Protein Kinase C Activation During Corneal Endothelial Wound Repair

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In previous studies, the authors have shown that the two forms of cell translocation that occur during corneal endothelial monolayer wound repair can be pharmacologically separated. Epidermal growth factor (EGF) enhanced the breaking of cell-cell contacts and movement of individual cells from the wound edge, while indomethacin, an inhibitor of PGE₂ synthesis, promoted cell enlargement and spreading of the confluent monolayer sheet into the wound defect. From these findings, the authors hypothesized that the two forms of cell translocation were stimulated by different but coordinately regulated second messenger systems. The current studies used selected protein kinase C (PKC) stimulators and inhibitors, Rh-phalloidin staining of actin filaments, and immunofluorescent localization of PKC to show that: (1) PKC acts as a mediator of the EGF-induced enhancement of the migratory response; (2) the enhanced migratory response results, at least in part, from short-term EGF stimulation of PKC; (3) PKC is a mediator of the EGF-induced alterations in the actin cytoskeleton; and (4) PKC becomes activated in cells at the wound edge during normal, endogenously stimulated wound repair. The results of these studies provide suggestive evidence that wounding of the corneal endothelial monolayer must produce an endogenous, EGF-like stimulation of PKC activity in cells at the wound edge. One effect of PKC activation that must contribute to stimulation of individual cell migration is the induction of cytoplasmic changes that lead to alterations in actin filament organization. Invest Ophthalmol Vis Sci 33:1958-1973, 1992

In vivo repair of defects in the corneal endothelial monolayer occurs via two forms of cell translocation.¹-⁴ One form of translocational response, which we term “migration,” occurs when cells at the wound edge break contacts with neighboring cells and move as individuals into the wound defect. The other form of translocation, which we call “spreading,” occurs when cells within the confluent monolayer adjacent to the wound edge and several rows back from the edge move as a sheet into the wound area. Our laboratory has developed an in vitro model of wound closure, using rabbit corneal endothelial cells, that mimics the behavior of adult human corneal endothelial cells in terms of their migration and spreading responses to wounding.⁵

Using this model, we have found that the migration and spreading forms of translocation can be pharmacologically separated. Epidermal growth factor (EGF) increases the number of individual cells migrating from the wound edge but inhibits spreading of the monolayer sheet during wound closure.⁵,⁶ Conversely, indomethacin, a nonsteroidal antiinflammatory agent, promotes spreading of the monolayer sheet but suppresses migration of individual cells from the wound edge.⁵,⁶ Exposure of corneal endothelial cells to these agents has opposing effects on actin filament organization. EGF induces diffuse actin filament organization consistent with that generally observed in motile cells, whereas indomethacin appears to induce or stabilize actin stress fiber formation, a characteristic of sessile, spread cells.⁵-⁷ The differential effects of EGF and indomethacin on actin filament organization correlate with the relative effects of these agents on the responses of corneal endothelial cells to wounding.

We hypothesize that the migration and spreading forms of translocation that occur in response to wounding are stimulated by at least two different but coordinately regulated transmembrane signalling pathways. Spreading may result primarily from alterations that occur within a cAMP-mediated pathway. Indomethacin, which inhibits the synthesis of prostaglandin E₂ (PGE₂) in cultured rabbit corneal endothelial cells,⁸ enhances the spreading response to wounding. Jumbllatt and Paterson⁹ showed that PGE₂ stimulates the synthesis of cAMP in these cells via activation of an EP₂-subtype plasma membrane re-
ceptron. Alteration of cAMP second messenger levels may lead to stimulation of the spreading response, as well as to other aspects of the wound healing process. EGF may stimulate migration of individual cells from the wound edge via activation of the phosphoinositide transmembrane signalling pathway, because EGF is known to stimulate this pathway in several cell types, including A431 cells. EGF appears to exert its effects via the tyrosine kinase activity of its receptor. In some cells, EGF induces tyrosine phosphorylation of phospholipase C-γ1, which causes the breakdown of phosphatidylinositol bisphosphate. This breakdown yields at least two second messengers, including inositol-1,4,5-trisphosphate, which releases calcium from intracellular stores, and 1,2-diacylglycerol, a cofactor that, together with phosphatidylserine and calcium, is necessary for activation of protein kinase C (PKC). The activity of one or more of these second messengers may result in stimulation of the corneal endothelial migratory response to wounding.

Jumblatt, Matkin, and Neufeld showed that when rabbit corneal endothelial cells were subcultured and grown to confluency in the presence of EGF, cell density increased, cells were elongated rather than polygonal, and actin filament organization was altered compared to control cultures grown in the absence of EGF. TPA, a phorbol ester that directly stimulates PKC, mimicked the effects of EGF on cell density and shape, and simultaneous exposure of cells to TPA and EGF did not result in a synergistic response. From these results, the authors concluded that EGF must induce cell division and elongation in rabbit corneal endothelial cells via activation of the phosphoinositide-PKC pathway.

