

Altered Morphology and Function of the Lacrimal Functional Unit in Protein Kinase α Knockout Mice

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PURPOSE. Protein kinase C (PKC) α plays a major role in the parasympathetic neural stimulation of lacrimal gland (LG) secretion. It also has been reported to have antiapoptotic properties and to promote cell survival. Therefore, the hypothesis for the present study was that PKC α knockout ($^{-/-}$) mice have impaired ocular surface-lacrimal gland signaling, rendering them susceptible to desiccating stress and impaired corneal epithelial wound healing. In this study, the lacrimal function unit (LFU) and the stressed wound-healing response were examined in PKC α $^{-/-}$ mice.

METHODS. In PKC α $^{+/+}$ control mice and PKC α $^{-/-}$ mice, tear production, osmolarity, and clearance rate were evaluated before and after experimental desiccating stress. Histology and immunofluorescent staining of PKC and epidermal growth factor were performed in tissues of the LFU. Cornified envelope (CE) precursor protein expression and cell proliferation were evaluated. The time course of healing and degree of neutrophil infiltration was evaluated after corneal epithelial wounding.

RESULTS. Compared with the PKC α $^{+/+}$ mice, the PKC α $^{-/-}$ mice were noted to have significantly increased lacrimal gland weight, with enlarged, carbohydrate-rich, PAS-positive acinar cells; increased corneal epithelia permeability, with reduced CE expression; and larger conjunctival epithelial goblet cells. The PKC α $^{-/-}$ mice showed more rapid corneal epithelial healing, with less neutrophil infiltration and fewer proliferating cells than did the PKC α $^{+/+}$ mice.

CONCLUSIONS. The PKC α $^{-/-}$ mice showed lower tear production, which appeared to be caused by impaired secretion by the LG and conjunctival goblet cells. Despite their altered tear dynamics, the PKC α $^{-/-}$ mice demonstrated more rapid corneal epithelial wound healing, perhaps due to decreased neutrophil infiltration. (*Invest Ophthalmol Vis Sci.* 2010;51:5592-5600) DOI:10.1167/iovs.09-4562

Protein kinase C (PKC) is an important intracellular signaling molecule that is present in mammalian cells as a family of at least 12 closely related isozymes with serine-threonine ki-

nase activity. The isoforms in this family are divided into three groups: classic PKC α , $-\beta$ I, $-\beta$ II, and $-\gamma$, which are activated by Ca^{2+} and diacylglycerol (DAG); newly identified PKC δ , $-\epsilon$, $-\mu$, $-\eta$, and $-\theta$, which are activated by DAG only; and Ca^{2+} and DAG-independent, atypical PKC ι/λ and $-\zeta$. PKC is involved in the intracellular transduction of a variety of signals related to proliferation, differentiation, migration, adhesion, transformation, and protection from apoptosis, that are triggered by guanine-nucleotide-binding, protein-coupled (G-protein) receptors; tyrosine kinase receptors; and other membrane-signaling molecules.¹ The PKC isoforms are activated in specific processes in a tissue-specific manner.

Lacrimal acinar cells have been reported to contain five different PKC isoforms: α , β , μ , γ , ϵ , and ι/λ . With the exception of ι/λ , all are activated by DAG, and some also require elevation of cytosolic Ca^{2+} .² Both Ca^{2+} and PKC α activate exocytic fusion of preformed secretory vesicles with the apical plasma membrane, so that the content proteins are released into the forming lacrimal gland fluid.^{3,4} It has been documented that the PKC- α and $-\mu$ isoforms control exocytosis in the lacrimal gland.^{5,6} The lacrimal gland is innervated by parasympathetic and sympathetic nerves, with the former predominating. Cholinergic agonists activate phospholipase C (PLC), to generate inositol 1,4,5-trisphosphate (IP3) and DAG. IP3 releases intracellular Ca^{2+} from IP3-sensitive stores and stimulates protein secretion by activating Ca^{2+} calmodulin-dependent protein kinase and PKC α ,⁵ whereas DAG stimulates protein secretion by activating PKC α . Both the Ca^{2+} and the PKC-dependent pathways are equally potent in stimulating protein secretion. Thus, in parasympathetic lacrimal secretion, PKC α accounts for more than 50% of cholinergic agonist-induced protein secretion.⁷ Acinar cells of the lacrimal gland secrete water, electrolytes, protein, and mucin into tear fluid, mostly in response to neural stimulation. The tears secreted by lacrimal glands contain supportive and protective factors for the ocular surface, including the cornea. In 1998, the ocular surface (cornea, conjunctiva, and meibomian glands), the lacrimal glands, and the neural network that connects them were described as the *lacrimal functional unit* (LFU),⁸ which controls secretion of the three major components of the tear film. The overall purpose of the LFU is to maintain the clarity of the cornea and quality of the image projected onto the retina.

Conjunctival goblet cell mucin secretion is under neural control, often stimulated by activation of afferent sensory nerves in the cornea. The efferent arc of this neural reflex is mediated by autonomic parasympathetic and sympathetic nerves that surround the goblet cells or by antidromic stimulation of the sensory nerves in the conjunctiva and collateral sensory nerves from the cornea.⁹ It has been documented that cholinergic muscarinic agonists, increased Ca^{2+} , and PKC activation stimulate conjunctival goblet cell secretion.^{10,11} However, the contributory role of the various PKC isozymes present in the goblet cells remains to be determined.

PKC α is activated by a variety of stimuli, including ligands binding to G-protein receptors and to tyrosine kinase recep-

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tors, and also by physical stresses such as hypoxia and mechanical strain. Therefore, this isotype plays an important role in the control of major cellular functions, including proliferation, apoptosis, differentiation, and motility, depending on where and when it is activated and what substrates it acts on.

The proliferative effect of PKC α is mediated via activation of the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) cascade. PKC α is also involved in cell differentiation and cell migration and adhesion by interacting with the integrin β 1, ezrin, radixin, and moesin (ERZ) proteins and the F-actin-binding proteins, which are involved in the maintenance of cell survivor and extension.¹² Some studies have reported that the overexpression of PKC α in human skin keratinocytes has no effect on proliferation or differentiation.¹³ The contribution of PKC α to corneal epithelial cell proliferation and differentiation after wound healing has not been evaluated.

