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Effect of Protein Kinase C Inhibitors and Activators on Corneal Re-epithelialization in the Rat

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Purpose. To examine the ability of protein kinase C (PKC) inhibitors and activators to influence the rate of corneal re-epithelialization in the rat.

Method. Rat corneas with 3 mm diameter central epithelial abrasions were organ-cultured in control medium or in medium with inhibitors or activators of PKC.

Results. In control corneas, the defect was completely re-epithelialized by 25 hr. In the presence of the PKC inhibitors staurosporine (100 nM), sphinganine (50 μmol/l), or H-7 (100 μmol/l) there were significantly larger epithelial defects than in controls after 5–25 hr of incubation. Re-epithelialization rates were similar to control corneas when the incubation medium contained HA1004 (100 μmol/l), an analogue of H-7 that is a potent inhibitor of cyclic adenosine monophosphate- and cyclic guanosine monophosphate-dependent protein kinases and a weak inhibitor of PKC. Two PKC activators, 1-oleoyl-2-acetyl-sn-glycerol (OAG) and phorbol 12-myristate 13-acetate (PMA), were unable to enhance the rate of epithelial wound healing.

Conclusions. Our results suggest that PKC activity is an important factor in regulating corneal epithelial wound healing, presumably by influencing cell migration. Moreover, the results with OAG and PMA suggest that PKC is maximally activated during re-epithelialization in this organ-culture assay. Invest Ophthalmol Vis Sci. 1993;34:216–221.

Protein kinase C (PKC) is a family of enzymes initially described in 1977.[1] PKC activity is calcium and phospholipid dependent, and the enzyme is activated by 1,2-diacylglycerol (DAG), one of the initial products of inositol phospholipid hydrolysis.[2] The role of PKC activation in signal transduction was first demonstrated for the release of serotonin from platelets.[3] Since then, the importance of this enzyme has been shown for the release and exocytosis of cellular constituents from a variety of endocrine, exocrine, and neuronal tissues, as well as for the modulation of membrane functions such as arachidonic acid release from platelets and enzyme release from lysosomes.[4] The discovery that tumor-promoting phorbol esters stimulate cellular responses by fulfilling the DAG requirement for activation of PKC indicated that cell proliferation may be linked to the activity of this enzyme.[5,6]

PKC activity has been demonstrated in the cornea,[7] but its role is uncertain. There have been no reports, to our knowledge, regarding a function of this enzyme in corneal wound healing. After corneal abrasion, epithelial healing involves a complex series of cellular changes that lead to the sliding of a sheet of epithelial cells to resurface the defect.[8] During movement of these epithelial cells, there is redistribution of

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actin filaments,[9,10] and interference with actin polymerization prevents re-epithelialization.[11] In other cell types, activation of PKC by phorbol esters diminishes contractility of the actin cytoskeleton;[12,13] enhances spreading;[14] stimulates actin polymerization;[15–17] provokes[18] or inhibits[19] membrane ruffling, depending on cell type; and alters adherence of cells to extracellular matrix[20,21] and other cells.[22] To implicate PKC activity as a regulator of corneal epithelial wound healing, we tested the ability of PKC inhibitors and activators to influence the healing rate of epithelial defects of organ-cultured corneas.

MATERIALS AND METHODS

Chemicals

Sphinganine, phorbol 12-myristate 13-acetate (PMA), and bovine serum albumin (BSA; essentially fatty-acid free; Cohn fraction V) were purchased from Sigma Chemical Co., St. Louis, Missouri. Staurosporine, H-7 (1-[5-isoquinolinesulfonyl]-2-methylpiperazine), and 1-oleoyl-2-acetyl-sn-glycerol (OAG) were from Biomol Research Laboratories, Inc., Plymouth Meeting, Penna., and HA1004 was from Seikagaku America, Inc., St. Petersberg, Florida.

To avoid cellular lysis, 100 mmol/l sphinganine in absolute ethanol was diluted slowly into a solution of 2.5 mmol/l BSA in Dulbecco's phosphate buffered saline (D-5773; Sigma Chemical Co.) to a final concentration of 2 mmol/l.[23] This then was added to the organ culture medium to achieve the final sphinganine concentration. Staurosporine and PMA were dissolved in dimethyl sulfoxide (DMSO) and diluted into the medium so the final concentration of DMSO was 0.05%. H-7 and HA1004 were prepared as concentrated stock solutions in distilled water and diluted 100-fold with medium. OAG initially was dissolved in chloroform/methanol (2:1, volume/volume), and after evaporation of the organic solvent, it was dissolved in medium.