Our current studies expand and extend the findings of Jumblatt, Matkin, and Neufeld and those of our own laboratory. These studies had the following objectives: (1) to determine whether PKC also acts as a mediator of the EGF-induced enhancement of the migratory response to wounding; (2) to ascertain whether the enhanced migratory response of cultured rabbit corneal endothelial cells results from direct, short-term EGF (PKC) stimulation or is the result of a feedback response to long-term exposure of the cells to these agents; (3) to determine whether PKC mediates the EGF-induced effects on the actin cytoskeleton previously observed in our laboratory; and (4) To determine whether PKC is activated in untreated, endogenously stimulated wounds.

To accomplish our first three research objectives, we pursued two lines of investigation. In one series of experiments, we compared the effects of TPA to those of EGF on the corneal endothelial migratory and spreading responses during long- and short-term exposure to these agents. We also compared the effects of TPA and EGF on actin filament organization in sparsely plated corneal endothelial cells. In a second series of studies, we tested the ability of PKC inhibitors to reverse the observed TPA and EGF effects on these responses.

To determine whether PKC is activated in untreated, endogenously stimulated wounds, we took advantage of the fact that PKC activation is generally accompanied by movement of the enzyme from the cytosol to the plasma membrane, where it becomes associated with phosphatidylserine, a necessary cofactor for activation. Taking an immunocytochemical approach, we used a commercially available monoclonal antibody, which specifically recognizes the α and β1, isoforms of PKC, to observe the relative change in localization of this enzyme upon exposure of sparsely plated cells to TPA or EGF. Comparison of the localization patterns obtained in these studies to those obtained using our wound closure model permitted us to determine whether PKC becomes activated in cells at the edge of untreated, endogenously stimulated wounds.

Results from our studies provide suggestive evidence that the EGF-induced enhancement of the migratory response in in vitro corneal endothelial wounds is mediated, at least in part, via activation of PKC. The increase in the number of migrating cells observed upon exposure to EGF or TPA appears to be the result of direct EGF (PKC) stimulation rather than that of a feedback response to long-term exposure to these agents. PKC also appears to be activated in endogenously stimulated, untreated wounds, indicating that wounding of the corneal endothelial monolayer must produce an endogenous, EGF-like stimulation of PKC activity in cells at the wound edge. One effect of PKC activation that must contribute to stimulation of the migratory response is the induction of cellular changes that lead to alterations in actin filament organization.

Materials and Methods

Primary Culture of Rabbit Corneal Endothelial Cells

Albino, male New Zealand rabbits, treated and housed in accordance with the ARVO Resolution on the Use of Animals in Research, were used as a source of corneal endothelial tissue. Corneal endothelial cells were isolated from Descemet's membrane and cultured as described previously. Briefly, Descemet's membrane with intact endothelial cells was dissected from excised rabbit corneas, and the endothelial cells were dissociated from the membrane by incubation in Dispase II (Boehringer Mannheim Biochemicals, In-
dianapolis, IN). After trituration of cell clumps, the cells were plated in primary culture in the presence of Medium 199 supplemented with 50 μg/ml gentamicin, 10% fetal calf serum (all from GIBCO, Grand Island, NY), and 25 ng/ml fibroblast growth factor (Biomedical Technologies, Stoughton, MA). Cells then were grown to confluency (approximately 7 d) in a 5% CO₂-95% air, humidified atmosphere at 37°C.

In Vitro Wound Closure Model

Confluent primary cultures were treated with trypsin-EDTA (GIBCO), counted with a hemacytometer, and subcultured onto 25 mm diameter sterile glass coverslips at 4 × 10⁴ cells/cover slip and grown to confluence in the culture medium described above minus fibroblast growth factor. Forty-eight hours prior to wounding, confluent cultures were weaned to Medium 199 supplemented with 50 μg/ml gentamicin and 2.5%, rather than 10%, fetal calf serum to decrease the potential influence of serum-derived factors on the wound repair process. (Subsequently, this medium formulation will be termed "Medium 199-2.5.") Twenty-four hours prior to wounding, cultures were treated with 10 μg/ml 5-fluorouracil (5-FU), a mitotic inhibitor that previously was found to inhibit rabbit corneal endothelial cell growth and, thus, permit analysis of cell movement alone during wound repair. Confluent cultures were wounded with a sterile 3 mm diameter, 0.2 μm pore size Millipore filter disc (Millipore Corp., Bedford, MA). This procedure removes cells but leaves the underlying extracellular matrix intact. The wounded cultures were maintained in Medium 199-2.5 ± one or a combination of pharmacologic agents (see below) for specific periods of time. They then were washed, fixed, and Giemsa-stained to determine the effect of the test agents on migration of individual cells and on monolayer spreading.