Programmed cell death occurs by apoptosis and cornification in the epithelia.¹⁴⁻¹⁶ PKC has been reported to increase expression of the cornified envelope precursor gene, *SRR1*, which may promote death by cornification.¹⁷

Besides its proliferative and cornification functions, PKC α has been noted to inhibit inflammation by negatively regulating NF κ B-induced cytokine expression—particularly, that of interleukin (IL)-1.¹⁸

We evaluated the morphology and secretory function of the LFU in PKC α ^{-/-} mice. The effects of PKC α knockout on tear production and tear concentrations of lacrimal growth factors, such as epidermal growth factor (EGF), that support the ocular surface were assessed after desiccating stress. Markers of proliferation, cornification, and apoptosis were compared in PKC α ^{-/-} mice and PKC α ^{+/+} wild-type mice. Furthermore, the effects of PKC α deficiency on corneal epithelial wound healing were evaluated. Specifically, corneal epithelial morphology, proliferation, inflammation, and healing rate after experimental corneal epithelial debridement, were compared in the two mouse strains.

MATERIALS AND METHODS

Animal and Genotype

PKC α gene-deficient mice were generated by homologous recombination and were provided as a gift from Michael Leitges (Laboratory for Lymphocyte Signaling, University of Cologne, Cologne, Germany).¹⁹ The PKC isoenzymes were adapted from the rat to the mouse. Targeted embryonic stem (ES) cells (PKC α ^{-/-}) were injected into NMRI albino mouse blastocysts to generate chimeras. Germ line-transmitting male chimeras were crossed to 129/SV females to give rise to F1 heterozygous offspring on a pure 129 background. Intercrosses of these mice were used to establish a homozygous, PKC α -deficient (-/-) mouse line. Male mice from this line were compared with age-matched male 129/SV PKC α control (+/+) mice.¹⁹ Coat color was used to detect germ line transmission in mice homozygous for PKC α deficiency. All studies were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all animal research protocols were approved by the Baylor College of Medicine Center for Comparative Medicine.

Twenty PKC α gene knockout mice (PKC α ^{-/-}) and 20 wild-type control mice (PKC α ^{+/+}) at age 2 months were used. Each set of 20 mice contained 10 female and 10 male mice. Genotypes of 1-month-old pups from homozygous parents were tested by using genomic DNA isolated from their tails (Genomic DNA Isolation kit; Sigma-Aldrich, St. Louis, MO). PCR amplification was performed on a thermal cycler (DNA Thermal Cycler 480; GeneAmp PCR kit; Applied Biosystems, Foster City, CA) with the *Pkca/Neo* gene primer pair, GGCGAACAGTTCGGCTGCGGAGCCCC and GAGCCCTGGGTTTCAAGTATAGA, yielding a 520-bp fragment from homozygous PKC α ^{+/+} mice and a

200-bp fragment from homozygous PKC α ^{-/-} mice, with both bands detected in the heterozygotes (+/-) (Fig. 1). Further confirmation of PKC deficiency was detected by the absence of PKC α protein expression shown by immunofluorescent staining in the cornea and lacrimal glands of the PKC α ^{-/-} mice (Fig. 2).

Experimental Dry Eye Model

A modification of a previously reported mouse model of experimental dry eye (EDE) was used.²⁰ Briefly, age and sex-matched mice were placed in a low-humidity (<35%) environment and exposed to an air draft for 18 hours per day. They received SC injections of 0.5 mg/0.2 mL scopolamine hydrobromide (Sigma-Aldrich, St. Louis, MO) in alternating hindquarters, four times per day (9 AM, Noon, 3 PM, and 6 PM) to inhibit tear secretion.

Aqueous Tear Production

Tear volumes were measured by using phenol red-impregnated cotton threads (Quick Zone; Oasis, Glendora, CA), held in the tear meniscus of the lateral canthus for 20 seconds. The measured uptake of tear fluid in millimeters was compared with a standard curve prepared from cotton threads of known uptake volumes of a stock basic solution (1500 mL of 0.9% saline and 5 mL of 5 N NaOH) over 20 seconds, with volumes in the range that would be expected in mouse tears.

Fluorescein Clearance Test

The fluorescein clearance test is a measure of total tear production, tear spread, and tear drainage.²¹ This test was performed by instilling 1 μ L of 2% sodium fluorescein into the conjunctival sac. After 15 minutes, 1 μ L of phosphate-buffered saline (PBS) was instilled, and the fluorescein-stained tear fluid was collected atraumatically from the lateral tear meniscus for 20 seconds under a surgical microscope with a 1- μ L volume glass capillary tube (Drummond Scientific Co., Broomhall, PA). The tear sample collected from the capillary tube was eluted into a 1.5-mL tube containing 99 μ L PBS and centrifuged for 5 minutes. The solution was transferred to a single well in a 96-well plate. The fluorescein concentration was measured with a fluorophotometer (CytoFluor II; Perseptive Biosystems, Framingham, MA), using 485-nm excitation and 530-nm emission filters.

Corneal Permeability to AlexaFluor Dextran

The corneal uptake of 10-kDa AlexaFluor dextran (AFD; Molecular Probes, Eugene, OR) was measured by using a modification of a previously reported technique.²² Briefly, 1 μ L of 0.3% AFD was instilled onto the ocular surface 15 minutes before euthanization. Excised corneas were rinsed four times with 200 μ L balanced salt solution (BSS; Alcon Laboratory, Inc., Fort Worth, TX) and placed in 200 μ L balanced salt solution. The solution containing the corneal tissue was protected from light and placed on an orbital shaker. The concentration of eluted AFD was measured with 485-nm excitation and 530-nm emission filters at 10, 20, and 60 minutes on a fluorophotometer (CytoFluor II; Perseptive Biosystems).

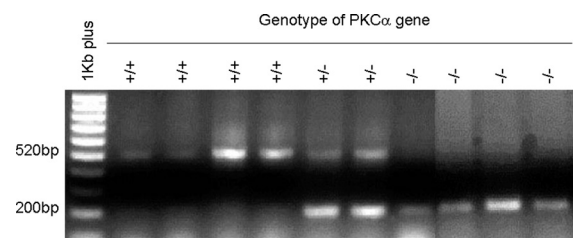


FIGURE 1. Genotype determination by PCR of mouse tail genomic DNA using PKC α -specific primer pairs. The PCR products were analyzed by 1.5% agarose gel electrophoresis. A 520-bp band was obtained from the wild-type mice (+/+), a 200-bp band from the PKC α knockout mice (-/-), and both bands from the heterozygotes (+/-).

