EGF-Induced ERK Phosphorylation Independent of PKC Isozymes in Human Corneal Epithelial Cells

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PURPOSE. To investigate the role of protein kinase C (PKC) isozymes in epithelial growth factor (EGF)-induced activation of extracellular signal-regulated kinase (ERK) and cell proliferation in cultured human corneal epithelial cells.

METHODS. Simian virus (SV)40 stably transfected human corneal epithelial (THCE) cells were cultured in keratinocyte growth medium. PKC isozymes and phosphorylation of ERK in THCE cells were assessed by Western blot analysis. Translocation of the PKC isozyme was determined by subcellular fractionation followed by Western blot analysis. Cell proliferation was measured by incorporation of [3H]-thymidine into DNA.

RESULTS. Six PKC isozymes—PKC-α, -βI, -βII, -δ, -ε, and -μ—were found in THCE cells. Phorbol 12-myristate 13-acetate (PMA) caused PKC-α, -βI, and -ε, initially present in the cytoplasm, to be translocated to the membrane and nuclear subcellular fractions and PKC-δ to be depleted from the cytoskeleton. The PKC inhibitor GF109203X inhibited PMA-induced, but not basal or EGF-induced, phosphorylation of ERK, whereas the EGF receptor inhibitor tyrophostin AG1478 blocked basal and EGF-, but not PMA-, induced phosphorylation of ERK. Depletion of PMA-sensitive PKC isozymes including PKC-α, -βI, -βII, -δ, and -ε, inhibited PMA-, but not EGF-, induced phosphorylation of ERK. Depletion of these PKC isozymes blocked PMA-, but not EGF-, induced cell proliferation.

CONCLUSIONS. Although activation of PKC by PMA results in phosphorylation of ERK, EGF-induced phosphorylation of ERK and/or cell proliferation is independent of the conventional and novel PKC isozymes PKC-α, -βI, -βII, -δ, and -ε in human corneal epithelial cells. (Invest Ophthalmol Vis Sci. 2002;43:3673–3679)

Protein kinase C (PKC) is composed of a family of serine-threonine kinases that modulate the function of a variety of signal transduction pathways that control cell growth, cell differentiation, and wound healing. The PKC gene family is divided into three subgroups based on sequence homology and cofactor requirements: classic-conventional PKC isozymes (PKC-α, -βI, -βII, and -γ), which are Ca2+-dependent and diacylglycerol (DAG)-stimulated kinases; novel PKC isozymes (PKC-δ, -ε, -η, and -θ), which are Ca2+-independent and DAG-stimulated kinases; and atypical PKC isozymes (PKC-ζ and -ι/λ), which are Ca2+- and DAG-independent kinases. Cell signal pathways involving the PKC family are initiated by binding of a ligand, such as a growth factor, to its respective cell surface receptor, which triggers the breakdown of phospholipids by phospholipases C and D and production of DAG. DAG binds to and activates most PKC isozymes that translocate to specific subcellular compartments that vary among PKC isozymes and among cell types. Phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), are potent tumor promoters and can substitute for DAG in stimulating PKC. There has been considerable interest in the potential role of PKC isozymes in growth factor-modulated biological processes, such as cell proliferation, extracellular matrix (ECM) remodeling, and wound healing.

A number of previous studies have demonstrated a role for PKC in corneal epithelial gene expression and wound healing. Increased PKC-α activity was observed in proliferating epithelium after wounding, suggesting a role for PKC-α in postinjury, long-term responses, such as gene expression and proliferation of corneal epithelial cells. In organ-cultured corneas, inhibition of PKC significantly delayed reepithelialization, suggesting that PKC activity is an important factor in regulating corneal epithelial wound healing, presumably by influencing cell migration. Moreover, antisense oligonucleotides specific to PKC-α appeared to inhibit epithelial wound closure in organ culture. Substance P, a neurotransmitter in the trigeminal nerve, which innervates the corneal epithelium, induced expression of E-cadherin, which is involved in the activation of PKC. In rabbit corneal epithelium, five isozymes—PKC-α, -γ, -δ/ε, -η, and -μ—were identified. Expression and/or distribution of these isozymes were altered in wounded corneas, implying their involvement in epithelial wound healing in vivo.