Animals/Epithelial Abrasion

All studies adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Sprague-Dawley male rats that weighed 300–350 g were purchased from Dominion Laboratories, Dublin, Virginia and were given food (Rodent Laboratory Chow, Ralston Purina Co., St. Louis, MO) and water ad libitum. Rats were rapidly asphyxiated in a carbon dioxide chamber, and corneal epithelial abrasions were made as described by Gipson and Anderson.[24] Briefly, a 3 mm diameter area was outlined on the central cornea using a 3 mm diameter Baker's biopsy punch (Holliday Surgical Supply, Winston-Salem, NC), and the epithelium within this area was abraded with a Beaver Mini-Blade 6400 (R. Beaver, Inc., Waltham, MA). Eyes were enucleated, corneas with scleral rims were excised, and the irides were removed. Preliminary studies using light microscopy of sections stained with hematoxylin-eosin and periodic acid-Schiff reagent after diastase digestion of glycogen showed that this procedure removed the full thickness of epithelium, but left the epithelial basement membrane largely intact.

Organ Culture

Organ culture of corneas was performed by the method of Gipson et al.[24,25] In brief, excised corneas were dip-rinsed in 10 changes of Eagle's minimum essential medium (MEM) with Earle's balanced salt solution, L-glutamine, and nonessential amino acids (M-0643; Sigma Chemical Co.). Corneas with scleral rims were cultured on paraffin posts[26] at 35°C with 5% CO_2, four per 60 × 15 mm plastic tissue culture dish, with or without inhibitors or activators in 10 ml MEM supplemented with trace elements and antibiotics.[25]

Quantitation of Epithelial Defects

Epithelial defects were stained with Richardson's stain made by mixing an equal volume of 1% azure II in distilled water and 1% methylene blue in 1% borax solution.[27] The area of the defects was measured by computerized image analysis with JAVA video analysis software (Jandel Scientific, Corte Madera, CA). Corneas with stained defects were left on their paraffin posts and transilluminated by placing them on a fluorescent light box (Ladd Research Industries, Inc., Burlington, VT). A magnified image (approximately ×20) was obtained using a television camera attached to a Zeiss (Hanover, MD) dissecting microscope. The actual magnification was determined by calibration with a stage micrometer (Buehler Scientific, Lake Bluff, IL). In preliminary studies with corneas 0, 5, 10, and 15 hr after epithelial abrasion (n = 4–8 for each time), we determined that defect areas measured with flattened corneas were 14.9 ± 1.4% (mean ± 1 standard error of the mean) less than areas measured when the corneas were left on posts ($P = 0.02$ [15 hr] to $P < 0.001$ [5 hr]; paired Student's t-test). Because the difference in defect area measurements was consistent for all incubation lengths, we measured defect areas using corneas on paraffin posts for the remainder of the studies.

Statistics

The ability of PKC inhibitors and HA-1004 to influence epithelial defect size at different incubation lengths was analyzed by logistic regression. The comparisons versus control corneas determined whether any shift in the epithelial defect healing curves to the right or left was significant ($P < 0.05$). For the dose-re-

sponse curves with PKC inhibitors, the concentration of drug that resulted in 50% inhibition (IC_{50}) of re-epithelialization was calculated by fitting the data to asymptotic regression curves. In all figures and tables, data are expressed as the mean ± 1 SEM.

RESULTS

Although the same procedure was used to create abrasions of the corneal epithelium, moderate variation in the areas of epithelial defects was observed. The coefficient of variation for defect areas of control corneas cultured on the same day or on different days was similar (10.6 ± 1.6% and 8.4 ± 2.5%, respectively). In control corneas, without or with 0.05% DMSO, the defects were re-epithelialized by 25 hr of incubation.