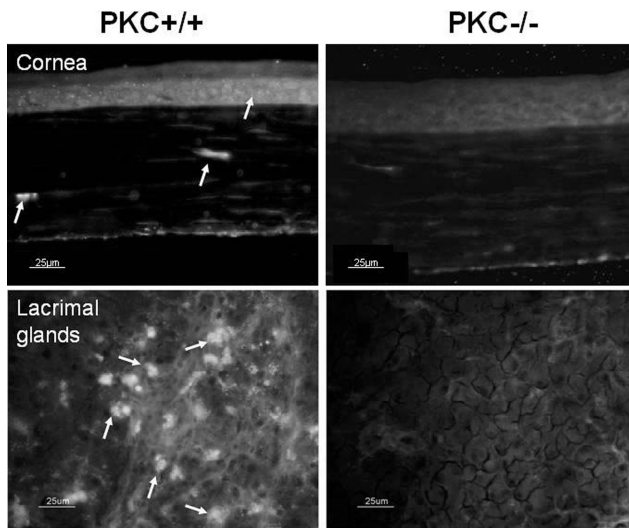


FIGURE 2. Immunofluorescent staining of PKC (arrow) in cornea (top) and lacrimal gland (bottom) from PKC $\alpha^{+/+}$ (left) and PKC $\alpha^{-/-}$ mice (right).

Tear Collection

PBS (1.5 μ L) containing 0.1% bovine serum albumin (BSA) was instilled into the conjunctival sac. The tear fluid and buffer were collected with a 1- μ L volume glass capillary tube (Drummond Scientific Co.) from the tear meniscus in the lateral canthus.

Collection of tear fluid to measure sodium concentration was performed before and after 3 days of desiccation stress. Briefly, 1 μ L of distilled water was instilled into the conjunctival sac for 20 seconds. The diluted tear fluid was collected with a 0.5- μ L glass capillary tube (Drummond Scientific Co.) from the tear meniscus in the lateral canthus and immediately added to 4.5 μ L of distilled water. The samples were then capped to prevent evaporation and frozen for further analysis.

Tear Sodium and Osmolarity Measurements

Tear sodium and osmolarity measurements were performed by using a published method,²⁰ with modifications. Briefly, 5 μ L of a red mitochondrial probe (CoroNa Red, cat. no. C-24431; Molecular Probes, Eugene, OR) in DMSO was added to each sample. The samples were placed in individual wells of a round-bottomed, 96-well plate (cat. no. 163320; Nunc, Rochester, NY). A multiwell plate reader (CytoFluor II; Perkin Elmer, Wellesley, MA) was then used to measure fluorescence intensity. A standard curve was created with the fluorescence values from 500 mM of the mitochondrial dye in DMSO placed in a 10- μ L sample with a known sodium concentration. The sodium concentration of the mouse tear sample was then calculated from the standard curve, by using a dilution factor based on the previously measured tear volume, to give the following formula: total tear sodium concentration (mM) = measured tear sodium concentration \times (1 + tear volume)/tear volume. In four different mice of each strain, tear sodium was measured in both eyes.

The osmolarity of the mouse tear sample derived from sodium concentration was then calculated by comparing the sodium concentration in the tear sample with the osmolarity of a standard curve established from samples of known sodium concentrations, whose osmolarity was determined by using a vapor pressure osmometer (model 3300; Advanced Instruments Inc., Norwood, MA) at the clinical core laboratory of the Department of Pathology at Methodist Hospital (Houston, TX).

The serum osmolarity of the two mouse strains was determined by taking blood from the orbital venous sinus immediately after euthanization. The serum was separated from the blood by centrifuging at 14,000 rpm (Microcentrifuge model 5417 C, 18,000g; Eppendorf, Fremont, CA) at room temperature for 10 minutes, and the osmolarity was measured (Table 1).

Lacrimal Gland Weight

After euthanization, both exorbital lacrimal glands were removed from PKC $\alpha^{+/+}$ and PKC $\alpha^{-/-}$ mice. The lacrimal glands' weight and volume and the mouse's body weight were measured. The lacrimal gland density (weight/volume) and the lacrimal gland/body weight ratio were compared between the two mouse strains.

Histology and Immunofluorescent Staining

After the mice were euthanized, the eyeballs, together with the eyelids and conjunctiva, were excised; embedded in a mixture of 75% (vol/vol) OCT compound (Sakura Finetek USA, Inc., Torrance, CA) and 25% (vol/vol) aqueous mounting medium (Immu-Mount; Thermo-Shandon, Pittsburgh, PA); and fresh frozen in liquid nitrogen. Sections (10 μ m thick) were cut and stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS), to detect the carbohydrate-rich cells, which stain for a high proportion of carbohydrate macromolecules (i.e., conjunctival goblet cells and granules in acinar cell). For immunofluorescent staining, sections were fixed with 100% methanol at 4°C for 10 minutes and blocked with 5% normal goat serum in PBS for 30 minutes. The primary antibody was applied for 1 hour at room temperature. These monoclonal antibodies and goat polyclonal antisera were reactive with mouse EGF (Monosan, Uden, Netherlands), PKC α (BD Transduction Laboratories, San Jose, CA), MUC-5AC (a gift from Marcia Jumblatt, University of Louisville, Louisville, KY) and SPRR2 (Apotech, Epalinges, Switzerland). After the sections were washed with PBS, the species-specific secondary antibody (AlexaFluor-488 conjugate; 1:100 dilution; Molecular Probes) was applied for 1 hour in a dark incubation chamber. After another wash with PBS, the sections were mounted with antifade medium (Gel-Mount; Fisher, Atlanta, GA) containing 1 μ g/mL Hoechst 33342 dye, and a coverslip was applied. The sections were examined and photographed with an epifluorescence microscope (Eclipse 400; Nikon, Tokyo, Japan) and a digital camera (model DMX 1200; Nikon).

