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# Role of cPKC*α* and nPKC*ε* in EGF-Stimulated Goblet Cell Proliferation

**Marie A. Shatos**[1], **Robin R. Hodges**[1], **Yoshia Oshi**[1], **Jeffrey A. Bair**[1], **Driss Zoukhri**[2], **Claire Kublin**[2], **Kameran Lashkari**[1], and **Darlene A. Dartt**[1]

1 *Schepens Eye Research Institute and Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts*

2 *Tufts University School of Dental Medicine, Boston, Massachusetts*

## Abstract

**Purpose—**The authors determined the role of the protein kinase C (PKC) isoforms cPKC*α* and nPKC*ε* in EGF-stimulated proliferation of cultured rat and human conjunctival goblet cells.

**Methods—**Rat and human conjunctivas were minced, and goblet cells were allowed to grow. Passage 1 cells were serum starved for 24 to 48 hours and were incubated with the PKC inhibitors calphostin C and Gö 6983 ($10^{-10}$-$10^{-7}$ M) for 20 minutes before stimulation with EGF ($10^{-7}$ M) for 24 hours. The presence and localization of PKC isoforms in cultured rat goblet cells were determined by Western blot analysis and immunofluorescence microscopy, respectively. Cultured rat goblet cells were serum starved and incubated with adenoviruses containing genes for dominant-negative cPKC*α*(Ad DNPKC*α*, $10^4$ pfu), dominant-negative nPKC*ε*(Ad DNPKC*ε*, $10^4$ pfu), and wild-type cPKC*α*(Ad WTPKC*α*, $10^7$ pfu), and proliferation was measured.

**Results—**In rat goblet cells, EGF-stimulated proliferation was completely inhibited by calphostin C, whereas Gö 6983 inhibited proliferation by 53% ± 15%. In human goblet cells, EGF-stimulated proliferation was completely inhibited by calphostin C. PKC*α*, -*β*I, -*β*II, -*δ*, -*ε*, -*ι*/*λ*, -*θ*, -*γ*, and -*ζ* were found in cultured rat goblet cells. Ad DNPKC*α* and Ad DNPKC*ε* inhibited EGF-stimulated proliferation in rat goblet cells by 78% ± 6% and 92% ± 8%, respectively. Incubation with Ad WTPKC*α* alone significantly increased proliferation.

**Conclusions—**cPKC*α* and nPKC*ε* play key roles in conjunctival goblet cell proliferation.

The protein kinase C (PKC) superfamily of lipid-regulated serine/threonine protein kinases includes 10 different isoforms.[1] Specific isoforms play critical roles in the signal transduction pathways that regulate cell proliferation, transformation, differentiation, and secretion. The PKC isoforms can be divided into three classes based on structure and cofactor requirements. Classical or conventional PKCs (cPKC*α*, -*β*I, -*β*II, and -*γ*) are activated by $Ca^{2+}$ and diacylglycerol. Novel PKCs (nPKC*δ*, -*ε*, -*η*, and -*θ*) are activated by diacylglycerol but not $Ca^{2+}$. Atypical PKCs (aPKC*ζ* and -*ι*/*λ*) are unresponsive to diacylglycerol and $Ca^{2+}$.

It is well established that PKC isoforms play important roles in cell proliferation[2–5] and are cell type specific.[6] Overexpression of PKC*α* has been shown to increase proliferation in thymocytes, MCF-7 cells, and U87 cells.[7] In contrast, in intestinal, pancreatic, and mammary cells, PKC*α* has been shown to have an antiproliferative effects.[6] nPKC*ε* increases the

Corresponding author: Darlene A. Dartt, Schepens Eye Research Institute, 20 Staniford Street, Boston, MA 02114; E-mail: darlene.dartt@schepens.harvard.edu.

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proliferation of lactotrophs through ERK1/2[8] and promotes survival in lung cancer cells.[6] Indeed, nPKC$\varepsilon$ has been shown to be an oncogene.[4]

The conjunctiva is an epithelium that surrounds the cornea and lines the eyelids. We previously showed that conjunctival goblet cells in vivo contained at least seven PKC isoforms.[9] Cholinergic agonists, which are known to activate PKC, stimulate mucin secretion, as do phorbol esters.[10] In this tissue, the phorbol ester PMA activates the nonreceptor tyrosine kinases Pyk2 and Src to phosphorylate the EGF receptor, which then activates ERK1/2 to cause secretion.[10]

Large oligomeric mucins, such as MUC5AC and MUC5B (two gel-forming mucins), are produced in the airway, gastrointestinal tract, and ocular surface and protect these wet-surfaced mucosa from the external environment.[11,12] Gel-forming mucins are synthesized and secreted by goblet cells located in the wet-surfaced epithelia in response to stimuli from the extracellular environment. The amount of mucin produced by the goblet cells is dependent on the number of cells present (proliferation or differentiation), the amount of mucin synthesized and stored in the secretory granules (synthesis), and the release of mucin from the secretory granules (secretion). Each tissue has its own unique response, leading to an increase or a decrease in mucin production.

Goblet cells in the conjunctiva are responsible for production of the large soluble mucin MUC5AC, the major soluble mucin of the tear film.[13] Ocular mucin is increased in allergy and inflammation but decreased in diseases of impaired corneal sensitivity such as herpes keratitis and anesthetic cornea.[14,15] Thus, the amount of ocular surface mucin is highly regulated. In the conjunctiva, the regulation of goblet cell mucin synthesis has not been investigated. It is known that the parasympathetic neuro-transmitters acetylcholine, vasoactive intestinal peptide and the nerve growth factor (NGF) and its family member brain-derived nerve factor (BDNF) induce goblet cell secretion.[16,17] It is also known that the epidermal growth factor (EGF) family of growth factors stimulates conjunctival goblet cell proliferation when measured in primary cell culture.[18,19]

EGF and its family members TGF$\alpha$ and HB-EGF are stimuli of conjunctival goblet cell proliferation.[10] EGF activates the EGF receptor and stimulates the ERK1/2 pathway, translocating ERK1/2 to the nucleus and causing proliferation in rat and human goblet cells.[10] In the present study, we investigated the role of PKC isoforms in EGF stimulation of goblet cell proliferation and found that EGF activation of cPKC$\alpha$ and nPKC$\varepsilon$ induces cell proliferation.