Pharmacologic Agents

Mouse submaxillary gland EGF (Collaborative Research Inc., Bedford, MA) was used at a concentration of 10 ng/ml (1.7 × 10⁻⁹ mol/l). This concentration of EGF was found to induce rabbit corneal endothelial cell elongation² and to increase the migration of individual cells from the edge of in vitro wounds.⁵,⁶ TPA (phorbol 12-myristate 13-acetate; Sigma Corp., St. Louis, MO) induces corneal endothelial cell elongation¹⁸ at a concentration of 10⁻⁸ mol/l (6 ng/ml). The same concentration was used for the current studies. H-7 (1-[5-isouquinolinesulfonyl]-2-methylpiperazine; Molecular Probes, Eugene, OR) and sphingosine (Sigma) were used as PKC inhibitors for these studies. H-7 was appropriately diluted in Medium 199-2.5, while sphingosine was solubilized in methanol, diluted in Medium 199-2.5, and then added to the cells at a final methanol concentration of 0.01%. The appropriate methanol-containing controls were included in all relevant experiments. The concentration of H-7 and sphingosine to be used in these studies was determined as indicated below.

Determination of PKC Inhibitor Concentrations

Rabbit corneal endothelial cells subcultured and grown in the presence of 10⁻⁸ mol/l TPA are elongated, rather than polygonal, at confluency. To determine the concentration of PKC inhibitors to be used in our studies, we tested the ability of H-7 and sphingosine to reverse the elongating effects of TPA. For these studies, rabbit corneal endothelial cells were subcultured at 1 × 10⁴ cells/well in 24-well tissue culture plates and grown in the presence of 10⁻⁸ mol/l TPA ± increasing concentrations of H-7 or sphingosine. At confluency, the cultures were washed, fixed, Giemsa-stained, and photographed on a Nikon UFX-II microscope (Donsanto Corp., Natick, MA). Prints, prepared at a final magnification of 120×, were used for morphometric analysis of cell shape using a Zeiss Videoplan-2 digitizer equipped with a MOP-Videoplan Image Analysis Systems software package developed by Zeiss (Thornwood, NY). The factor, P/DMax (perimeter/maximum diameter), was calculated for 200 cells per treatment condition. This factor is an indicator of cell elongation.¹⁸ Polygonal-shaped cells should have a P/DMax factor close to π (3.14). Numbers less than π indicate cellular elongation. H-7 and sphingosine reversed TPA-induced elongation in a dose-dependent manner. A concentration of each of the PKC inhibitors was chosen that inhibited the elongation activity of TPA and returned the confluent cells to polygonal shape (data not shown). H-7 was used at a concentration of 10 μmol/l and sphingosine at 2 μmol/l.

Analysis of Cell Migration

Twenty-four hours after wounding, cultures were Giemsa-stained and observed directly on the microscope at 100× final magnification. Individual cells migrating ahead of the advancing monolayer were counted to determine the effect of the test agents on the migratory response. As in previous studies, a cell was considered migratory when it was located ahead of the advancing wound edge and retained no cell-cell contacts, or when it was located at the advancing wound edge but retained only tenuous contact with neighboring cells and had formed lamellipodia or anterior ruffled membranes and a posterior uropod. The mean number of migrating cells was determined in an average of 24 wounds per treatment condition. The
standard error of the mean was calculated using an RS/Explore software program developed by BBN Software Products Corp. (Cambridge, MA).

Planimetry Measurements of Monolayer Spreading

For planimetry measurements, the same Giemsa-stained wounded cultures used for analysis of cell migration were observed in the microscope at 30 X final magnification. Using a glass reticle with 0.01 mm divisions inserted within the projection lens, measurements were made, as described previously,\textsuperscript{6} to determine the area within the original wound defect covered by the confluent monolayer sheet. Two measurements were made per wound, and an average of 20 wounds were measured per treatment condition. Standard error of the mean was calculated as above.

Sparse Plating of Corneal Endothelial Cells

In some experiments, cells were plated at low cell densities to determine the effects of pharmacologic agents on cells that had not formed cell-cell contacts. Twenty-four hours prior to subculture, primary cultures of rabbit corneal endothelial cells were weaned to Medium 199-2.5. Cells then were seeded at $2 \times 10^4$ cells onto two-chamber glass slides (Lab Tek; Miles Scientific, Naperville, IL). After subculture, cells were maintained for 24 hr prior to experimentation in the above medium supplemented with 10 µg/ml 5-FU. Cells were exposed for specific periods of time to Medium 199-2.5 ± one of the test agents. Then they were treated with Rh-phalloidin (Molecular Probes), according to previously described methods,\textsuperscript{5,6} for staining of actin filaments or were prepared for immunocytochemistry as indicated below.