BrdU Incorporation

BrdU is a nucleoside that can be incorporated into DNA in place of thymidine to measure DNA synthesis and cell proliferation. Mice were injected with 100 μ g BrdU/g body weight intraperitoneally for 1 to 2 hours before euthanization. BrdU was detected by immunostaining frozen sections that were fixed in cold methanol at 4°C for 10 minutes,

TABLE 1. Comparison of Tear Sodium, Tear Osmolarity, and Serum Osmolarity in Two Strain Mice before and after 3 Days EDE Treatment

	Tear Sodium (mM)		Tear Osmolarity (mOsm/L)		Serum Osmolarity (mOsm/L)	
	Baseline	EDE	Baseline	EDE	Baseline	EDE
PKC $\alpha^{+/+}$	171 \pm 60	264 \pm 89*	317 \pm 112	491 \pm 165*	324 \pm 3.9	330 \pm 2.7
PKC $\alpha^{-/-}$	194 \pm 42	303 \pm 71*	359 \pm 77	561 \pm 132*	324 \pm 5.4	331 \pm 7.3

Data are expressed as the mean \pm SD.

* $P < 0.05$, Mann-Whitney test.

followed by incubation with 2 N HCl at 37°C for 1 hour, to denature the DNA, and neutralization in boric acid (pH 8.5) for 20 minutes. Anti-BrdU polyclonal antibody (1:100) was applied, followed by incubation with anti-rabbit secondary antibody (1:300) with 3,3'-diaminobenzidine (DAB) and hematoxylin counterstaining. The BrdU labeling index was assessed by point-counting positively stained cells through an inverted microscope (TE200; Nikon), equipped with a 40 \times objective lens. A total of 500 to 951 nuclei were counted in six to eight representative fields because this number was considered to be the minimum requirement to observe representative labeling.²³ The labeling index was expressed as the number of positively labeled nuclei/total number of nuclei \times 100%.

Corneal Epithelial Wound Healing

A central corneal wound was made as previously described.^{24,25} Age- and sex-matched PKC $\alpha^{+/+}$ and PKC $\alpha^{-/-}$ mice were used in the experiments. Briefly, the central corneal epithelium was marked with a 3-mm trephine and then debrided with a golf club-shaped spatula (Accutome, Malvern, PA) under a dissecting microscope. After the corneal wound was created, the rate of closure was measured in digital images of fluorescein-stained corneas obtained every 6 hours.

Immunofluorescent Staining of Whole Mount Corneas

A protocol described in another publication²⁴ was used for morphometric analysis of the corneal response to injury. Immunofluorescent staining using anti-Gr-1-FITC (PharMingen, San Diego, CA) to detect neutrophils and anti-tubulin (Sigma, St. Louis, MO) to detect cells undergoing mitosis was performed in excised corneas with the limbus retained. Nuclear morphology and cell division were assessed by staining with 1 μ M 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO) in flat, whole mount corneas. The pattern of microscopic analysis included counting inflammatory cells (i.e., neutrophils) or healing (dividing epithelial cells) in nine microscopic fields (40 \times objective; field of view diameter, 0.53 mm) across the cornea from limbus to limbus. Digital images were captured and saved for analysis (DeltaVision; Applied Precision, Issaquah, WA). At least six corneas were examined in each group, and the average number of cells in each field was calculated and used for statistical analysis. The limbus was defined as the intervening zone between the cornea and sclera, and was considered the most peripheral field.

Statistical Analysis

Based on the normality of the data distribution, the *t*-test or Mann-Whitney test was used for statistical comparison of assay results between groups. $P < 0.05$ was regarded as statistically significant.

Tear Production

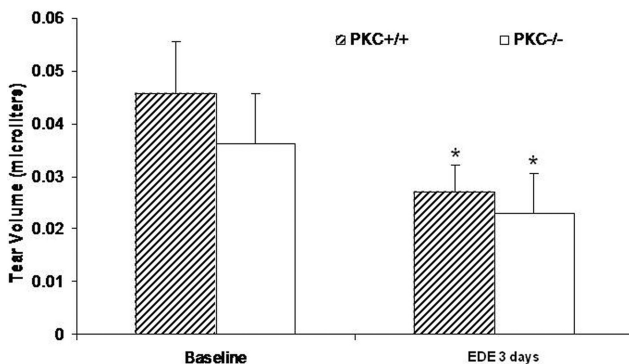


FIGURE 3. Tear production in PKC $\alpha^{+/+}$ and PKC $\alpha^{-/-}$ mice without (baseline) and after EDE for 3 days. *Significant difference between EDE and baseline in each mouse strain ($P < 0.05$), Mann-Whitney test.

Tear Clearance

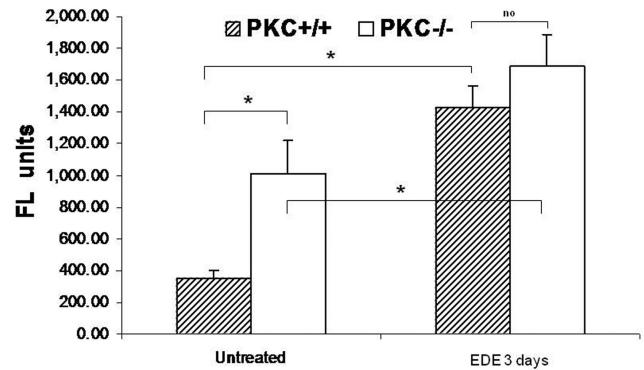


FIGURE 4. Comparison of tear fluorescein clearance between PKC $\alpha^{+/+}$ and PKC $\alpha^{-/-}$ mice before and after experimental dry eye. Data show mean and SD (error bars) fluorescence units. * $P < 0.05$; Mann-Whitney test.

RESULTS

Phenotype of PKC $\alpha^{-/-}$ Mice

Aqueous Tear Production and Clearance. Aqueous tear production was reduced in the PKC $\alpha^{-/-}$ mice (mean \pm SD, $n = 11$) compared with that in age-matched PKC $\alpha^{+/+}$ mice (mean \pm SD, $n = 16$), with or without imposition of EDE for 3 days, but the difference between strains did not reach statistical significance in either situation (Fig. 3). However, there was a significant difference between EDE and baseline in each of the mouse strains ($P < 0.05$; Fig. 3). PKC $\alpha^{-/-}$ mice had significantly delayed tear fluorescein clearance compared with age-matched PKC $\alpha^{+/+}$ mice, with or without EDE ($P < 0.05$; Fig. 4).