## Materials and Methods

Human EGF was purchased from PeproTech (Rocky Hill, NJ). Calphostin C and Gö 6983 were from EMD (Madison, WI). Cell proliferation reagent WST-8 came from Dojindo Molecular Technologies (Gaithersburg, MD). RPMI 1640 media, L-glutamine, penicillin-streptomycin, and trypsin-EDTA solution were from Lonza (Walkersville, MD); fetal bovine serum was from Hyclone Laboratories (Logan, UT).

### Antibodies

Antibodies specific to the individual anti-rabbit PKC isoforms and horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibody used for immunofluorescence microscopy was Cy3 conjugated to rabbit IgG and was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). The secondary antibody for Western blot analysis was anti-rabbit IgG conjugated to HRP and was purchased from Millipore (Billerica,

MA). A monoclonal antibody against $\beta$-actin was purchased from Sigma Chemical (St. Louis, MO).

## Animals

Male Sprague-Dawley rats weighing between 125 and 150 g were obtained from Taconic Farms (Germantown, NY). Rats were anesthetized with $CO_2$ for 1 minute and decapitated, and the nictitating membranes and fornix were removed from both eyes and minced. The procedure for removal of the conjunctiva was performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the Schepens Eye Research Institute Animal Care and Use Committee.

## Isolation and Culture of Goblet Cells

Human conjunctival tissue was obtained from patients during ocular surgery according to a protocol that adhered to the tenets of the Declaration of Helsinki. The protocol was approved by the Massachusetts Eye and Ear Infirmary and Schepens Eye Research Institute Human Subjects Internal Review Boards. The tissue, which was normally discarded during surgery, was donated by seven patients (five men, two women; average age, 68 years) after informed consent. Diagnoses for the patients were retinal detachment (four patients), vitrectomy for age-related macular degeneration with subretinal hemorrhage (one patient), vitrectomy for aqueous misdirection (one patient), and vitrectomy for nonclearing vitreous hemorrhage (one patient). The tissue was immediately placed in Optisol-GS (Bausch & Lomb, Rochester, NY) for transportation to the laboratory. Before culture, the tissue was placed in 1× phosphate-buffered saline (PBS) consisting of 3× (300 $\mu$g/mL) penicillin-streptomycin.

Goblet cells from rat and human conjunctival pieces were grown in organ culture, as described previously.[20,21] Briefly, minced pieces of conjunctiva were placed in culture with RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 100 $\mu$g/mL penicillin-streptomycin. After cells were observed growing out from the plug, the plug was removed and goblet cells were allowed to grow. Cells were identified as goblet cells by the following characteristics: their morphology as visualized by light microscopy, their positive staining with the lectin *Ulex europaeus* agglutinin and antibody to cytokeratin 7 viewed by immunofluorescence microscopy, and their negative staining for the stratified squamous cell markers the lectin *Griffonia* (*Bandeiraea*) *simplicifolia* lectin I and antibody to cytokeratin 4. First-passage goblet cells were used in all experiments.

## Measurement of Goblet Cell Proliferation

Human or rat conjunctival goblet cells in primary culture were trypsinized, and 200 cells/well were seeded on 48-well culture plates. Cells were grown to subconfluence for 24 to 48 hours and were serum starved in RPMI with 0.35% bovine serum albumin (BSA) for 24 hours (rat) or 48 hours (human) before the start of the experiment. Cells were then pretreated for 20 minutes with no additions or with the inhibitors calphostin C or Gö 6983 at $10^{-10}$ to $10^{-7}$ M. EGF ($10^{-7}$ M) was then added for 24 hours. Either inhibitor was present for the entire 24-hour incubation time. Incubation was terminated by the removal of supernatant, and cell proliferation was determined by WST-8 assay, a colorimetric assay for the quantification of cell number and viability. This assay is based on the cleavage of the tetrazolium salt WST-8 by mitochondrial dehydrogenases in viable cells. Absorbance was read at 465 nm after 60-minute incubation at 37°C.

## Western Blot Analysis of PKC Isoforms

The presence of PKC isoforms in rat goblet cells was determined by Western blot analysis. Cultured goblet cells grown in RPMI 1640 and 10% FBS were removed from the culture dishes

by scraping and were homogenized in RIPA buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS and 1 mM EDTA) protease inhibitors (phenylmethylsulfonyl fluoride 0.6 mM, aprotinin 0.2 U/mL, and sodium orthovanadate 100 nM). Cells were sonicated and centrifuged at 2000*g* for 15 minutes at 4°C. Twenty micrograms of proteins in the supernatant were separated by SDS-PAGE on a 10% gel and transferred onto nitrocellulose membranes. The nitrocellulose membranes were blocked overnight at 4°C in 5% nonfat dried milk in buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween-20 (TBST) and then were incubated with primary antibody for 1 hour at room temperature at a dilution of 1:500 or overnight at 4°C, followed by incubation with HRP-conjugated secondary antibody at 1:5000. Immunoreactive bands were detected by the enhanced chemiluminescence method. Homogenized conjunctiva and brain served as the positive controls.

### Immunofluorescence Microscopy of PKC Isoforms

The cellular location of PKC isoforms was analyzed by immunofluorescence microscopy. Primary cultures of rat conjunctival goblet cells were trypsinized, seeded onto glass coverslips, and grown to subconfluence in RPMI medium supplemented with 10% FBS. Cells were fixed with ice-cold 4% paraformaldehyde. Fixed cells were blocked in 3% BSA and 0. 3% Triton X-100 for 1 hour before incubation for 1.5 hours at room temperature with primary antibody at a dilution of 1:100 and 1 hour at room temperature with secondary antibody at a dilution of 1:300. The same antibodies were used in Western blot analysis and immunofluorescence experiments. Coverslips were mounted on glass slides using PVA/DABCO mounting medium, and the cells were viewed under a microscope (Eclipse E80i; Nikon, Tokyo, Japan) with a microscope digital camera (SPOT; Diagnostic Instruments Inc., Sterling Heights, MI). Omission of primary antibody served as the negative control.