Phorbol esters, which directly activate most PKC isozymes, are often used experimentally to induce activation of PKC. The translocation of a PKC isozyme from the cytosol to the membrane has been used as an indication of its activation. However, considering a role in regulation of gene expression during cell proliferation and differentiation, an involvement of PKC, either directly or indirectly, in nuclear events is suggested. In addition, because activation of PKC induces cytoskeletal reorganization, and several PKC-binding proteins have been shown to bind to the actin filaments, association of PKC isozymes with cytoskeleton has also been indicated. Thus, determining the subcellular structure to which each PKC isozyme translocates after activation would provide information needed for understanding the role of each isozyme in cells.

Epidermal growth factor (EGF) and its related family members including TGF-α and heparin-binding EGF have been implicated in stimulation of corneal epithelial cell migration, proliferation, and synthesis of basement membrane and ECM components. EGF is known to activate PKC and mobilize intracellular Ca2+, initial important signaling events that contribute to the final physiological effects in a variety of cells. It is well established that EGF can activate the mitogen-activated protein kinase (MAPK) signaling pathway, a major signaling pathway leading to cellular proliferation in many cell types, through the Ras-Raf-MAPK kinase (MEK)-extracellular signal-regulated kinase (ERK) cascade initiated by stimulating the intrinsic tyrosine kinase activity to the EGF receptor. It is, however, evident that EGF-induced MAPK signaling may also

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be regulated through PKC isozymes in a cell-specific manner.\textsuperscript{11,21–25} For example, in human colonic epithelial cells, EGF was found to induce a rapid translocation of PKC-\(\varepsilon\) from cytoplasm to membrane, which led to inhibition of carbachol-induced chloride secretion.\textsuperscript{23} In addition, EGF stimulated MAPK through a PKC-\(\varepsilon\)-dependent pathway by acting upstream of MEK and inducing DNA synthesis. This was effective in rat hippocampal H19-7 cells, but not in embryonic rat brain hippocampal cell cultures.\textsuperscript{24,25} We recently observed that in simian virus (SV)40-transformed human corneal epithelial (THCE) cells both PMA and EGF induces ectodomain shedding of amyloid precursor-like protein (APLP)-2, a process associated with corneal epithelial wound healing.\textsuperscript{26} This suggests a potential role for PKC in modulation of EGF-induced APLP2 shedding in human corneal epithelial cells. Therefore, it was of interest to investigate the role of the PKC signaling pathway in EGF-treated corneal epithelial cells.

In this study, PMA induced translocation of PKC-\(\alpha\), -\(\beta\)-I, -\(\delta\) and -\(\varepsilon\) in THCE cells from cytoplasm to other specific subcellular fractions, whereas stimulation with EGF resulted in little translocation of PKC isozymes. Furthermore, PKC depletion blocked PMA, but not EGF-induced phosphorylation of ERK and epithelial cell proliferation. These results suggest that although induction of PKC activity activates MAPK in this cell line, EGF-induced MAPK signaling and cell proliferation are independent of PMA-sensitive PKC isozymes.

**Materials and Methods**

**Materials**

EGF was purchased from R&D Systems (Minneapolis, MN). GF109203X (bisindolylmaleimide I) was from Calbiochem (San Diego, CA). Poly-ADP ribose-polymerase (PARP) and c-fos antibodies specific to PKC-\(\alpha\), -\(\beta\)-I, -\(\delta\), -\(\varepsilon\), -\(\gamma\), -\(\eta\), -\(\iota\), -\(\mu\), -\(\theta\), and -\(\zeta\) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). [Methyl\(^{3}H\)]-thymidine was purchased from Perkin Elmer–NEN Life Science Products (Boston, MA). Keratinocyte basic medium (KBM) and keratinocyte growth medium (KGM, KBM supplemented with growth factors) were from BioWhittaker (Walkersville, MD). All other chemicals were purchased from Sigma (St. Louis, MO).