Tear and Serum Osmolarity. Tear sodium concentration in PKC $\alpha^{+/+}$ ($n = 4$) and PKC $\alpha^{-/-}$ mice ($n = 4$), respectively, measured 171 ± 60 (mean \pm SD) and 194 ± 42 mM at baseline and 264 ± 89 and 303 ± 71 mM after 3 days of EDE. The increase in sodium concentration after 3 days of EDE was significantly different from baseline in the PKC $\alpha^{+/+}$ and the PKC $\alpha^{-/-}$ mice ($P < 0.05$); however, there was no difference between strains in either case.

Tear osmolarity, calculated from the sodium ion concentration, increased significantly after EDE in both strains, to an average of 491 ± 165 mOsm/L in the PKC $\alpha^{+/+}$ mice and 561 ± 132 mOsm/L in the PKC $\alpha^{-/-}$ mice, compared with a baseline of 317 ± 112 osm/L ($P < 0.05$) and 359 ± 77 osm/L ($P < 0.05$), respectively.

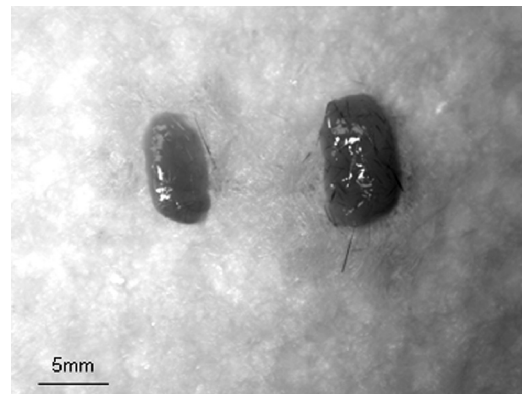


FIGURE 5. Lacrimal gland appearance in PKC $\alpha^{+/+}$ (left) and PKC $\alpha^{-/-}$ (right) mice.

The serum osmolarity of the $PKC\alpha^{+/+}$ mice ($n = 4$) was 324 ± 3.9 mOsm/L at baseline and 330 ± 2.7 mOsm/L after 3 days of EDE, whereas the serum osmolarity of the $PKC\alpha^{-/-}$ mice ($n = 4$) was 324 ± 5.4 mOsm/L at baseline and 331 ± 7.3 mOsm/L after 3 days of EDE. There was no significant difference between baseline and EDE measurements in either strain.

Lacrimal Gland Weight. The lacrimal glands of the $PKC\alpha^{-/-}$ mice weighed more (0.069 ± 0.03 g, $n = 4$) than those of the age-matched $PKC\alpha^{+/+}$ mice (0.038 ± 0.018 g, $n = 4$, $P < 0.05$; Fig. 5). Similarly, lacrimal gland density (weight/volume) in the $PKC\alpha^{-/-}$ mice (2.76 ± 0.6 mg/mm³) was higher than that in the age-matched $PKC\alpha^{+/+}$ mice ($1.21 \pm$

0.071 , $P < 0.05$), as was the ratio of lacrimal gland to body weight (2.13 ± 0.57 in the $PKC\alpha^{-/-}$ mice, 1.21 ± 0.049 in the $PKC\alpha^{+/+}$ mice; $P < 0.05$).

Corneal Epithelial Permeability to AFD. Decreased corneal epithelial barrier function is a key feature of human dry eye. We assessed it by measuring corneal permeability to AFD. There was no difference in AFD permeability in the corneas of the $PKC\alpha^{-/-}$ and the age-matched $PKC\alpha^{+/+}$ mice as three males and two females at baseline. After EDE for 3 days, the corneas of the $PKC\alpha^{-/-}$ mice were significantly more permeable to AFD (475 ± 25 U, $n = 5$) than were those of the age-matched $PKC\alpha^{+/+}$ mice (300 ± 25 U, $n = 5$, $P < 0.05$, by Mann-Whitney test). This finding