### Expression of Wild-Type and Dominant-Negative PKC Isoforms

Replication defective adenovirus (Ad) constructs expressing green fluorescent protein (Ad GFP), wild-type cPKC$\alpha$(Ad WT PKC$\alpha$), dominant-negative cPKC$\alpha$(Ad DN PKC$\alpha$), and dominant-negative nPKC$\varepsilon$(Ad DN PKC$\varepsilon$) were used. Virus constructs were a kind gift from George King (Joslin Diabetes Center, Boston, MA). First-passage goblet cells were serum starved for 24 hours and then incubated with Ad constructs at either $1 \times 10^7$ pfu (Ad GFP), $1 \times 10^7$ pfu (Ad WTPKC$\alpha$), or $1 \times 10^4$ pfu (Ad DN PKC$\alpha$ and Ad DN PKC$\varepsilon$) for 24 hours. The amount of PKC isoform synthesized was determined by Western blotting using an antibody for the appropriate PKC isoform. The amount of protein loaded was standardized with the use of an antibody for actin at a dilution of 1:1000. For measurement of proliferation, cells were serum starved for 24 hours before Ad was added. Proliferation was measured after 24 hours using WST-8, as described. Blots were scanned and analyzed by NIH ImageJ (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/).

### Data Presentation and Statistical Analysis

Data are expressed as the fold increase over the basal value, which was standardized to 1.0. Results are expressed as the mean ± SEM. Data were analyzed by Student's *t*-test. $P < 0.05$ was considered statistically significant.

## Results

### Effect of Inhibition of PKC on EGF-Stimulated Rat and Human Goblet Cell Proliferation

We previously showed that EGF at $10^{-7}$ M caused a significant increase in ERK activation and inhibition of ERK inhibited goblet cell proliferation.[19,22] To determine whether PKC is also involved in this process, rat goblet cells were preincubated with the PKC inhibitors

calphostin C and Gö 6983 at $10^{-10}$ to $10^{-7}$ M and were stimulated with EGF at $10^{-7}$ M. Calphostin C did not alter basal goblet cell proliferation (Fig. 1A). In the absence of inhibitor, EGF significantly stimulated proliferation 1.4 ± 0.1-fold above basal ($n$ = 7; Fig. 1A). Increasing concentrations of calphostin C inhibited EGF-stimulated proliferation in a concentration-dependent manner with a significant inhibition of 100% obtained at $10^{-8}$ M calphostin C. Calphostin C at $10^{-7}$ M, with or without EGF, decreased proliferation below basal levels, suggesting a toxic effect at this concentration. Gö 6983 increased basal levels of proliferation ($n$ = 5; Fig. 1B). EGF significantly stimulated proliferation 3.8 ± 0.6-fold above basal, which was decreased by Gö 6983 in a concentration-dependent manner, with maximum inhibition of 53% ± 15% obtained at $10^{-7}$ M.

The effect of calphostin C was tested on human conjunctival goblet cells. Calphostin C alone increased basal goblet cell proliferation with significant increases at $10^{-8}$ and $10^{-7}$ M ($n$ = 4; Fig. 2). EGF significantly stimulated proliferation 1.7 ± 0.2-fold above basal. Increasing concentrations of calphostin C blocked EGF-stimulated proliferation in a concentration-dependent manner, with 100% inhibition obtained at $10^{-6}$ M. In these cells calphostin C at $10^{-5}$ M, with or without EGF, decreased proliferation below basal levels.

Data from Figures 1 and 2 suggest that EGF activates PKC to stimulate conjunctival goblet cell proliferation in rats and humans.

### Identification of PKC Isoforms in Rat Conjunctival Goblet Cells

The presence of PKC isoforms in rat conjunctival goblet cells was determined by Western blot analysis and confirmed by fluorescence microscopy. With the use of Western blot analysis, we found PKC$\alpha$, -$\beta$I, -$\beta$II, -$\delta$, -$\varepsilon$, and -$\iota/\lambda$ in rat conjunctival goblet cells ($n$ = 3; Fig. 3). PKC$\gamma$, -$\theta$, and -$\zeta$ were not detected. Each PKC isoform identified was present as a major band at the appropriate molecular weight. For some isoforms, additional minor bands were detected. Each PKC isoform, including PKC$\gamma$, -$\theta$, and -$\zeta$, was also detected in the positive controls rat conjunctiva and brain. Given that we previously showed that PKC$\eta$ was not present in conjunctival goblet cells,[9] we did not determine whether it was present in cultured goblet cells. We also detected the closely related protein kinase D (formally known as PKC$\mu$; data not shown).

The cellular location of each PKC isoform was determined in cultured goblet cells. All PKC isoforms were detected in the goblet cells by immunofluorescence ($n$ = 3; Fig. 4). PKC$\alpha$, -$\beta$I, -$\varepsilon$, -$\gamma$, and -$\theta$ were detected in the cytoplasm. PKC$\beta$II, -$\delta$, -$\iota/\lambda$, and -$\zeta$ were found predominantly in the nuclei, with some expression in the cytoplasm. PKC$\gamma$, -$\theta$, and -$\zeta$ were detected by immunofluorescence microscopy but not by Western blot analysis. Thus, multiple PKC isoforms of the classical, novel, and atypical subtypes are present in conjunctival goblet cells.

### Effect of Inhibition of cPKC$\alpha$ and nPKC$\varepsilon$ on EGF-Stimulated Rat Conjunctival Goblet Cell Proliferation

Adenovirus-containing GFP was used to measure the effectiveness of adenovirus transduction of conjunctival goblet cells. Cells were incubated with Ad GFP ($1 \times 10^7$ pfu) for 24 hours. With the use of microscopy, we found that all cells expressed GFP and, hence, were transduced by adenovirus (Fig. 5).