**Cell Culture and Treatments**

THCE cells that continue to grow and exhibit a cobblestone-like appearance were generously provided by Kaoru Araki-Sasaki.\textsuperscript{27} THCE cells were grown in KGM in a humidified 5% CO\(_2\) incubator at 37°C. Before experiments, cells were cultured onto plastic dishes precoated with fibronectin-collagen coating mix (Biological Research Faculty and Facility Company, Ljamsville, MD) and grown to 60% confluence in KGM. Before treatment, KGM was replaced with KBM for 16 hours (growth factor starvation) overnight. At the time of treatment, culture medium was replaced with fresh KBM containing indicated test substances. To deplete PMA-sensitive PKC isozymes, cells were treated with PMA (1 \(\mu\)M) for 24 hours.

**Subcellular Fractionation**

To identify which PKC isozymes were present, cultured THCE cells were lysed and subjected to Western blot analysis with 10 PKC isozyme–specific antibodies. To determine subcellular distribution of PKC isozymes, subcellular fractionation was performed by Maloney et al.\textsuperscript{11} with modifications. Growth factor-starved THCE cells were treated with PMA for 1 to 10 minutes, untreated as control. Cells were washed twice with ice-cold PBS and scraped into a homogenization buffer containing 25 mM Tris/HCl (pH 7.4), 2 mM EDTA, 10 mM \(\beta\)-mercaptoethanol, 10% glycerol, and a proteinase inhibitor mixture containing 10 \(\mu\)g/mL aprotinin, 10 \(\mu\)g/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF) and then homogenized (Dounce homogenizer; Bellco Glass Co, Vineland, NJ). The homogenates were centrifuged at 500g for 5 minutes and the low-speed supernatant was centrifuged at 100,000g for 30 minutes. The high-speed supernatant constituted the cytosolic fraction. The high-speed pellet was washed three times and extracted in ice-cold homogenization buffer containing 1% Triton X-100 for 30 to 60 minutes. The Triton-soluble component (membrane fraction) was separated from the Triton-insoluble material (cytoskeletal fraction) by centrifugation at 100,000g for 30 minutes. The cytoskeletal fraction was washed three times with homogenization buffer, resuspended in the same buffer, and dispersed by sonication. The low-speed pellet containing nuclei and unbroken cells was resuspended in a nuclear buffer containing 25 mM Tris/HCl (pH 7.4), 3 mM MgCl\(_2\), 1 mM PMSF, 10 mM \(\beta\)-mercaptoethanol, and 0.05% Triton X-100 and homogenized (Dounce; Bellco Glass Co.). To remove contaminating membrane components, the low-speed pellet homogenate was centrifuged at 500g, resuspended in the nuclear buffer without Triton X-100, layered over 45% sucrose, and centrifuged at 1900g for 30 minutes in a microfuge (Eppendorf, Fremont, CA). The purified nuclei were resuspended in the homogenization buffer containing 1% Triton X-100. The small amount of insoluble material was removed by centrifugation at 100,000g for 15 minutes at 4°C. Protein concentration was determined with a kit (MicroBCA Protein Assay Kit; Pierce, Rockford, IL) with bovine serum albumin as a standard.

**Western Blot Analyses**

An equal amount of protein of cell lysates was applied to a 5% to 15% gradient SDS-polyacrylamide gel and electrophoresed. For fractionation study, the same amount of cytosolic protein (10 \(\mu\)g) and its corresponding portion of fractions were used for SDS-PAGE (i.e., if one tenth of total cytosol was 10 \(\mu\)g, the same portion—one tenth of the total volume—of other cellular fractions was used). The gel-separated proteins were then transferred to nitrocellulose membrane. Efficiency of protein transfer was assessed by staining the nitrocellulose membranes withponceau S. The membranes were blocked with 5% nonfat skim milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) for 1 hour and then incubated with isozyme-specific anti-PKC antibodies to PKC-\(\alpha\) (1:1000 dilution), -\(\beta\)-I (1:200), -\(\delta\) (1:400), -\(\varepsilon\) (1:1000), -\(\eta\) (1:400), -\(\iota\) (1:400), -\(\mu\) (1:400), -\(\theta\) (1:400), and -\(\zeta\) (1:200) at room temperature for 1 hour, or at 4°C overnight. Nitrocellulose membranes were washed three times with TBST and then incubated with horseradish peroxidase–conjugated secondary antibodies for 1 hour. An enhanced chemiluminescence detection system from Pierce (Rockford, IL) was used to visualize the labeled protein bands. Molecular mass was estimated by comparison of sample bands with prestained molecular mass markers (Bio-Rad, Hercules, CA). For quantitative studies, the bands on x-ray films were scanned onto a computer (BDS Image System; Biological Detection System, Pittsburgh, PA) and analyzed with NIH Image software (version 1.61; NIH Image; W. Rasband, National Institutes of Health; available by ftp from zippy.nih.nih.gov or on floppy disc from NTIS, Springfield, VA, part number PB95-500195GEI).