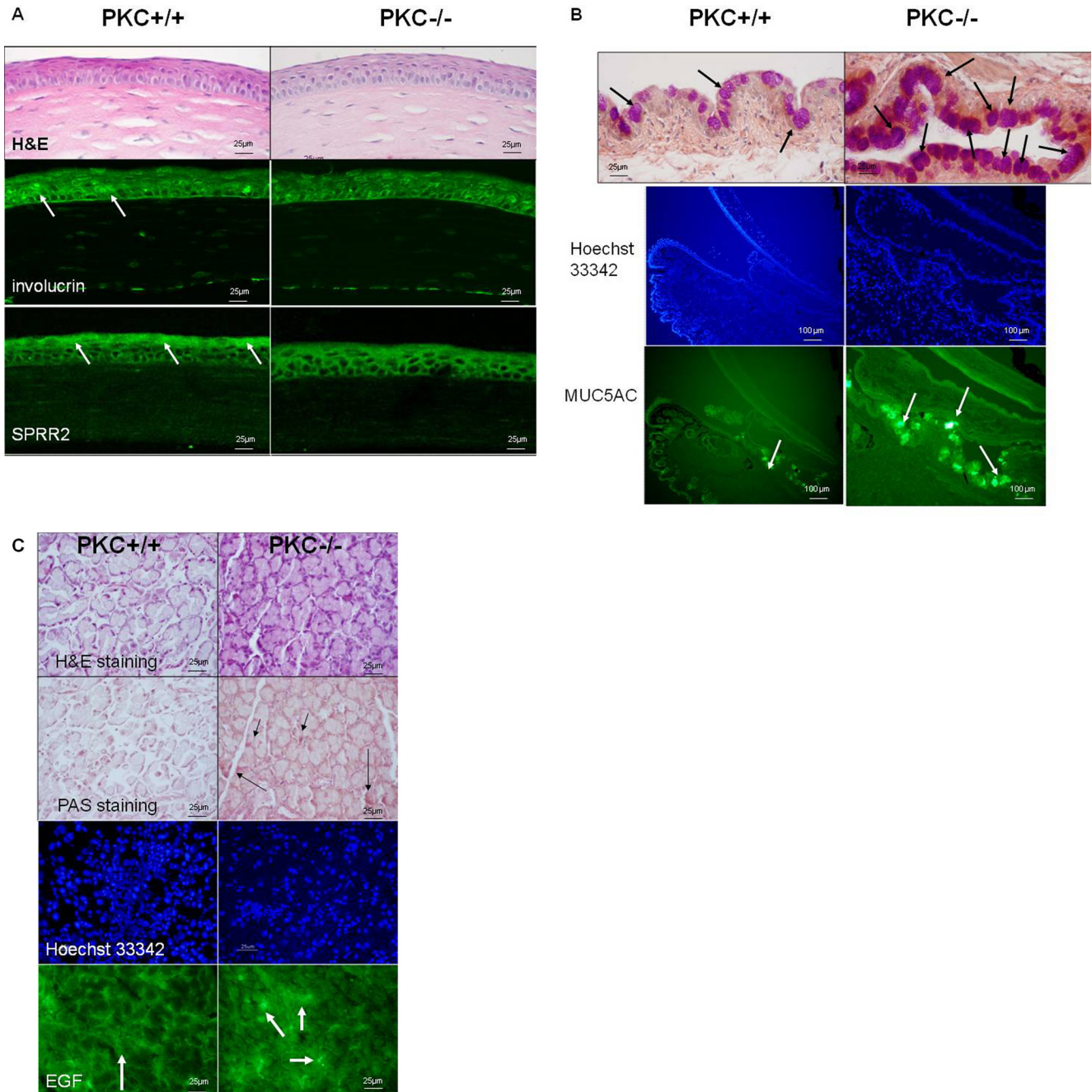


FIGURE 6. Histology and immunofluorescent (IF) staining of cornea, conjunctiva, and lacrimal gland of $PKC\alpha^{+/+}$ and $PKC\alpha^{-/-}$ mice. (A) Corneal epithelia: H&E staining and IF for involucrin and SPRR2 in mice subjected to EDE for 3 days (arrows, IF⁺ staining). (B) Conjunctiva: PAS staining (top, arrows, secretory granules) and IF for MUC5AC (arrows, IF⁺ staining) with Hoechst 33342 as the counterstain. (C) Lacrimal glands: H&E and PAS staining (arrows, secretory granules) and IF for EGF (arrows, IF⁺ staining) with Hoechst 33342 as the counterstain.

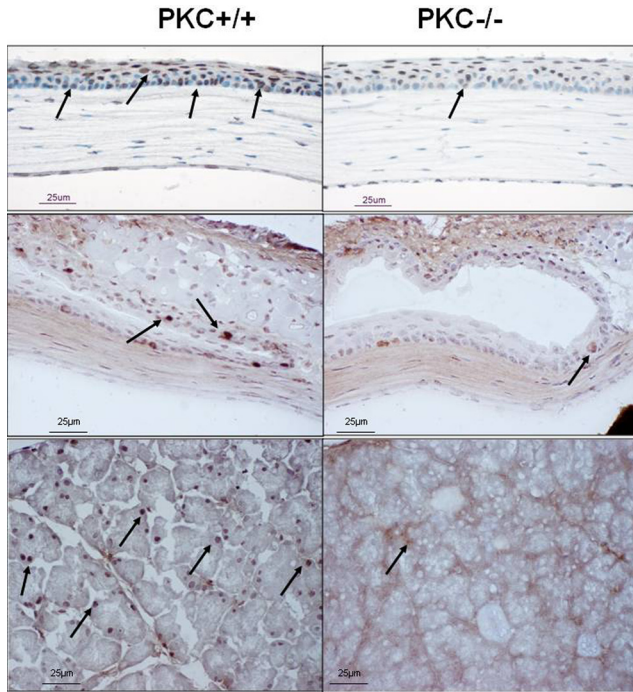


FIGURE 7. BrdU incorporation (arrows) in corneal (top) and conjunctival (middle) epithelia and lacrimal gland (bottom) in PKC $^{+/+}$ and PKC $^{-/-}$ mice ($n = 4$).

suggests that PKC $^{-/-}$ mice are more susceptible to corneal barrier disruption after experimental desiccating stress.

Morphology of the LFU. There was no difference in corneal epithelial morphology between the 2-month-old PKC $^{-/-}$ mice and the age-matched wild-type mice (Fig. 6A). Immunofluorescent staining of mouse corneal frozen sections was performed to evaluate the expression by corneal epithelial cells of the cornification markers involucrin and SPRR2, which may have a role in corneal barrier functioning. The intensity of both involucrin and SPRR2 immunofluorescent staining in the corneal epithelia of nonstressed mice was lower in the PKC $^{-/-}$ than in the PKC $^{+/+}$ mice (Fig. 6A).

In the conjunctiva, enlarged carbohydrate-rich goblet cells with stronger PAS staining were observed in the PKC $^{-/-}$ mice compared with those in the PKC $^{+/+}$ mice at baseline (before EDE). Correspondingly, greater MUC5AC staining was detected in the conjunctiva of the PKC $^{-/-}$ than in the PKC $^{+/+}$ mice (Fig. 6B).

Lacrimal gland histologic sections stained with H&E and PAS showed larger carbohydrate-rich acinar cells containing more PAS-positive granules in samples from the PKC $^{-/-}$

mice. The number of lacrimal gland acinar cells in the PKC $^{-/-}$ samples was similar to that in the wild-type, but they appeared to be filled with secretory granules that were not being discharged into the lumen. Furthermore, the lacrimal glands of the PKC $^{-/-}$ mice had stronger staining for EGF, a growth factor produced by the acinar cells and secreted into the tears, than in the PKC $^{+/+}$ mice, but the difference was without statistical significance (immunofluorescent staining intensity: PKC $^{+/+}$ mice: 50.35 ± 17.62 ; PKC $^{-/-}$ mice: 52.95 ± 18.17 ; $P > 0.05$; Fig. 6C).

BrdU Incorporation Index. BrdU incorporation was evaluated as a marker of cell proliferation in tissues of the LFU. The PKC $^{+/+}$ mice ($n = 4$) had a greater percentage of BrdU-positive cells in the cornea, conjunctival epithelia, and lacrimal gland acinar cells ($21.25\% \pm 5.78\%$, $8.49\% \pm 2.83\%$, and $11.11\% \pm 4.37\%$, respectively) than did the PKC $^{-/-}$ mice ($n = 4$, $10.85\% \pm 3.92\%$, $2.83\% \pm 0.95\%$, and $3.39\% \pm 1.49\%$, respectively; all $P < 0.05\%$). These findings indicate that PKC α is essential in initiating proliferation in the cells of the LFU (Fig. 7).