Initial experiments used concentrations of inhibitors reported to abolish PKC activity in other tissues (Fig. 1). In the presence of 100 nM staurosporine, re-epithelialization was markedly retarded ($P < 0.001$); defects persisted even after 40 hr of culture. The inhibition of re-epithelialization by 100 nM staurosporine was reversible, as demonstrated by incubation in medium with 100 nM staurosporine for 10 hr, rinsing of the corneas with fresh medium, and incubation for 20 hr in medium with or without 100 nM inhibitor. Epithelial defect size was 5.4 ± 0.2 mm^2 (n = 4) when the first and second incubations contained 100 nM staurosporine, whereas the defect size was only 1.6 ± 0.3 mm^2 (n = 4) when the second incubation was in medium without inhibitor ($P < 0.0001$ using Student's t-test). Corneas cultured with 50 μmol/l sphinganine or 100 μmol/l H-7 also had significantly delayed resurfacing of the epithelial defects ($P < 0.001$ for both agents). The re-epithelialization rate was similar to control corneas when the medium contained HA1004, an analogue of H-7 that is a potent inhibitor of cyclic adenosine monophosphate (cAMP)- and cyclic guanosine monophosphate (cGMP)-dependent protein kinases and a weak inhibitor of PKC.

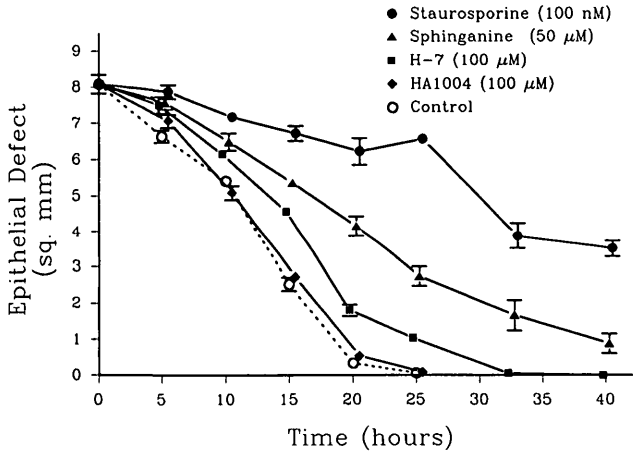

FIGURE 1. Effect of incubation length on epithelial defect size in the absence or presence of protein kinase inhibitors. The protein kinase C inhibitors staurosporine, sphinganine, and H-7 significantly delayed re-epithelialization ($P < 0.001$ for each inhibitor when compared to the control corneas). HA1004, a potent inhibitor of cAMP- and cGMP-dependent protein kinases and a weak inhibitor of PKC, did not affect the rate of epithelial wound healing. Results are the mean ± 1 SEM for eight corneas from 0–25 hr and four corneas for 32.5 and 40 hours.

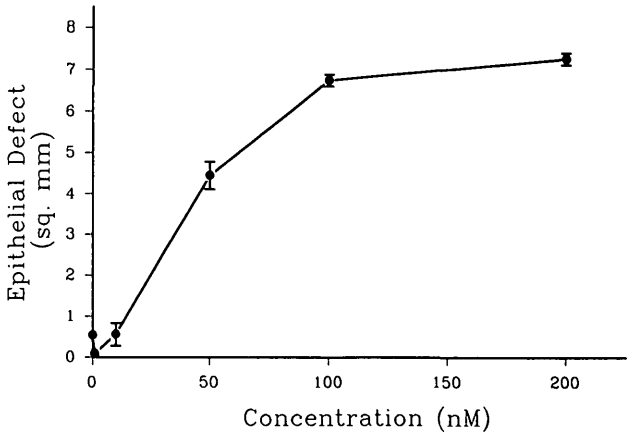

FIGURE 2. Effect of staurosporine concentration on epithelial defect size after 20 hr of organ culture. In the presence of 100 or 200 nM staurosporine, the original epithelial defect decreased in size only about 14%, whereas in control corneas the defect decreased in size by 94%. Results are the mean ± 1 SEM for four corneas at each concentration.