Immunocytochemical Localization of PKC

A mouse monoclonal antibody that specifically recognizes the α- and β\textsubscript{II} isoforms of PKC was obtained from Amersham Corp. (Arlington Heights, IL). According to the manufacturer, the epitope for this antibody has been mapped to residues 296-317 of bovine α-PKC. This epitope is similar in the α- and β\textsubscript{II} isoforms of PKC but is not present in the γ-isofrom.\textsuperscript{24} Rabbit cerebellum was chosen as an enriched source of PKC to test the ability of the monoclonal antibody to detect this enzyme in rabbit tissue. For these studies, a triton X-100 extract of rabbit cerebellum was prepared and chromatographed on a diethylaminoethyl (DEAE) cellulose column as described.\textsuperscript{20} DEAE chromatography was a necessary step to enrich for PKC in our extracts. Peak fractions from the DEAE column were concentrated on a Centricon-10 membrane (Amicon, Beverly, MA) and then electrophoresed on a 5-15% gradient Lammeli\textsuperscript{25} polyacrylamide gel. The resulting separated polypeptides were electrophoretically transferred to nitrocellulose for Western blots using techniques previously described.\textsuperscript{26,27} Figure 1, Lane 3 shows that a single, major peptide band was specifically recognized by the anti-PKC antibody. The relative molecular weight of this band was approximately 76 kD, the expected molecular weight of PKC.\textsuperscript{23} Nonspecific binding was determined in similar blots using nonimmune mouse IgG in place of anti-PKC (Fig. 1, Lane 4). Western blots of the triton X-100 extract prepared without subsequent DEAE cellulose chromatography and concentration were negative (data not shown). Triton X-100 extracts of cultured rabbit corneal endothelial cells were prepared and chromatographed as described above. West-
ern blots of the DEAE cellulose peak showed a single, very light positive band when probed with the anti-PKC antibody. However, the photographic contrast of this Western blot was insufficient for inclusion in Figure 1.

PKC was localized in rabbit corneal endothelial cells in two separate studies. In one study, cells were sparsely plated as described above and exposed to TPA or EGF for specific periods of time prior to PKC localization. In the second study, cells were subcultured onto two-chamber glass slides and grown to confluency in Medium 199 supplemented with 10% fetal calf serum and 50 μg/ml gentamicin. At confluency, cultures were weaned to Medium 199-2.5, mitotically inhibited, and wounded as described above. Wounded cultures then were maintained in Medium 199-2.5 alone for specific periods of time, after which they were washed and fixed for PKC localization.

For immunocytochemistry, all incubation steps were carried out at room temperature, unless stated otherwise. Cells were washed with Dulbecco’s phosphate buffered saline (PBS) and fixed for 30 min in 4% formaldehyde (freshly prepared from paraformaldehyde) in PBS. Aldehydes were quenched for 30 min with 0.15 mol/l glycine in half-strength PBS. Cells were permeabilized for 30 min with 0.1% triton X-100 in PBS supplemented with 2% bovine serum albumin and 0.05% sodium azide at 4°C and incubated overnight at 4°C with primary antibody at a 1/5 dilution in permeabilization buffer. After being washed with 0.1% triton in PBS for 30 min with several changes of buffer, the cultures were incubated for 2 hr with a 1/200 dilution of biotin-conjugated goat anti-mouse IgG (Amersham), washed several times in triton buffer, incubated for 1 hr in streptavidin-rodamine (Pierce, Rockford, IL) diluted 1/100, and washed over 1 hr in triton buffer. Specimens then were mounted in 1:1 PBS:glycerol supplemented with 5% n-propyl gallate, and observed with a Nikon UFX-II microscope. Negative controls omitted the primary antibody or both the primary and secondary antibodies.

**Evaluation of the Actin Filament and PKC Immunolocalization**

For the Rh-phalloidin staining of actin filaments and the PKC immunolocalization studies, random photographs were taken on a Nikon UFX-II photomicroscope. Multiple areas containing two or more cells were photographed in two individual chambers per microscope slide. Two slides were prepared per experimental condition, and each experiment was repeated at least three times. Photographs of the experimentally treated cells then were compared to photos of control cells to qualitatively determine the effects of the test agents on actin filament organization or on the cellular localization of PKC.

**Results**

**PKC Effects on Cell Migration and Monolayer Spreading**

Phase contrast video microscopy and light microscopic observation of Giemsa-stained wounds have revealed that, within 30 min after wounding, cells at the wound edge begin to extend lamellipodia and ruffling membranes into the defect area. After 6–7 hr, cells break contact with their neighbors and migrate into the defect. Initially, the response to wounding is sporadic, with only scattered single cells or small groups of cells moving into the wound area. By 24 hr after wounding, a sufficient number of cells have responded to permit evaluation of the effects of test agents on the wound closure pattern and to more accurately count the number of individual cells migrating ahead of the leading edge. For this reason, the effects of PKC stimulators and inhibitors on the migration response were evaluated 24 hr after wounding. For planimetry measurements of monolayer spreading, a 48 hr time point was chosen, because, by that time, the monolayer sheet had moved into the wound area a distance sufficient to permit comparison of the effect of the test agents on the spreading response. For the sake of brevity, only the effects of the PKC inhibitor, H-7, will be discussed. Sphingosine produced effects similar to those of H-7 on all parameters tested. The 0.01% methanol vehicle used for maintaining sphingosine solubility had no observable effect on cell viability or on the ability of the cells to respond to wounding.