To determine whether individual PKC isoforms were used to stimulate goblet cell proliferation, cPKC$\alpha$ or nPKC$\varepsilon$ activity was prevented in rat conjunctival goblet cells with Ad DN PKC$\alpha$ and Ad DN PKC$\varepsilon$. Through Western blot analysis, we found that these viruses caused a 4.0-fold and a 4.8-fold over-expression of cPKC$\alpha$ and nPKC$\varepsilon$, respectively ($n$ = 3; Fig. 6A). EGF ($10^{-7}$ M) stimulated goblet cell proliferation 3.2 ± 0.7-fold above basal ($n$ = 5; Fig. 6B).

Incubation of Ad DN PKC*α* and Ad DN PKC*ε*, alone or together, did not alter basal goblet cell proliferation (Fig. 6B). Expression of Ad DN PKC*α* and Ad DN PKC*ε* significantly blocked EGF-stimulated goblet cell proliferation by 78% ± 6% and 92% ± 8%, respectively. Transduction with Ad DN PKC*α* and Ad DN PKC*ε* together completely blocked EGF-stimulated goblet cell proliferation. These data show that EGF uses two PKC isoforms, cPKC*α* and nPKC*ε*, to stimulate rat conjunctival goblet cell proliferation.

### Effect of Overexpression of cPKC*α* on Rat Conjunctival Goblet Cell Proliferation

Adenovirus-expressing wild-type cPKC*α* was used to determine whether overexpression of this PKC isoform alone could stimulate goblet cell proliferation. With the use of Western blot analysis, we found that Ad WT PKC*α* was overexpressed by 14.0-fold (Fig. 7A). With fluorescence microscopy, the overexpressed Ad WT PKC*α* was located in the perinuclear area, similar to the location of cPKC*α* in nontransduced cells (Fig. 7B). In addition, cPKC*α* was present on filaments throughout the cytoplasm in cells treated with Ad WT PKC*α*. When cultured goblet cells were incubated for 24 hours with Ad WT PKC*α*, goblet cell proliferation was increased 2.1 ± 0.4-fold above basal, the same proliferative response as for EGF ($n = 5$; Fig. 7C). Thus, overexpression of cPKC*α* alone can stimulate rat conjunctival goblet cell proliferation.

## Discussion

The present study demonstrates that EGF stimulates conjunctival goblet cell proliferation by activating cPKC*α* and nPKC*ε*. EGF regulation of goblet cell proliferation has not been studied in goblet cells from tissue other than the conjunctiva, though Nadel et al.[23] showed that EGF can stimulate airway goblet cell MUC5AC synthesis. In tumor cells, nPKC*ε* is thought to be proliferative and to induce cell survival, whereas cPKC*α* can be antiproliferative and can cause apoptosis or proliferation, depending on the cell type.[6] In contrast to cancer cells and similar to goblet cells, in corneal endothelial cells, serum stimulation of cell proliferation uses cPKC*α* and nPKC*ε* to stimulate proliferation.[24] It has been shown that EGF induces the translocation of cPKC*α* and the induction of cell proliferation in corneal epithelial cells.[25] In conjunctival goblet cells, cPKC*α* and nPKC*ε* play major roles in the stimulation of proliferation.

In this study, we showed that cultured conjunctival goblet cells contain multiple PKC isoforms in addition to cPKC*α* and nPKC*ε*. The roles of the other PKC isoforms in cell proliferation were not directly determined. Interestingly, inhibition of EGF-stimulated proliferation with the PKC inhibitors Gö 6983 in rat goblet cells and calphostin C in human goblet cells increased basal proliferation. It is possible that additional PKC isoforms play a negative role in proliferation (i.e., they inhibit proliferation by acting as a mechanism to stop proliferation). Removing this inhibitory pathway would increase proliferation. Therefore, it is possible that multiple PKC isoforms play a role, positive or negative, in goblet cell proliferation.

cPKC*α* is a classical isoform and is activated by $Ca^{2+}$ and diacylglycerol, whereas nPKC*ε* is a novel isoform and is activated by diacylglycerol only. Inhibition of either cPKC*α* or nPKC*ε* using dominant-negative adenovirus blocked EGF-stimulated proliferation. Inhibition of both PKC isoforms caused even greater inhibition than inhibition of each of the PKC isoforms alone. These results imply that cPKC*α* and nPKC*ε* have separate or incompletely overlapping mechanisms of action. In conjunctival goblet cells, cPKC*α* and nPKC*ε* had similar subcellular localizations. In goblet cells within the intact conjunctiva, these two isoforms were also present in similar locations.[9] However, nPKC*ε*, but not cPKC*α*, is known to have an actin-binding domain.[26] Actin polymerization is well known to play a role in cell proliferation because depolymerization causes cell cycle arrest in the G1 phase.[27] Thus, the activation of nPKC*ε*, but not cPKC*α*, could stimulate cell proliferation by inducing actin polymerization in

a $Ca^{2+}$-independent manner. Differential activators of the two PKC isoforms plus differential actin binding could provide the basis for the distinct pathways activated by the two PKC isoforms.

Cultured rat conjunctival goblet cells provide an excellent model for studying human goblet cell function. In the present study, EGF-stimulated cell proliferation was inhibited by the PKC inhibitor calphostin C in human and rat goblet cells, suggesting that EGF activates PKC isoforms to cause proliferation in both species. In related studies, EGF activated the EGF receptor, which, in turn, stimulated ERK1/2 activity to induce proliferation in goblet cells from both species.[22] In human and rat cultured conjunctival goblet cells, cholinergic agonists and EGF activated MAPK with a similar time dependence. This activation was receptor mediated, and cholinergic agonists transactivated the EGF receptor.[22] In the present study, the concentration dependence of calphostin C in inhibiting EGF-stimulated proliferation varied between rat and human goblet cells. Human goblet cells were 100-fold less sensitive than rat goblet cells to the concentration of calphostin C, which caused 100% inhibition. Furthermore, calphostin C at $10^{-7}$ M appeared to be toxic to rat, but not human, goblet cells. This suggests that human goblet cells are more robust than rat goblet cells. Additional evidence for this is that human goblet cells require 48-hour serum starvation to reduce proliferation to minimal levels, but only 24 hours are required for rat goblet cells. In spite of these minor differences, cultured rat conjunctival goblet cells can be used as a model to study human conjunctival goblet cells because all the signaling pathways studied to date are present in human and rat goblet cells.

