, 15(S)HETE, has been shown to form 15(S)HETE-esterified diacylglycerols that specifically activate PKCα in human tracheal epithelial cells (37).

Several reports in the literature indicate that there are specific cell surface receptors for 12(S)HETE (38–40). In support of this view, addition of 12(S)HETE has been shown to activate PKC, PLCγ, MAPK, and PI3K in a variety of cell types (38, 40, 41). In contrast, we found that 12(S)HETE had no effect on signaling via MAPK, PI3K, or intracellular calcium and was unable to activate PKCγ without the cooperative action of EGF. The difference between these findings and those reported for other cell types suggests that 12(S)HETE may have multiple modes of action. Indeed, binding curves for 12(S)HETE are complex and provide evidence for both high affinity and low affinity receptors, further supporting the possibility that 12(S)HETE may have various modes of action (39, 40). Moreover, the apparent lack of downstream signaling in response to 12(S)HETE in the present study raises the possibility that some of its effects may be receptor-independent and may result from its ability to modify the lipid environment of the membrane.

In most adherent cell types, PKC is highly phosphorylated in both the activation loop and C terminus, even under unstimulated conditions (10, 15) or after serum starvation (15). Under these circumstances, cPKC translocation is sufficient for its activation, because membrane binding induces the conformational change needed to release the catalytic core from the autoinhibitory pseudosubstrate (10, 12, 34). In contrast, serum-deprived lens epithelial cells have very low levels of PKCβ phosphorylation, making it possible to separate translocation and activation. Our data suggest a model in which serum-deprived lens epithelial cells contain both phosphorylated and unphosphorylated PKCβ, which is distributed between the membrane and cytoplasm, with the bulk of the enzyme in the cytoplasm. Upon addition of exogenous 12(S)HETE, both phosphorylated and unphosphorylated PKCβ are translocated from the cytoplasm to the membrane (Fig. 9A). The phosphorylated PKCβ present in this fraction produces an increase in membrane-associated kinase activity. However, because there is no new phosphorylation of PKCβ, there is no increase in the specific enzymatic activity. On the other hand, if endogenous

![Synergistic Effect of 12(S)HETE and EGF on PKC Activation](image1)

Fig. 9. Schematic of the synergistic effect of 12(S)HETE and EGF on PKC activation. A, when endogenous 12(S)HETE synthesis is blocked, most PKCβ is unphosphorylated and cytoplasmic. Application of exogenous 12(S)HETE (300 nM) recruits PKCβ to the cytoplasmic membrane without changing its phosphorylation state. B, under the same experimental conditions, application of EGF (15 ng/ml) triggers an asymmetric autophosphorylation of EGF receptor (EGFR) leading to activation of PI3K and MAPK signaling pathways but not the PLCγ pathway. This increases phosphorylation of PKCβ that was already membrane-bound (presumably through the action of PDK-1); however, because most PKCβ is cytoplasmic, there is little increase in PKCβ activity. Previous results have shown that cell proliferation does not occur under these conditions, although MAPK signaling is activated (6–8). C, application of exogenous 12(S)HETE (300 nM) and EGF (15 ng/ml) together leads to 12(S)HETE-dependent translocation of PKCβ as well as EGF-dependent PI3K activation. The resulting synthesis of PtdIns(3,4,5)P3 may recruit PDK-1 to the membrane, increasing the probability of interaction between PDK-1 and PKCβ. Under these conditions, a large proportion PKCβ is activated by phosphorylation and mitogenesis occurs (6–8).
12(S)HETE synthesis is blocked and EGF is added, activation of PI3K signaling may phosphorylate the small amount of unphosphorylated PKCβ associated with the membrane fraction of the serum-deprived cells but is unable to promote translocation (Fig. 9B). Phosphorylation of PKCβ in the membrane increases both the activity of the membrane fraction and the specific enzymatic activity, but the effect is small, because the amount of enzyme associated with the membrane is small. Previous results have shown that cell proliferation does not occur under these conditions, although MAPK membrane is small. Previous results have shown that cell proliferation does not occur under these conditions, although MAPK

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