**Long-term PKC stimulation:** In an initial series of studies, one-half of the confluent cultures were exposed to H-7 for 24 hr prior to wounding. This exposure had no detrimental effect on the cells, as evidenced by normal morphology and cell counts (data not shown). All cultures were then wounded and exposed for 24 hr to TPA or EGF ± H-7. Figure 2 illustrates the relative effects of these agents on wound closure patterns. Figures 3a and b provide quantitative information on the effects of PKC stimulators and inhibitors on individual cell migration and monolayer spreading, respectively. In control, untreated cultures 24 hr after wounding, individual cells migrated ahead of the spreading monolayer sheet (Fig. 2a). Exposure of wounded cultures to TPA or EGF alone increased the relative number of cells that migrated as individuals into the wound area, as indicated by the closure patterns in Figures 2c and e and by the cell counts in Figure 3a. On the other hand,
Fig. 2. Edge of Giemsa-stained corneal endothelial wounds 24 hr after wounding and exposure to Medium 199-2.5 alone (a), $10^{-5}$ M H-7 (b), $10^{-4}$ M TPA (c), TPA + H-7 (d), 10 ng/ml EGF (e), or EGF + H-7 (f). Vertical arrows indicate the position of the original wound edge. Arrowheads designate representative migrating cells, and curved arrows indicate the relative position of the leading edge of the confluent monolayer sheet. ECM = extracellular matrix within defect area. Bar = 100 μm.
back response to PKC activation. To determine the effects of short-term PKC stimulation on the migratory response of corneal endothelial cells, confluent cultures were wounded, immediately exposed to TPA or EGF for 0, 5, 15, 30, or 60 min, washed with Medium 199-2.5, and maintained in this medium for 24 hr prior to Giemsa-staining and counting of migrating cells. Figures 4a and b show the results of short-term TPA or EGF stimulation on the migration response and compares this response to that obtained after 24 hr exposure. Exposure of cultures to TPA or EGF for 5–15 min immediately after wounding is sufficient to increase cell migration from the wound edge when compared to controls. The extent of the increase is similar to that observed when wounded cultures are exposed to these agents for 24 hr prior to counting.

PKC Localization in Sparsely Plated Endothelial Cells

Figure 5 illustrates the patterns of PKC localization in sparsely plated, nonconfluent rabbit corneal endothelial cells. Sparsely plated cells were used for these

TPA and EGF suppressed spreading of the intact monolayer into the defect area (Fig. 3b). Exposure of wounded cultures to H-7 alone inhibited migration of individual cells when compared to untreated controls and promoted monolayer spreading above control levels (Figs. 2b, 3a, 3b). H-7, when added in the presence of TPA or EGF, reversed the effects of these agents (Figs. 2d, 2f, 3a, 3b). The relative number of individual migrating cells was greatly decreased under these conditions, and spreading of the confluent monolayer was restored to control levels. When EGF and TPA were added together, the number of individual cells migrating from the wound edge was very similar to that found in wounds treated with TPA or EGF alone (data not shown).

**Short-term PKC stimulation:** In the initial studies, it could not be determined whether the enhanced migratory response observed upon 24 hr exposure of wounded cultures to TPA or EGF was a direct effect of PKC stimulation or the result of a long-term feed-
Fig. 5. Immunofluorescence localization of PKC in sparsely plated corneal endothelial cells after short-term exposure to TPA or EGF. (a) Control minus primary antibody, (b) Medium 199-2.5 alone, (c) 5-min exposure to $10^{-8}$ M TPA, (d) 10-min exposure to TPA, (e) 5-min exposure to 10 ng/ml EGF, (f) 10-min exposure to EGF. Arrows indicate cell periphery. Bar = 10 μm.
studies to eliminate any effects on PKC localization caused by the presence of cell-cell contacts within the confluent monolayer. Negative controls, in which the primary antibody alone or both primary and secondary antibodies, but not the streptavidin-rhodamine, were omitted, appeared unstained, as seen in Figure 5a. Phase-contrast microscopy revealed that cells maintained in Medium 199-2.5 alone generally were flat and well spread (data not shown). Relatively few cells exhibited membrane ruffles or morphology suggestive of active motility. In these cells, PKC was localized in a punctate pattern within the cell center, whereas the nucleus was generally unstained (Fig. 5b). Little-to-no positive staining was visible at the cell periphery. Exposure of cells to TPA for 5 min or longer resulted in an apparent increase in the number of cells exhibiting elongated shapes, membrane ruffles, and actively motile morphologies. After only 5 (Fig. 5c) or 10 min (Fig. 5d) exposure to TPA, PKC was localized in a broad punctate pattern at the edge of ruffling membranes and at the leading edge of motile cells, as well as within the cell center. Similar evidence of membrane activity and shape change and similar PKC patterns were observed in cells exposed to EGF for short periods of time (Fig. 5e and f).