In conclusion, cPKC$\alpha$ and nPKC$\varepsilon$ play pivotal roles in conjunctival goblet cell proliferation because overexpression of PKC$\alpha$ and -$\varepsilon$ isoforms increased goblet cell proliferation, and inhibition of these isoforms inhibited EGF-stimulated proliferation.

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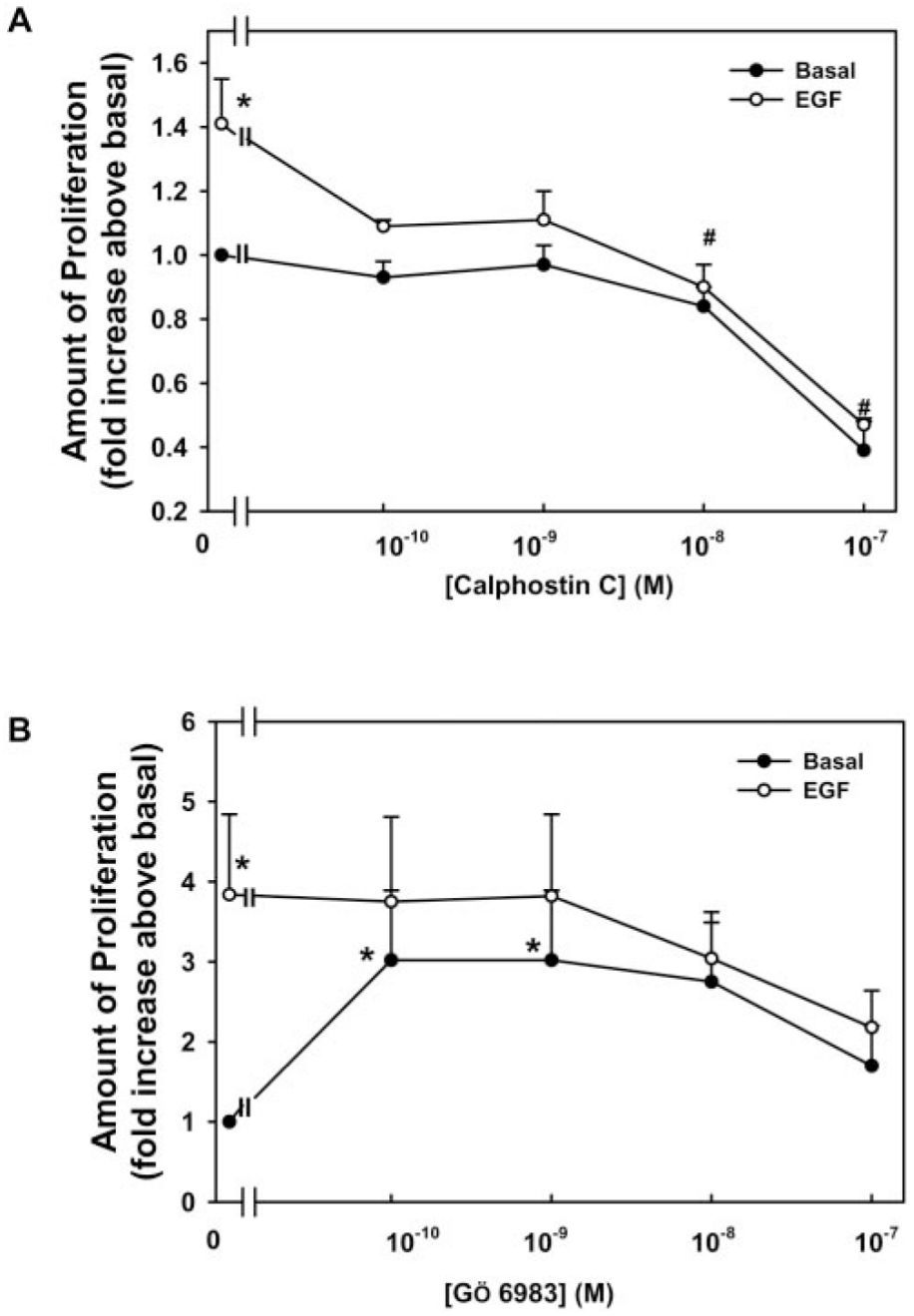


**Figure 1.**

Inhibition of PKC blocks EGF-stimulated cultured rat goblet cell proliferation. Cultured rat goblet cells were serum starved for 24 hours before the addition of the PKC inhibitors calphostin C (**A**) or Gö 6983 (**B**) at the indicated concentrations for 20 minutes and before the addition of EGF ($10^{-7}$ M) for 24 hours. Goblet cell proliferation was determined using WST-8. Data are mean ± SEM from either seven (calphostin C) or five (Gö 6983) independent experiments. *Significant difference from basal; #significant difference from EGF.