**Determination of ERK and ERK Phosphorylation**

ERK2 and phosphorylation of ERK were determined using monoclonal antibodies against ERK2 (1:2000) or phosphorylated ERK 1 and 2 (p+4 and p+12, 1:200 dilution), respectively. To determine the effects of PKC and EGF receptor inhibitors on phosphorylation of ERK, THCE cells were pretreated with GF109203X and tyrphostin AG1478 for 10 minutes and then treated with PMA and EGF for 10 minutes. For time-course studies, cells with or without PMA pretreatment were stimulated with PMA or EGF for up to 2 hours. The stimulation was stopped by the addition of 5 volumes of ice-cold KBM. The cells were lysed and processed for Western blot analysis for ERK2 and phosphorylated ERK.

**Measurement of Incorporation of [\(^{3}H\)]-Thymidine into DNA**

An equal number of THCE cells were seeded in each well in 24-well plates and allowed to grow to 60% confluence. After 24 hours' starva-
ion in KBM, cells were pretreated with either PMA or equivalent concentrations of the solvent (dimethyl sulfoxide) in KBM for 24 hours. PMA or EGF were then added for another 24 hours. [3H]thymidine (2.5 μCi/ml) was added 16 hours before the end of the incubation. The cells were quickly washed three times with ice-cold PBS, incubated with 1 mL 10% (wt/vol) trichloracetic acid (TCA) for 10 minutes, and washed twice with 10% TCA and three times with 95% ethanol. The acid-insoluble precipitate was incubated in 200 μl 0.2 N NaOH for 1 hour and neutralized with HCl. The radioactivity was determined by liquid scintillation counting.

Statistical Analysis
Statistical parameters were ascertained by computer (StatView; Abacus Concepts, Inc., Berkeley, CA) and results were expressed as the mean ± SE. Statistical significance was determined by Student’s t-test, and P < 0.05 was considered significantly different.

RESULTS
Expression of PKC Isozymes in THCE Cells
As a first step to study the involvement of PKC signaling in corneal epithelial cells, we investigated the expression of PKC isozymes in THCE cells by Western blot analysis with antibodies against 10 PKC isozymes. As shown in Figure 1, six PKC isozymes—three classic, PKC-α, -βI, and -βII, and three novel, PKC-δ, -ε and -μ—were identified in THCE cell lysate. Whereas the band intensity for these isozymes varied greatly, antibodies against PKC-ε, -η, -λ/ι, -δ, and -γ exhibited no immunoreactivity.