PKC Effects on Actin Filament Organization

As mentioned above, we had observed previously that EGF induced changes in actin filament organization in sparsely plated, nonconfluent cells and in cells at the edge of in vitro wounds. Because EGF and TPA both promoted cell elongation and stimulated movement of cells from the wound edge, we asked whether the EGF-induced changes in actin organization in sparsely plated cells resulted, at least in part, from PKC activity. To answer this question, sparsely plated, mitotically inhibited rabbit corneal endothelial cells were exposed to TPA or EGF ± H-7 for 0, 5, 10, 15, 30, or 60 minutes or for 24 hr. After the cells were washed, they were fixed and stained with Rh-phalloidin for visualization of actin filaments.

Figure 6 illustrates the effects on actin filament organization of 24 hr exposure of sparsely plated cells to PKC stimulators and inhibitors. In untreated, control cells (Fig. 6a), actin formed thin stress fibers that extended throughout the cytoplasm in multiple directions. These stress fibers appeared to insert into focal contacts at the cell-substrate interface. Actin also formed narrow bundles around the cell periphery. Incubation of cells in H-7 alone (Fig. 6b) appeared to have relatively little effect on actin filament organization. However, in some cells, the stress fibers seemed more numerous than in controls. Exposure of cells for 24 hr to TPA (Fig. 6c) or EGF (Fig. 6e) induced an apparent decrease in the number of actin stress fibers and an increase in diffuse actin staining in the majority of treated cells. Stress fibers that remained after exposure to these agents generally appeared thinner than those in control cells. H-7 reversed the effects of TPA and EGF (Figs. 6d, f), returning stress fiber staining and organization to control levels. In some cells, addition of H-7 caused the formation of long actin cables in narrow structures resembling uropods and in narrow, branched arborizations.

The effects of TPA and EGF on actin filament organization are quite rapid. Figure 7 compares normal actin stress fiber organization in untreated controls (Figs. 7 a, b) with actin organization after 5 or 15 min of exposure to TPA (Figs. 7 c, d) or EGF (Figs. 7 e, f). In both cases, a decrease in Rh-phalloidin staining of actin stress fibers can be detected after 5 min treatment of sparsely plated cells. Increased diffuse actin staining with concomitant loss of stress fibers is observable within 15 min after exposure to TPA or EGF.

PKC Localization at the Wound Edge

PKC was localized in untreated, endogenously stimulated cultures 15 min, 1, 2, 4, and 24 hr after wounding. Figure 8 presents representative examples of the results. In all cells, regardless of their relative distance from the wound edge or time after wounding, PKC was localized in a generally punctate pattern within the perinuclear region, while nuclei remained unstained. This perinuclear pattern was consistently observed in confluent cells distal to the wound edge (Fig. 8a). Little-to-no PKC staining was associated with the plasma membrane in confluent cells at any time during the period of observation.

By 15 min after wounding (Fig. 8b), a few cells had begun to extend thin cytoplasmic processes into the defect area. Over time (Figs. 8b–f), more and more cells at the wound edge extended lamellipodia or long, thin filopodia. In a few instances, such as in Figure 8d, individual cells began to break cell-cell contact and migrate into the wound area. Within 15 min after wounding, light punctuate PKC staining was visible within the cytoplasmic processes. One hour after wounding (Fig. 8c), a more intense punctate staining was unevenly distributed at or near the plasma membrane of these processes. In the few apparently actively motile cells, PKC formed a punctate pattern within the cytoplasm of the leading lamellipodium (Fig. 8d). Within 1 (Fig. 8c) to 2 hr (Fig. 8d) after wounding, some cells at or near the wound edge also contained intensely stained linear structures in the perinuclear region, and, in some cases, at the bases of lamellipodia. These structures were less evident in cells 4 (Fig. 8e) or 24 hr (Fig. 8f) after wounding. At
Fig. 6. Rh-phalloidin staining of actin filaments in sparsely plated corneal endothelial cells exposed for 24 hr to TPA or EGF ± H-7. 
(a) Medium 199-2.5 alone, (b) 10⁻⁵ M H-7, (c) 10⁻⁸ M TPA, (d) TPA + H-7, (e) 10 ng/ml EGF, (f) EGF + H-7. Bar = 10 μm.
Fig. 7. Rh-phalloidin staining reveals the effects on actin filament organization of short-term exposure of cells to TPA or EGF. Untreated, sparsely plated cells in (a) and (b) show normal organization of actin-containing stress fibers. Cells exposed to TPA for 5 (c) or 10 min (d) or to EGF for 5 (e) or 10 min (f) show decreased stress fiber organization and increased diffuse actin staining. Bar = 10 μm.
Fig. 8. PKC immunofluorescence localization in endogenously stimulated corneal endothelial wounds. (a) Confluent cells distal to the wound edge, (b–f) cells at the wound edge 15 min (b), 1 hr (c), 2 hr (d), 4 hr (e), and 24 hr (f) after wounding. Arrowheads indicate position of punctate PKC staining in cytoplasmic extensions. Arrows show intense PKC localization in linear structures within cytoplasm. Note punctate staining in circular structures toward the tip of the lamellapodia, particularly in (c, e, f). Bar = 50 μm.
these later time points, cells were intensely stained for PKC along the plasma membrane and within the cytoplasm at the tips of lamellipodia and filopodia and in circular structures resembling close contacts just distal to the tips of these processes.