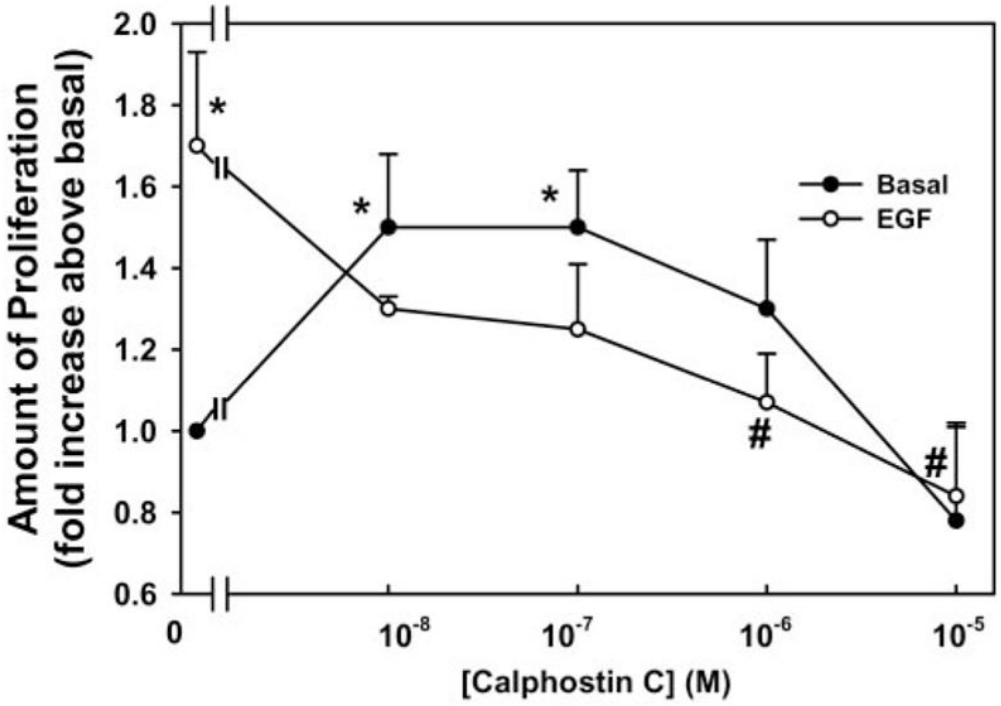


**Figure 2.**

Inhibition of PKC blocks EGF-stimulated cultured human goblet cell proliferation. Cultured human goblet cells were serum starved for 48 hours before the addition of the PKC inhibitor calphostin C at the indicated concentrations for 20 minutes and before the addition of EGF ($10^{-7}$ M) for 24 hours Goblet cell proliferation was determined using WST-8. Data are mean ± SEM from four independent experiments. *Significant difference from basal; #significant difference from EGF.

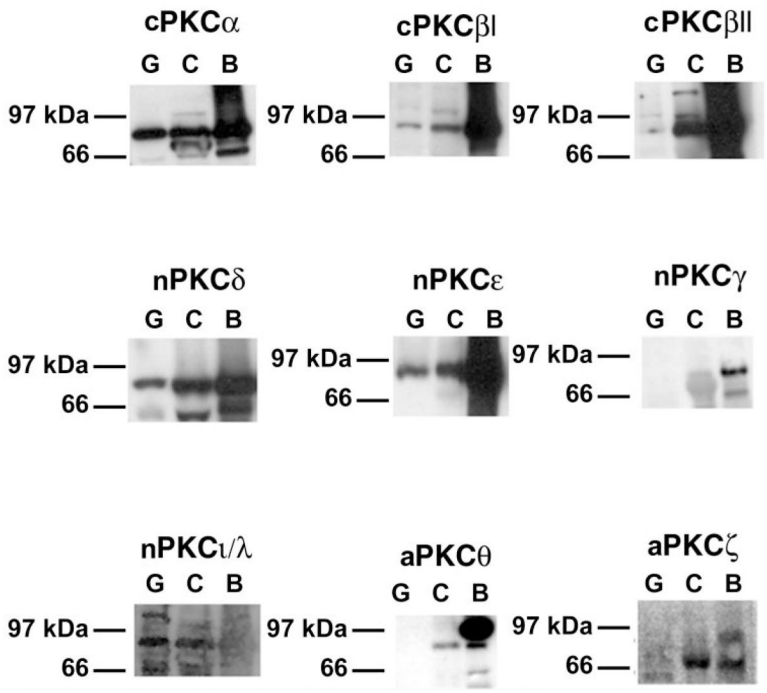


**FIGURE 3.**

Presence of PKC iso-forms in cultured rat goblet cells. Cultured rat goblet cells (G), rat conjunctiva (C), and rat brain (B) were homogenized. Proteins were separated by SDS-PAGE, and Western blot analysis was performed with antibodies specific to the PKC isoforms. Blots are representative of three independent experiments.