Translocation of PKC Isozymes in Response to Stimulation with PMA
In an initial experiment, we found that four PKC isozymes, PKC-α, -βI, -δ, and -ε, was redistributed from the soluble to the insoluble fraction after stimulation with PMA in THCE cells. PKC isozymes are known to be associated with membrane, nuclei, and cytoskeleton where they may play distinct roles. Consequently, we fractionated cells into four subcellular fractions and assessed the isozyme translocation using Western blot analysis; the results are shown in Figure 2. In unstimulated cells, PKC-α was either present in the cytoplasm or associated with the cytoskeleton (42.3% and 57.5%, respectively). Stimulation with 1 μM PMA induced rapid translocation of PKC-α from the cytosol to the membrane fraction and slow translocation to the nuclei, whereas the level of the cytoskeleton-associated protein slowly declined from 57.7% to 32.8% of total immunoreactivity. The translocation of PKC-α to membrane and nuclear fractions was evident at 1 minute, and only a very small amount of PKC-α (7.7%) remained in the cytosol at 10 minutes. In untreated cells, PKC-βI was distributed in the cytosol (57.8%), cytoskeleton (39.2%) and a small portion in the nuclear fraction (2.96%). PMA induced translocation of PKC-βI from the cytosol to the membrane and nucleus fractions within 1 minute, thereafter the level of PKC-βI in the membrane fraction declined. Concomitant with the decrease in cytosol, the level of nucleus-associated PKC-βI increased dramatically, reaching 53.7% of total immunoreactivity at 10 minutes, whereas in the cytosolic fraction it was undetectable (4.3%). In resting state, PKC-δ was distributed in all four fractions. Incubation of PMA for 1 minute induced an increase in membrane-associated PKC-δ molecules, concomitant with a decrease in both the cytosol-and cytoskeleton-associated ones. The increase in membrane-associated PKC-δ was transient, whereas the level of nucleus-associated PKC-δ continued to increase (41.6% at 10 minutes). PKC-ε was distributed primarily in the cytoplasm (52.3%), but was detectable in other fractions in resting THCE cells. PMA induced a rapid increase of PKC-ε in the membrane and nuclear fractions and a decrease in the cytosol at 1 minute (40% and 39.5%, respectively). After 1 minute, the level of PKC-ε gradually decreased in the nuclei, increased steadily in the membrane fraction (54.6% at 5 minutes and 60% at 10 minutes), and remained at low level in the cytosol (~6%). PKC-βII and -μ were found mostly associated with membrane fractions in unstimulated THCE cells, and PMA treatment did not significantly affect their subcellular localization (data not shown). A similar pattern of PKC translocation was also observed in human telomerase-immortalized human corneal epithelial cells (data not shown).

To determine whether PKC was activated after stimulation with EGF, PKC isozyme translocation was examined. Unlike PMA, EGF induced no significant changes of PKC isozyme levels in both the soluble and insoluble fractions (data not shown).

Effects of PKC and Tyrosine Kinase Inhibitors on ERK Phosphorylation in THCE Cells
Phosphorylation of ERK is indicative of activation of the MAPK pathway. Figure 3 shows PMA- or EGF-stimulated phosphorylation of ERK in THCE cells. In 16-hour growth factor-starved THCE cells, there was basal phosphorylation of ERK (KBH). The band intensity of phosphorylated ERK in these cells, however, was lower than that in cells cultured in growth medium (KGM). As we have shown,26 activation of PKC by PMA and stimulation with EGF increased the level of phosphorylated ERK in THCE cells. A similar pattern of phosphorylation of ERK was observed in telomerase-immortalized human corneal epithelial cells (data not shown). To assess the relationship between PKC- and EGF-induced activation of ERK, we determined phosphorylation of ERK in the presence of PKC or tyrosine kinase inhibitors of EGF receptor in THCE cells, using antibodies against the phosphorylated ERK. As shown in Figure 4, there was basal phosphorylation of MAPK in untreated, growth factor-starved THCE cells (lane 1), and the presence of PKC inhibitor GF109203X (5 μM) had minimal effect on the basal phosphorylation of ERK (lane 2). Addition of PMA to the cells resulted in an increase in phosphorylation of ERK (lane 3) and the presence of GF109203X inhibited PMA-induced phosphorylation of ERK (lane 4). Addition of EGF receptor inhibitor tyrphostin AG1478 (1 μM) reduced the level of phosphorylation of ERK in the presence of PMA (lane 5). Because tyrphostin AG1478 inhibited basal phosphorylation of ERK (lane 6), the observed decrease in PMA-induced phosphorylation of ERK by tyrphostin AG1478 (compare lane 3 with lane 5) was probably due to its inhibition of basal phosphorylation, suggesting that inhibition of EGF receptor activation had no inhibitory effect on PMA-induced phosphorylation of ERK. EGF also induced phosphorylation of ERK (lane 7), and GF109203X had no effect on this induction (lane 8), suggesting that PKC activ-
ity is not necessary for EGF to stimulate phosphorylation of ERK. Addition of tyrphostin AG1478, however, inhibited both basal and EGF-induced phosphorylation of ERK in THCE cells (lane 9). Taken together, these data indicate that PKC and EGF independently induced MAPK signaling in THCE cells.