Dose-response studies were performed for the PKC inhibitors staurosporine, sphinganine, and H-7 using corneas cultured for 20 hr (Figs. 2 and 3). There was almost total inhibition of epithelial wound healing

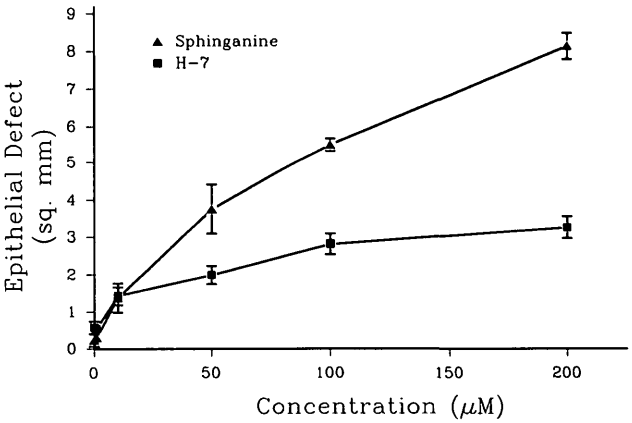

FIGURE 3. Effect of sphinganine and H-7 concentration on epithelial defect size after 20 hr of organ culture. In the presence of 200 μmol/l sphinganine, there was no significant healing of the epithelial defect, whereas in the presence of 100 or 200 μmol/l H-7, the original epithelial defect decreased in size by about 62%. In control corneas, the defect decreased in size by 96–98%. Results are the mean ± 1 SEM for four corneas at each concentration.

TABLE 1. Effect of Protein Kinase C Activators on Epithelial Defect Size

	Length of Incubation		
*Treatment**	*10 hr*	*15 hr*	*20 hr*
Control	4.7 ± 0.2	1.9 ± 0.1	0.3 ± 0.1
Phorbol 12-myristate 13-acetate (100 nM)	4.5 ± 0.1	1.8 ± 0.1	0.3 ± 0.1
Treatment†	*10.5 hr*	*15 hr*	*20 hr*
Control	3.8 ± 0.1	2.0 ± 0.1	0.3 ± 0.1
1-Oleoyl-2-acetyl-sn-glycerol (100 μmol/l)	3.8 ± 0.2	1.9 ± 0.1	0.3 ± 0.1

* Corneas were incubated with phorbol 12-myristate acetate (PMA) or 0.05% dimethylsulfoxide (DMSO; vehicle) for 2.5 hr and transferred to fresh medium that did not contain PMA or DMSO. Transfer to fresh medium was done to minimize the possibility of down regulation of PKC. Values are the mean ± 1 standard error of the mean for epithelial defect areas (mm^2) of eight corneas. There were no statistically significant ($P < 0.05$) differences between controls and PMA-treated corneas at any of the time points.

† Corneas were cultured in medium containing 1-oleoyl-2-acetyl-sn-glycerol (OAG) for 10.5, 15, or 20 hr. Values are the mean ± 1 SEM for epithelial defect areas (mm^2) of six to eight corneas. There were no statistically significant differences between controls and OAG-treated corneas at any of the time points.

in the presence of 100 nM or more staurosporine (Fig. 2) or 200 μmol/l sphinganine (Fig. 3). H-7 was maximally effective at 100 μmol/l, but the inhibition of re-epithelialization was only about half that observed with the two other inhibitors (Fig. 3). The concentration of each compound that gave 50% inhibition of re-epithelialization (IC_{50}) was 38.7 nM for staurosporine and 62.4 μmol/l and 40.5 μmol/l for sphinganine and H-7, respectively.

We also attempted to enhance the rate of wound healing using the PKC activators PMA and OAG. Table 1 shows that when corneas were incubated with 100 nM PMA for 2.5 hr to activate PKC and then transferred to fresh medium devoid of PMA to prevent down-regulation, there was no significant effect on the re-epithelialization rate compared to controls. Similarly, incubation with 100 μmol/l OAG did not increase the rate of epithelial wound healing (Table 1). Because OAG may be rapidly metabolized, we performed a control experiment in which corneas were cultured with 100 μmol/l OAG for a total of 10 hr but with fresh OAG added at 3.3 and 6.7 hr. There was no difference in epithelial defect size in the presence (4.7 ± 0.1 mm^2; n = 8) or absence (4.8 ± 0.2 mm^2; n = 8) of OAG.