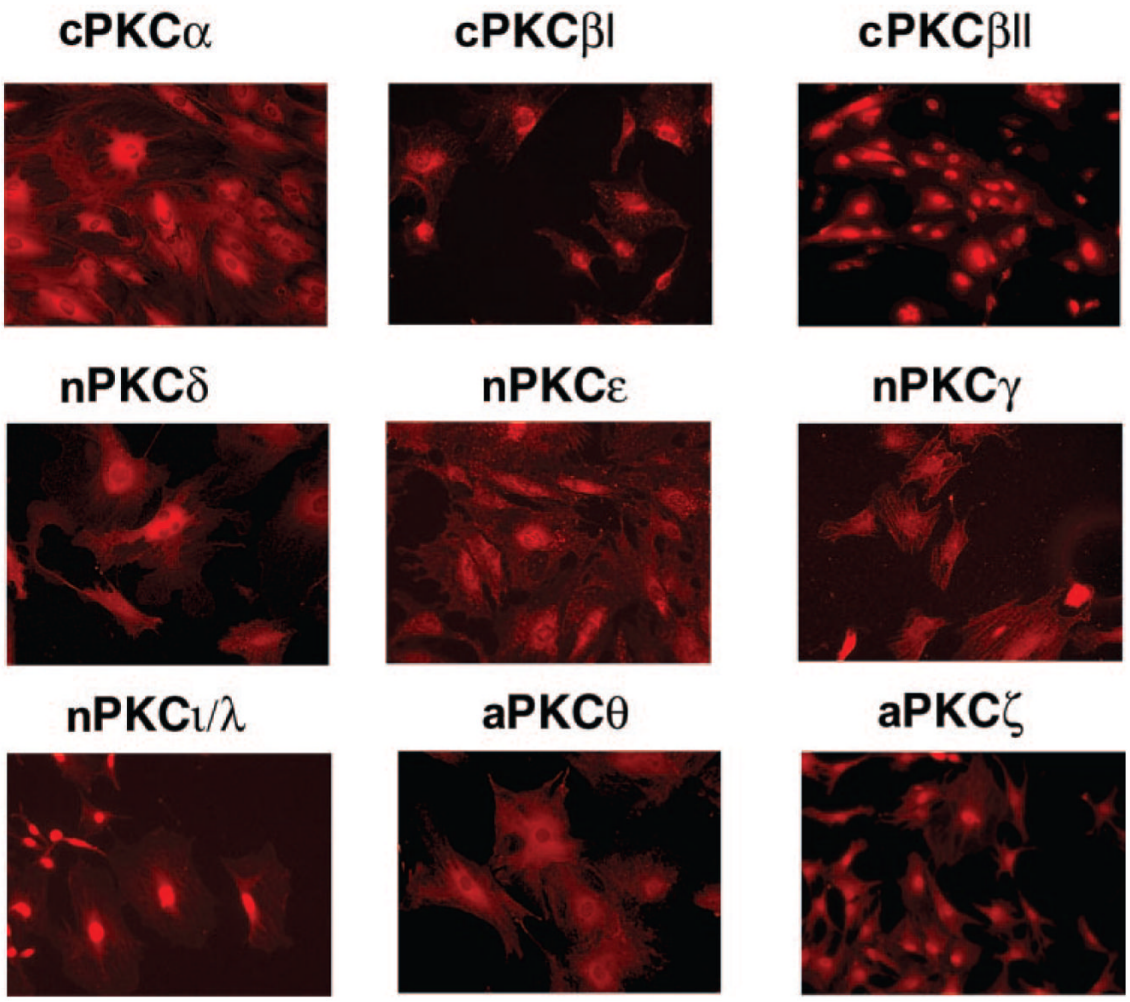


**Figure 4.**
Localization of PKC isoforms in cultured rat goblet cells. Cultured rat goblet cells were grown on glass coverslips, and immunofluorescence was performed with antibodies specific to the PKC isoforms. Images are representative of three independent experiments. Original magnification, 200×.

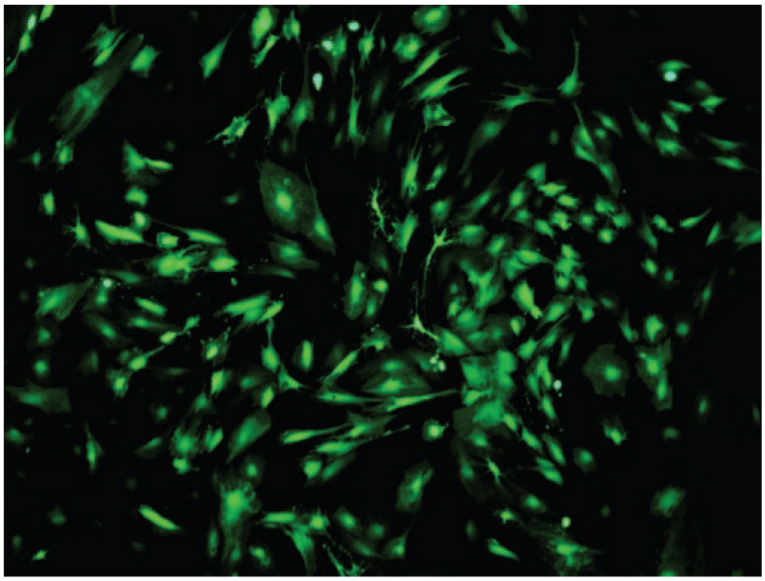

**Figure 5.**

Cultured rat goblet cells can be transduced by adenovirus containing green fluorescent protein. Cultured rat goblet cells grown on glass coverslips and serum starved for 24 hours before incubation with adenovirus containing the gene for green fluorescent protein ($1 \times 10^7$ pfu) for 24 hours. Cells were viewed by fluorescence microscopy. Original magnification, 200×.