**FIGURE 2.** PMA-induced translocation of PKC isozymes in THCE cells. (A) Representative Western blot analysis of PKC isozymes translocated in response to PMA in subcellular fractions of THCE cells. THCE cells were cultured in KBM overnight (growth factor–starved) and then stimulated with 1 μM PMA for 1, 5, and 10 minutes. The same amount of cytosolic protein (10 μg) and corresponding volume of other fractions were subjected to SDS-PAGE and probed with PKC isozyme antibodies for Western blot analysis (C, cytosol; M, membrane; N, nucleus; Ck, cytoskeleton). (B) Percentage changes of PKC isozymes among subcellular fractions after stimulation with PMA. The relative amount of PKC isozymes in each fraction was quantitated by gel scanning and expressed in square pixels (pixels in a defined area of the band). The total square pixels from four fractions was calculated as 100%. Data are the mean ± SE of results in three to four independent experiments.

**FIGURE 3.** Phosphorylation of ERK in THCE cells. THCE cells were cultured in KBM overnight (growth factor–starved) or KGM, the growth factor–starved cells were also stimulated with 1 μM PMA or 20 ng/mL EGF for 10 minutes. Five micrograms of protein from THCE cell lysate were separated by SDS-PAGE and analyzed by Western blot analysis with anti-ERK2 and anti-phosphorylated ERK1 and 2. ERK2 staining was used to show that the alteration in phosphorylation of ERK was not due to changes in expression of the ERK gene and to normalize the protein loading.

**FIGURE 4.** Assessment of PKC- and EGF-induced activation of ERK/MAPK. Growth factor–starved THCE cells were treated with 1 μM PMA (lanes 3–5) or 20 ng/mL EGF (lanes 7–9) in the absence or presence of the tyrosine kinase inhibitor of EGF receptor, tyrphostin AG1478 (AG, lanes 5, 6, and 9) or the PKC inhibitor GF109203X (GF, lanes 2, 4, and 8), with no treatment as the control (lane 1). Cell lysates were probed with antibodies against phosphorylated ERK (upper panel) or ERK2 (lower panel). The levels of ERK2 remained constant under all experimental conditions, whereas the presence of GF109203X (5 μM) inhibited PMA; but not EGF–, induced phosphorylation of ERK. AG1478 blocked both basal and EGF, but not PMA–, induced phosphorylation of ERK.
Effects of Downregulation of PKC on Phosphorylation of ERK and Proliferation of Epithelial Cells

Because prolonged treatment with PMA has been shown to deplete cells of the expression of PKC,28 we analyzed the expression of DAG-sensitive PKC isozymes after 24 hours of treatment with PMA (Fig. 5). PMA completely depleted PKC-α, -βI, -δ, and -ε, and greatly downregulated PKC-βII and -μ in THCE cells, as assessed by Western blot analysis. Prolonged incubation had a limited effect on PKC-ε (also termed PKD) in THCE cells. This is consistent with an early report that DAG-sensitive PKC-ε cannot be depleted from cells by prolonged treatment with PMA.29

The time course of phosphorylation of ERK was also examined in THCE cells, with or without treatment of PMA for 24 hours (Fig. 6). Addition of EGF to THCE cells, regardless of pretreatment with PMA, resulted in a rapid increase in phosphorylation of ERK, with the maximum level reached within 5 minutes and sustained for at least 20 minutes after EGF exposure (Figs. 6A, 6C). By 2 hours, the level of phosphorylated ERK in PMA-treated THCE cells declined from its peak level.

Similarly, addition of PMA to untreated THCE cells led to an increase in phosphorylation of ERK, with the maximum level reached within 5 minutes (Fig. 6B). The PMA-stimulated phosphorylation of ERK appeared to be less and decreased at a rate faster than EGF-stimulated one (comparing Fig. 6B with 6A). However, in PMA-pretreated cells, there was little phosphorylated ERK when cells were further stimulated with PMA (Fig. 6D). Taken together, these data indicate that PMA- but not EGF- stimulated phosphorylation of ERK was inhibited by depletion of PMA-sensitive PKC isozymes in THCE cells.