Discussion
EGF, PKC Activation, and the Migratory Response to Wounding

Previous studies in our laboratory demonstrated that exposure of cultured rabbit corneal endothelial cells to EGF at the time of wounding increases the number of individual cells that migrate from the wound edge. Individual cell migration is one of the two forms of cell translocation that contribute to endothelial wound closure, the other form being spreading or sliding of the confluent monolayer sheet to cover the wound defect. Our studies have provided information on the ability of pharmacologic agents to stimulate one of these two forms of translocation. They have not evaluated the effects of these agents on the rate at which cells translocate.

Our ability to pharmacologically separate the two forms of cellular translocation provides an excellent tool for studying how corneal endothelial wound repair is regulated. EGF is known to stimulate the phosphoinositol signalling pathway in other cell types, and PKC has been found to act as a mediator of EGF-induced increases in cell density and shape change in rabbit corneal endothelial cells. In the present study, we wished to determine whether a direct relationship exists between EGF stimulation, PKC activation, and the cell biologically relevant migratory response of rabbit corneal endothelial cells to wounding.

Comparison of the effects of TPA and EGF and of the effects of PKC inhibitors, H-7 and sphingosine, on the responses of corneal endothelial cells to wounding provides suggestive evidence that PKC acts as a mediator of the EGF-induced enhancement of the migratory response. In our studies, TPA, like EGF, increased the number of individual cells migrating from the wound edge and suppressed spreading of the monolayer sheet into the wound area. Addition of H-7 or sphingosine to the TPA- or EGF-containing medium returned the migration and spreading responses to control levels.

A note regarding the use of H-7 and sphingosine is necessary. H-7 and sphingosine both were used in these studies, because they have different modes of activity and because neither is an absolutely specific PKC inhibitor. Sphingosine exerts its effects by binding to the regulatory portion of the PKC molecule and prohibiting association of diacylglycerol or TPA with PKC at the plasma membrane, thus preventing activation of the enzyme. H-7 is an isoquinoline sulfonamide derivative that binds to the catalytic portion and inhibits the activity of cAMP-dependent protein kinase, cGMP-dependent protein kinase, protein kinase C, and myosin light chain kinase, with apparent \( K_i \) values of 3.0, 5.8, 6.0, and 97 \( \mu \text{mol/l} \), respectively. For our studies, H-7 was used at a final concentration of 10 \( \mu \text{mol/l} \), a concentration insufficient to effect myosin light chain kinase. Because TPA specifically stimulates PKC, the reversal of its effects by H-7 can be attributed to inhibition of PKC activity rather than to its effects on cAMP- or cGMP-dependent protein kinase. The TPA-induced responses to wounding were inhibited by H-7 and sphingosine in our model system. This result acted as a positive control for studies of the effects of these inhibitors on the EGF-induced responses. That these inhibitors reversed the EGF-induced effects on the migration and spreading responses to wounding is additional evidence that EGF exerts some of its effects through activation of PKC.

Enhancement of the migratory response upon exposure of cells to EGF (or TPA) appears to result, at least in part, from direct, short-term stimulation of PKC activity and not from a long-term feedback mechanism. Exposure of cultures to EGF or TPA for as little as 5-15 min immediately after wounding induced an increase in the number of individual cells migrating from the wound edge several hours after wounding. PKC also localized within ruffling membranes and at the leading edge of cells exhibiting motile morphologies within 5 to 15 min after addition of EGF or TPA to the culture medium of sparsely plated cells. This result correlates well with the wound closure studies and provides additional suggestive evidence for a direct, short-term stimulation of PKC activity by EGF (or TPA).

PKC activation by TPA or EGF promotes changes in actin filament organization. Both TPA and EGF altered the actin cytoskeleton from the normal stress fiber pattern to a more diffuse pattern in sparsely plated rabbit corneal endothelial cells. These changes were prevented by adding H-7 or sphingosine to the EGF- or TPA-containing medium. The observed actin changes probably are not the result of a long-term feedback response, because these alterations occurred within 5 min of exposure to either agent. The ability of EGF or TPA to alter actin cytoskeletal organization has been observed in many cell types. EGF induces changes in actin structure in human epidermoid carcinoma A-43135 and KB cells36 and in human retinal pigment epithelial cells,37 while similar changes have
been stimulated by TPA in epithelial African green monkey kidney (BSC-1) cells,38 chick embryo fibroblasts,39 various transformed fibroblast cell lines,40 canine thyroid epithelial cells,41 and human T lymphocytes.42 In all these cell types, changes in actin organization were accompanied by increased membrane ruffling, increased lamellipodial and filopodial extension and, in some cases, by increased cell motility. Results of these studies are consistent with our observations and, in some cases, by increased cell motility.