DISCUSSION

In this study, we demonstrated that three PKC inhibitors prevent normal epithelial wound healing in the rat cornea. Structurally unrelated inhibitors with two different mechanisms of action were used in an attempt to circumvent the limitations inherent in studies that employ protein kinase inhibitors.[28]

H-7, an isoquinolinesulfonamide derivative,[29,30] binds at or near the adenosine triphosphate (ATP)-binding site of PKC and reversibly inhibits enzyme activity.[31] H-7 is a potent inhibitor of PKC, but it also inhibits cAMP- and cGMP-dependent protein kinases.[29] In our study, H-7 significantly delayed re-epithelialization at concentrations higher than necessary for inhibiting growth factor-induced cytosketetal reorganization in human epidermoid carcinoma cells,[19] but lower than required to retard locomotion of human polymorphonuclear leukocytes.[32] HA1004, a structural relative of H-7, served as control for H-7 specificity because it is a potent inhibitor of cyclic nucleotide-dependent protein kinases but a weak inhibitor of PKC activity.[29] HA1004 had no effect on corneal epithelial wound healing, thus implicating PKC inhibition as the manner by which H-7 exerted its effect.

Staurosporine[33] and sphinganine[34] are PKC inhibitors developed more recently than H-7. Staurosporine reversibly inhibits PKC by competing with ATP for its binding site[35] and typically exerts effects at nanomolar concentrations.[17,33,36] The IC_{50} we observed for inhibition of corneal re-epithelialization by staurosporine (38.7 nM) is similar to that for blockade of antigen-induced increased F-actin in rat basophilic leukemic cells (60 nM),[17] suggesting a similar mechanism. However, our results do not exclude the possibility that part of the inhibition of epithelial wound healing by staurosporine may have been the result of disruption of actin filaments by a PKC-independent mechanism.[37] It is unlikely that the inhibition of re-epithelialization by staurosporine was the result of diminished cell viability, because the inhibitory effect was reversible when medium without inhibitor was added to the organ cultures.

The inhibition of PKC by sphinganine is competitive with diacylglycerol and phorbol ester and noncompetitive with calcium.[38,39] In addition to inhibiting PKC, sphingolipids such as sphinganine may exert a variety of PKC-independent effects, including inhibition of calmodulin-dependent enzymes.[39,40] If sphinganine inhibited calmodulin-dependent enzymes in the epithelium of our organ cultured corneas, this undoubtedly would have contributed to the delay in re-epithelialization.[41,42] We suspect that the sphinganine inhibition of corneal re-epithelialization was the result of multiple mechanisms, not just inhibition of PKC, because the dose-response curve deviated from the classical sigmoidal shape expected for inhibitors with a single mode of action.

In contrast to our ability to delay or prevent cor-

neal re-epithelialization using PKC inhibitors, we were unable to increase the rate of healing using the PKC activators OAG and PMA. The lack of effect of these PKC activators is unlikely to have been a methodologic problem, because PMA was left in the media for only 2.5 hr to minimize down-regulation of PKC,[43] and multiple applications of OAG were tested to reduce the possibility that rapid metabolism of this compound was negating a possible effect.[44] We hypothesize that rapid and maximal activation of PKC occurs in the cells surrounding an epithelial abrasion and is necessary for normal rates of re-epithelialization. Such a scenario would explain the ability to delay wound healing using PKC inhibitors and the inability to speed healing using PKC activators. Further experiments that measure PKC activity or assess PKC immunohistochemically will be needed to validate or refute this theory.

Re-epithelialization in organ-cultured rat corneas after a 3 mm diameter abrasion depends on migration, but not division, of the remaining epithelial cells.[11] Protein and glycoprotein synthesis are markedly increased in the migrating epithelial cells,[26,45,46] and inhibition of glycoprotein synthesis prevents complete closure of the epithelial defect.[45] Other factors crucial for normal rates of epithelial migration are actin polymerization[10,11,41] and proper interaction of the migrating cells with the extracellular matrix.[24] Our results do not help distinguish which of these events may be modulated by PKC, but they should provide a useful framework for future studies to clarify the cellular mechanisms requisite for epithelial wound healing in the rat cornea.

Key Words

cornea, cytoskeleton, epithelium, phorbol esters, protein kinase C, sphinganine, staurosporine, wound healing, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7), 1-oleoyl-2-acetyl-sn-glycerol.

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