Stimulation of the ERK pathway in many cells is known to correlate with increased cell proliferation. Consequently, it was of interest to determine the effects of downregulation of PKC on EGF-stimulated proliferation of THCE cells. Addition of 10 nM PMA or 20 ng/mL EGF to THCE cells increased incorporation of [3H]-thymidine into DNA (Control, Fig. 7). We chose 10 nM PMA, because we observed that further incubation with a higher concentration for another 24 hours after prolonged exposure to PMA caused cell detachment. Pretreatment of the cells for 24 hours with 500 nM PMA significantly reduced PMA- but not EGF- induced incorporation of [3H]-thymidine. In fact, the same amount of EGF stimulated a significantly larger amount of [3H]-thymidine incorporation into DNA in PKC-depleted THCE cells than in non-PMA–treated cells. Consistent with PMA pretreatment, GF109203X incubation did not affect EGF-induced THCE cell proliferation (data not shown). Thus, EGF-induced cell proliferation in human corneal epithelial cells was independent of DAG-sensitive PKC isozymes.

**DISCUSSION**

The present study was initiated to investigate the involvement of PKC in EGF-induced signal transduction pathways. Several important observations emerged from our studies. First, PMA-induced differential translocation of four PKC isozymes (PKC-α, -βI, -δ, and -ε) from cytoplasm to membrane and/or nuclear subcellular fractions, and activation of PKC isozymes by PMA resulted in activation of MAPK. Second, addition of a PKC inhibitor blocked only PMA-induced phosphorylation of ERK, whereas the EGF receptor inhibitor blocked basal and...
EGF, but not PMA, induced phosphorylation of ERK. Third, prolonged treatment with PMA resulted in downregulation of five PKC isozymes (PKC-α, -βI, -βII, -δ, and -ε) and abolished PMA, but not EGF, induced phosphorylation of ERK and proliferation of epithelial cells. Although both PMA and EGF activated cell proliferation, they appeared to use different signaling pathways. Our results suggest that EGF-induced activation of the ERK signaling pathway and cell proliferation is independent of the PKC isozymes, PKC-α, -βI, -βII, -δ, and -ε.

We previously observed that the expression of APLP2 is greatly enhanced in the basal cells of wounded epithelium. Furthermore, APLP2 has been found to be released (ectodomain shedding) onto the wound bed by migratory and proliferative epithelia in vivo. Using THCE cells, we showed that both activation of PKC by PMA and stimulation with EGF increases ectodomain shedding of APLP2 through activation of MAPK. In this study, we used the same cell line to elucidate a potential role for PKC isozymes in EGF-stimulated signaling pathways by investigating MAPK activation and cell proliferation. However, it should be mentioned that SV40 transformation, known to prevent replicative senescence of cells in culture, has strong effects on proteins that participate in the cell cycle, growth arrest, and apoptosis; thus, these cells may have a compromised cell-cycle control. THCE cells had basal phosphorylation of ERK in untreated, growth factor-starved cells, and addition of the growth medium EGF or PMA stimulated phosphorylation of ERK to different extents. Similarly, serum-starved THCE cells appeared to be able to proliferate, albeit at a low rate, in the absence of exogenously added EGF (Fig. 7). The observed proliferative response is most likely due to autocrine secretion of growth-promoting substances by cells in the culture medium. When treated with EGF or PMA, the cells exhibited an increase in cell proliferation. Thus, the THCE cells respond to exogenous stimulation and therefore were suitable for our study to characterize the role of PKC isozymes in EGF-induced MAPK activation as well as cell proliferation.

The relative importance of PKC in activation of the MAPK pathway has been shown to depend on the agonist and the cell type. In rabbit corneal epithelial cells, five PKC isozymes, PKC-α, -βI, -γ, -ε, and -λ, were identified. Tseng and Li, using immunohistochemistry, detected PKC-α, -β (weakly), -λ, -δ (weakly), and -γ, but not -ε, in human corneal epithelium. In this study, we used Western blot analysis and detected six isozymes, PKC-α, -βI, -βII, -δ, -ε, and -λ, in THCE cells. Our results differ from theirs in immunoreactivity of several PKC isoforms, including -λ, -γ, and -ε. This discrepancy may be due to differences in staining methods or the antibodies used. It has been suggested that each isozyme may perform distinct functions on cell activation through its translocation to discrete regions within the cell. In the present study, we investigated this possibility by first examining changes in the ratio of PKC isozymes in the cytosolic and noncytosolic fractions (data not shown). We observed significant translocation of PKC-α, -βI, -δ, and -ε, among six isozymes after stimulation of THCE cells with PMA. We then investigated translocation of these isozymes to different subcellular fractions after stimulation with PMA. PMA induced the translocation of PKC-α, -βI, and -δ from the cytosol to the membrane and nuclear fractions; however, PMA-induced translocation of PKC-βII to the membrane was much less in quantity and was transient. Thus, although all three isozymes may be involved in modulation of gene expression, PKC-α and -ε, two major isozymes translocated from the cytosol to the membrane fraction after stimulation with PMA, may participate in regulation of membrane-associated cell activities in response to stimulation with PMA. PKC-α and -βI were present in the cytoskeletal fraction of resting cells, and PMA did not cause a depletion of these PKC isozymes in the cytoskeleton. Unlike other PKC isozymes, PKC-δ was found in all subcellular fractions, and PMA caused depletion of the isozyme in the cytoskeleton. A similar pattern of PKC-α, -βI, -δ, and -ε translocation in response to stimulation with PMA was also observed in telomerase-immortalized human corneal epithelial cells (data not shown). Thus, the presence of multiple isozymes and their spatially and temporally distinct cellular localization after stimulation with PMA suggest diverse functions of PKC in cell signaling in corneal epithelial cells.