Microscopic observation of Rh-phalloidin-stained actin filaments can provide only a qualitative assessment of the effects of EGF or TPA on actin filament structure. To provide a more quantitative measure of the effects of these agents on the actin cytoskeleton, our laboratory is planning experiments to biochemically detect and measure the relative amount of filamentous versus soluble actin in control versus experimentally treated rabbit corneal endothelial cells.

**Endogenous PKC Stimulation at the Wound Edge**

Our wound closure studies have provided evidence that PKC is activated in cells at the edge of wounds under endogenously stimulated conditions. The fact that exposure of cultures to H-7 or sphingosine alone decreased the number of cells migrating from the wound edge to below control levels and promoted spreading of the monolayer sheet into the wound area suggests that PKC must be active in cells in untreated wounds.

Immunolocalization studies provided additional strong suggestive evidence that PKC is activated in cells at the edge of endogenously stimulated wounds. Localization of PKC in sparsely plated cells showed similar staining patterns when cells were exposed to TPA or EGF. In both cases, PKC was found to be associated with the plasma membrane, a characteristic commonly found in cells in which PKC has been activated. The association with the membrane was not uniform, however. Staining was most intense in focal areas of cellular activity, such as at the edge of ruffling membranes, in leading lamellipodia, and at the tips of filopodia. Little staining was evident in the remainder of the cell periphery in these cells or anywhere within the periphery of well-spread, apparently nonmotile cells. Very similar results were obtained when PKC was localized in untreated, endogenously stimulated wounds. PKC localized within filopodia and lamellipodia of cells at the wound edge, but was found only at the cell center in confluent, spread cells distal to the wound. The similarity of PKC localization in cells activated by TPA and EGF and in cells at the edge of untreated wounds indicates that PKC does become activated during the normal response of cells to wounding. Our laboratory is planning to measure the relative amount of PKC activity in the cytosolic and membrane fractions of cells exposed for various periods of time to EGF or TPA to obtain more quantitative evidence for EGF-induced activation of PKC in rabbit corneal endothelial cells. Comparison of these results with those obtained from endogenously stimulated wounded cultures should provide additional information regarding endogenous PKC stimulation in response to wounding.

As was observed with sparsely plated cells, PKC appears to be activated in cells at the wound edge relatively soon after wounding. Within 1 hr after wounding, many cells at the edge of untreated, endogenously stimulated wounds had extended lamellipodia or filopodia, and, within that same time frame, PKC was found within those structures. Changes in actin filament organization could be observed in sparsely plated cells within 5 min after exposure to TPA or EGF. Therefore, it can be assumed that PKC activation in cells at the wound edge by an EGF-like endogenous stimulator helps produce alterations in actin cytoskeletal organization that contribute to the migratory response.

The studies discussed above took advantage of the fact that EGF and TPA were able to enhance one aspect of the normal responses of corneal endothelial cells to wounding. The intracellular signaling pathways that are stimulated upon exposure of cells to EGF may be the same as those that respond to the endogenous stimulation of wounding. EGF or transforming growth factor-α (TGF-α), both of which bind to EGF receptors, may act as autocrine stimulators of wound healing. Messenger RNA for EGF and its specific receptor has been detected in cultured human corneal endothelial cells.43 TGF-α has been detected in the aqueous humor of cats after wounding of the corneal endothelium.44 Perhaps this growth factor, as well as EGF itself, is released from corneal endothelial cells upon wounding and can stimulate healing in an autocrine fashion. Fibroblast growth factor also may act as an endogenous, autocrine stimulator of corneal endothelial wound healing. Basic-fibroblast growth factor (b-FGF) mRNA has been detected in these cells,45 and acidic and b-FGF stimulate the corneal endothelial cell cycle.46,47 Our laboratory has shown that b-FGF is released from corneal epithelial cells and is deposited in Bowman’s membrane upon mechanical or chemical injury to the plasma membrane of the epithelial cells.48 Descemet’s membrane also readily binds b-FGF.49 It is possible that injury to cor-
neal endothelial cells may result in b-FGF release in a manner similar to that observed in the epithelium. Thus, binding of b-FGF to Descemet’s membrane may stimulate wound healing in these cells in an autocrine manner.

Clearly, EGF must elicit a larger number of cellular responses than the phorbol ester, TPA, because its effects must be transmitted via a receptor-mediated process. The tyrosine kinase activity of the EGF receptor and the possible formation of multiple second messengers via stimulation of the phosphoinositol pathway also must contribute to the cascade of events that leads to the migratory response to wounding. Further investigation using our in vitro wound closure model should help to determine: (1) how closely our observed EGF-induced stimulation parallels the endogenous stimulation that occurs upon wounding; (2) whether multiple intracellular signalling pathways contribute to the migration and spreading responses to wounding; and (3) what role the second messengers produced upon stimulation of these pathways play in the regulation of monolayer wound repair.

**Key words:** corneal endothelium, wound healing, protein kinase C, epidermal growth factor, cell migration

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