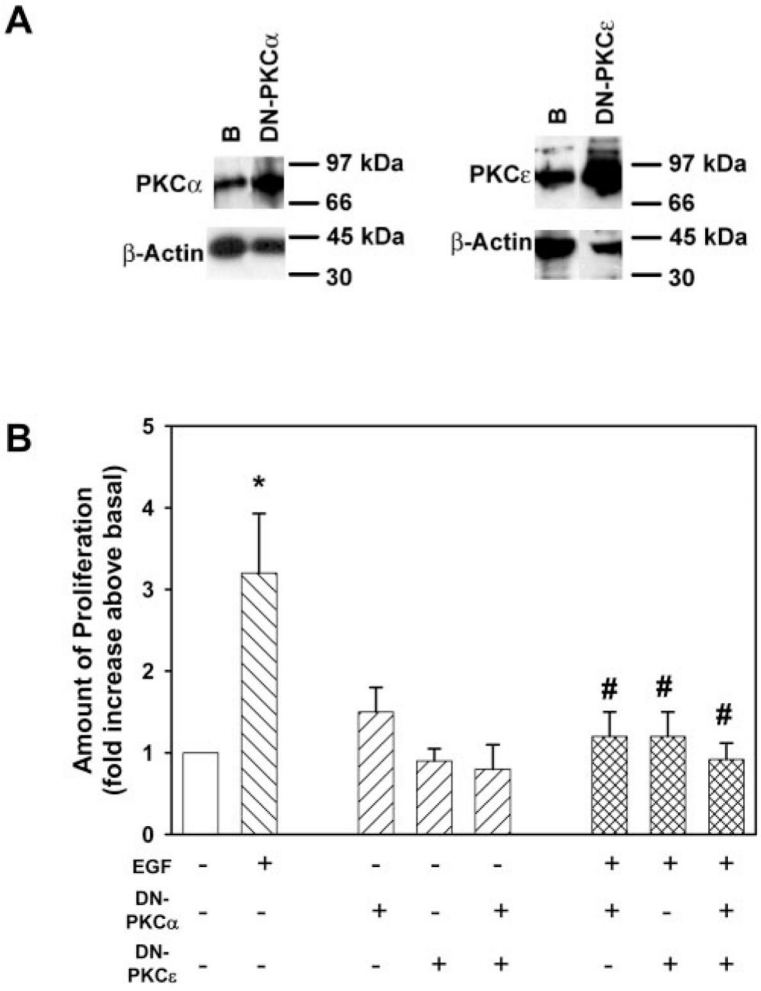


**Figure 6.**

Expression of dominant-negative cPKC*α* and nPKC*ε* inhibit EGF-stimulated cultured rat goblet cell proliferation. Cultured rat goblet cells were serum starved for 24 hours before incubation with EGF ($10^{-7}$ M) or adenovirus containing either the gene for dominant-negative cPKC*α*(DN-PKC*α*) or nPKC*ε*(DN-PKC*ε*; $1 \times 10^4$ pfu) for 24 hours. Western blot analysis was performed and shown (**A**) to ensure that transduction had occurred. Western blot analysis was performed for the presence of *β*-actin to indicate the total amount of protein in each sample. Blots are representative of three independent experiments. Goblet cell proliferation was measured by WST8 (**B**). Data are mean ± SEM of five independent experiments. *Open bar*: untreated cells; *left hatched bars*: cells treated with EGF; *right hatched bars*: cells treated with virus; *crosshatched bars*: cells treated with both. *Significant difference from basal; #significant difference from EGF.

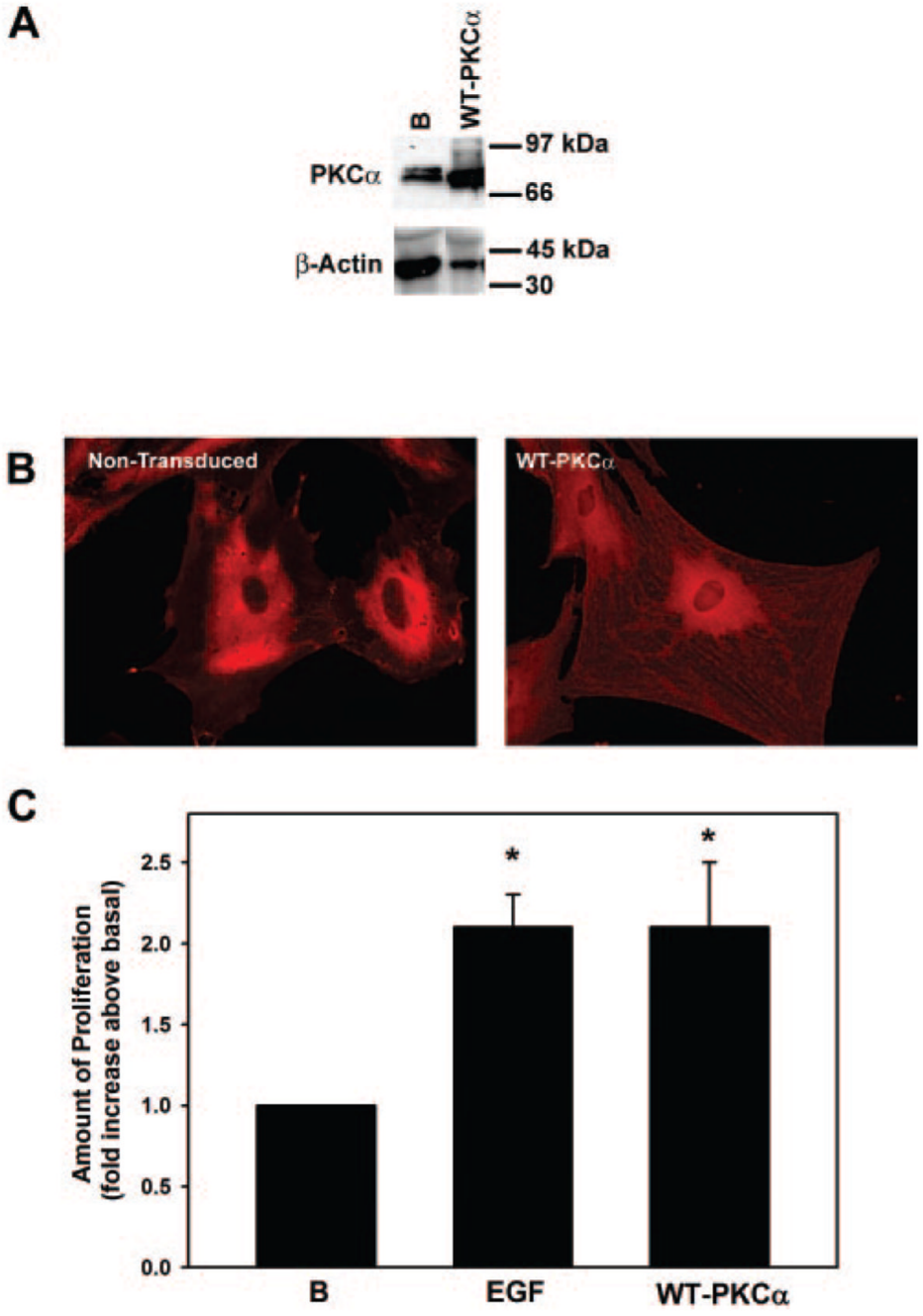


**Figure 7.**

Expression of wild-type PKC*α* stimulates cultured rat goblet cell proliferation. Cultured rat goblet cells were serum starved for 24 hours before incubation with adenovirus containing the gene for wild-type PKC*α* (WT-PKC*α*; $1 \times 10^7$ pfu) for 24 hours Western blot analysis was performed to ensure that transduction had occurred. (**A**) Blots are representative of two independent experiments. Western blot analysis was performed for the presence of *β*-actin to indicate the total amount of protein in each sample. (**B**) Two immunofluorescence micrographs of goblet cells grown on glass coverslips and incubated with antibody specific to cPKC*α*(*red staining*). Magnification, 200×. (**C**) Goblet cell proliferation was measured by WST8. Data

are mean ± SEM of five independent experiments. *Significant difference from basal; #significant difference from EGF.