Wound Healing

Effect of PKC α Deficiency on Corneal Re-epithelialization after Wounding. A standard model of corneal wound healing was established by removal of a 3-mm-diameter area of epithelium from the center of the cornea. The epithelial healing was monitored by fluorescein staining of the exposed stroma in the PKC $^{-/-}$ and PKC $^{+/+}$ mice. At 6, 12, and 18 hours after wounding, the size of the wounded areas in PKC $^{+/+}$ and the PKC $^{-/-}$ mice were similar. At 24 and 36 hours after creation of the epithelial defect, wound areas in the PKC $^{-/-}$ mice were reduced to 2% and 0% of the original area, respectively. In contrast, wound areas in the PKC $^{+/+}$ mice were reduced to 17% and 7% (Fig. 8). Complete epithelialization was accelerated between 18 and 36 hours in the post-wound PKC $^{-/-}$ mice compared with that in the wild-type mice.

PKC α has been observed to have a regulatory role in cell proliferation that is crucial to corneal wound healing.²⁶ Epithelial basal cell division was noted to be decreased in the PKC $^{-/-}$ mice at 18 and 30 hours after epithelial debridement compared with that in the wild-type mice (Fig. 9).

Effect of PKC α Deficiency on Neutrophil Accumulation in the Cornea. Experimental neutropenia induced by depletion of neutrophils has been noted to accelerate corneal epithelial wound closure.^{27,28} PKC α activation in keratinocytes is a crucial event that orchestrates cutaneous neutrophil responses by increasing the concentration of two chemotactic factors: cytokine-induced neutrophil chemoattractant and macrophage inflammatory protein (MIP)-2 in murine plasma.²⁹⁻³¹ To determine whether the accelerated corneal epithelial heal-

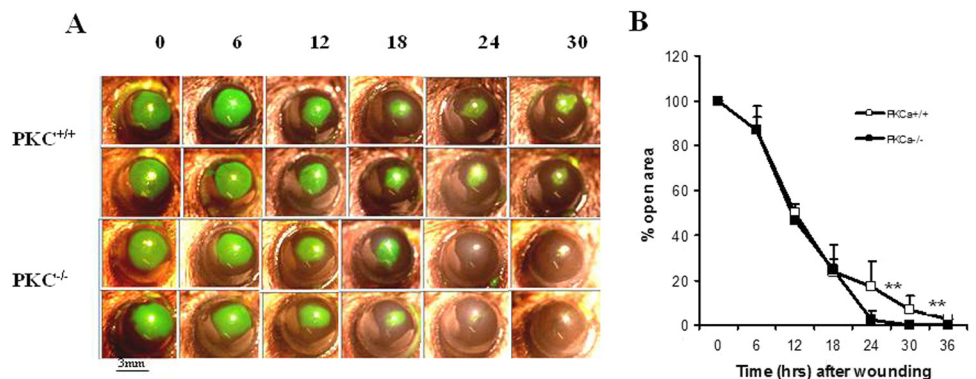


FIGURE 8. Accelerated corneal epithelial wound healing in PKC $^{-/-}$ mice. (A) Fluorescein-stained corneal images after corneal epithelial debridement in PKC $^{+/+}$ and PKC $^{-/-}$ mice. (B) Percentage of the original wound area is plotted over time and was calculated from the area of fluorescein staining at each time point. Data are mean \pm SD ($n = 6$, $**P < 0.01$).

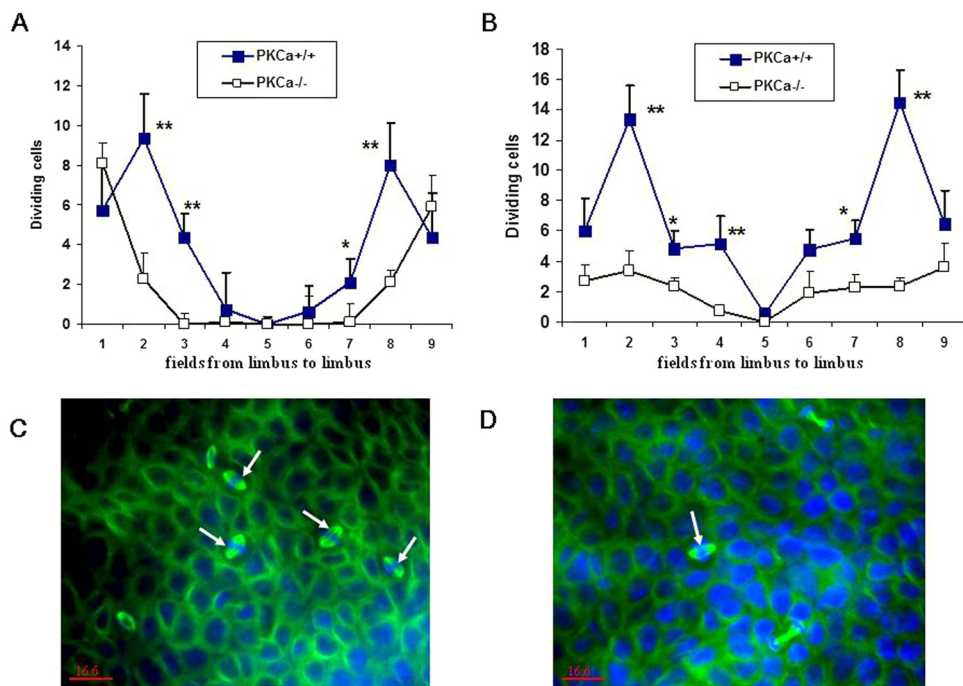


FIGURE 9. Effects of PKC α deficiency on dividing basal cells after corneal epithelial debridement. Dividing basal epithelial cells in each region of the cornea at 18 (A) and 30 (B) hours, respectively, after epithelial debridement. Representative photos of dividing cells (arrow) stained by tubulin (green) in PKC $\alpha^{+/+}$ (C) and PKC $\alpha^{-/-}$ mice (D) at 18 hours after wounding. DAPI (blue) was used for counterstaining. * $P < 0.05$ and ** $P < 0.01$.

ing observed in the PKC $\alpha^{-/-}$ mice is linked to reduced neutrophil migration, we analyzed neutrophil accumulation at 18 and 30 hours after epithelial debridement. Neutrophil migration into the avascular cornea stroma in the PKC α -deficient and wild-type mice was similar at 18 hours (Fig. 10A). In contrast to the response of the wild-type mice, the PKC $\alpha^{-/-}$ mice had a markedly blunted accumulation of neutrophils in every field from limbus to limbus at 30 hours after wounding ($P < 0.005$, Fig. 10B). These results demonstrate that neutrophil recruitment that occurs during the inflammatory phase after corneal epithelial wounding is markedly reduced in the absence of PKC α .