Our data also show that EGF produced a sustained activation of ERK, in accordance with the well-established mitogenic effect of this growth factor in epithelial cells. However, the data are conflicting regarding the role of PKC in the EGF-stimulated ERK pathway. Our data reported herein suggest that EGF-stimulated MAPK activation and cell proliferation occur independent of phorbol ester–sensitive PKC isoforms in THCE cells. First, EGF failed to induce translocation of PKC isoforms, whereas treatment with PMA resulted in membrane-directed translocation of four isozymes in THCE cells. However, our study cannot rule out the possibility that EGF induces translocation between different membrane fractions, such as translocation from intracellular membranes to the plasma membrane or that EGF activates PKC isozyme(s) without inducing its translocation. Second, we showed that the PKC inhibitor GF109203X inhibited PMA-, but not EGF-, induced or basal phosphorylation of ERK. In contrast, the tyrosine kinase receptor inhibitor tyrphostin AG1478 inhibited both basal and EGF-, but not PKC-, induced phosphorylation of ERK. These pharmacologic studies suggest that GF109203X-sensitive PKC isoforms are not required for EGF stimulation of MAPK activity. Third, the observation that PKC depletion had no effect on EGF-induced phosphorylation of ERK in THCE cells suggests that the phorbol ester–sensitive PKC isoforms have a minimal, if any, role in the ERK pathway induced by EGF. Fourth, concomitant with the changes in phosphorylation of ERK, depletion of phorbol ester–sensitive PKC isoforms was shown to inhibit PMA-stimulated corneal epithelial phosphorylation, but exerted no effects on EGF-induced incorporation of [3H]thymidine in THCE cells. Taken together, our results suggest that EGF-induced phosphorylation of ERK and cell proliferation are independent of phorbol ester–sensitive PKC isoforms in corneal epithelial cells, even though activation of PKC by phorbol esters stimulates these functions. Because at the present time it is difficult to obtain enough primary cultured human corneal epithelial cells for the outlined study, due to the limited availability of human corneas for research and to the limited number of cells generated from each cornea, whether EGF receptor activation and PKC signaling independently regulate proliferation of epithelial cells in vivo remains to be determined.

How might PKC activation lead to MAPK signaling? PKC may activate MAPK through EGF receptor-Ras-Raf-MEK1/2/ERK1/2 or Raf1–MEK1/2/ERK1/2 pathways. We showed that inhibition of tyrosine kinase exhibited no effect on PMA-induced phosphorylation of ERK, suggesting that activation of EGF receptors may not be involved in PKC-induced MAPK signaling. It has been suggested that activation of Raf-1 occurs through the translocation of Raf-1 to the plasma membrane, where it is subsequently phosphorylated by kinases such as PKC. Recently, PKC-α and -ε have been shown to phosphorylate and activate Raf-1. Consistent with these observations, we showed that, in corneal epithelial cells, these two isoforms, in response to stimulation with PMA, were translocated to membrane fraction where they might phosphorylate Raf-1. Thus, one or both of these isoforms are possible candidates for Raf-1/ERK activation in THCE cells. Which isozyme(s) is involved in these biological processes remains to be determined.
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