DISCUSSION

Effects of PKC α Deficiency on the Morphology and Function of the Lacrimal LFU

PKC α gene knockout was noted to affect the morphology and function of the LFU. These changes included reduced tear

production, secretory paralysis of the lacrimal gland acinar and conjunctival goblet cells, increased permeability of corneal epithelia after experimental desiccating stress, and decreased cornification of the corneal epithelia.

Loss of PKC α appears to block the parasympathetic pathway of lacrimal gland acinar and conjunctival goblet cell secretion. This effect was signified by the enlarged cell size and stronger carbohydrate-rich cells stained in both tissues, and the failure of exocytosis, due to the lack of the PKC α gene in the exocytic process of preformed secretory vesicles with an apical plasma membrane. The PKC $\alpha^{-/-}$ mice showed an elevated ocular surface response to experimental desiccating stress, with increased disruption of corneal epithelial barrier function. These changes may be directly due to the loss of the PKC α gene or may be secondary to the reduced secretion of the corneal supportive factors by the lacrimal gland and conjunctival goblet cells. Secretion of one such factor, EGF, was noted to be decreased.

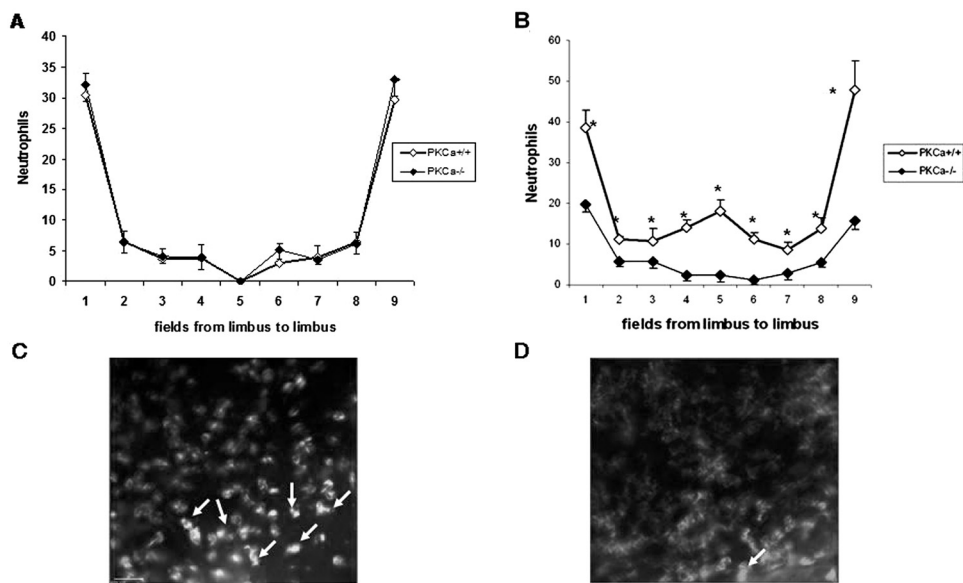


FIGURE 10. Neutrophil infiltration in PKC α -deficient mice after epithelial debridement. Neutrophil migration to corneal stroma in each region of the cornea at 18 (A) and 30 (B) hours, respectively, after epithelial debridement. * $P < 0.005$. Representative photos of neutrophils (arrow) stained by anti-Gr-1-FITC in PKC $\alpha^{+/+}$ (C) and PKC $\alpha^{-/-}$ mice (D) at 30 hours after wounding.

Effect of PKC α Deficiency on Corneal Epithelial Wound Healing and Inflammation

Traumatic corneal epithelial abrasion is a very common condition encountered in clinical practice. To further characterize the healing response after corneal epithelial debridement, we used our standardized epithelial wound model to evaluate the healing rate, proliferation, and inflammatory cell infiltration of the corneal epithelium. PKCs are key intracellular signal transduction mediators that are involved in various cell functions throughout the body, including cell growth and differentiation. Their known functions suggest that they have a pivotal role in corneal wound healing. Studies have already established the presence of PKC α in normal rabbit corneal epithelium.³²⁻³⁴ Its expression increased during epithelial wound healing. An oligonucleotide-targeting rabbit PKC α was found to inhibit the wound-healing process in vitro by more than 50%.³⁴ In contrast to the predictions made on the basis of these previously reported studies, we found that PKC $\alpha^{-/-}$ mice show accelerated corneal epithelial wound healing and faster re-epithelialization compared with PKC $\alpha^{+/+}$ mice.

The mechanism of these conflicting results is not clear. One factor that influences healing rate is the local inflammatory response that may have a positive or negative impact on the healing rate, as debated in a recent review.²⁷ The neutrophil is the predominant cell type in the acute phase of inflammation, soon followed by a second wave of monocyte infiltration. Although it is widely recognized that the infiltration of neutrophils into injured tissue protects wounds from invading pathogens, more recent studies suggest that neutrophils inhibit the wound repair process, including that in skin²⁸ and corneal epithelium.^{25,29} The present study showed that neutrophil infiltration into the injured cornea was markedly decreased in the PKC $\alpha^{-/-}$ mice and is consistent with the finding of a study linking neutrophil infiltration to PKC α activation in the epidermis.^{30,31,35} Therefore, deficient neutrophil infiltration is one of several possible explanations for the accelerated corneal epithelial re-epithelialization in the present study.

PKC α -modulated events in the cornea appear to have greater effects on epithelial healing than PKC α deficiency has on lacrimal gland function. A reduced corneal epithelial healing rate would be expected based on the reduced tear production that was observed in these mice. In summary, PKC α has essential functions in regulating tear secretion by the LFU and the ocular surface response to desiccating stress and corneal epithelial trauma. The details of the mechanism should be further investigated.

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