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Stimulatory Role of PKC α in Extracellular Regulated Kinase 1/2 Pathway in Conjunctival Goblet Cell Proliferation

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Abstract

Purpose—To determine whether a constitutively active protein kinase C (PKC)- α stimulates rat and human conjunctival goblet cell proliferation through activation of ERK 1/2.

Methods—Conjunctivas from rat and human were minced and goblet cells were allowed to grow. Goblet cells were serum starved and incubated with an adenovirus containing a constitutively active form of PKC α (Ad-myr-PKC α , 1×10^7 pfu), EGF (10^{-7} M), or both. The location of myrPKC α was determined by immunofluorescence microscopy. Cultured goblet cells were preincubated with the PKC inhibitor calphostin C (10^{-10} – 10^{-7} M) or the ERK 1/2 inhibitor U0126 (10^{-9} – 10^{-6} M) before incubation with Ad-myr-PKC α . Cell proliferation was measured.

Results—Transduction of rat goblet cells with Ad-myr-PKC α did not change PKC location compared with nontransduced cells. Incubation with Ad-myr-PKC α caused an increase in cell proliferation by 2.5 ± 0.3 -fold, whereas EGF increased proliferation by 2.1 ± 0.2 -fold. Simultaneous addition of Ad-myr-PKC α and EGF did not further increase proliferation. U0126 inhibited Ad-myr-PKC α -stimulated proliferation a maximum of 70%. In human goblet cells, incubation with Ad-myr-PKC α caused an increase in cell proliferation by 2.3 ± 0.3 -fold, whereas EGF increased proliferation by 3.1 ± 0.4 -fold. Simultaneous addition of Ad-myr-PKC α and EGF decreased proliferation compared with either compound alone. Ad-myr-PKC α caused ERK 1/2 to translocate to the nucleus in rat and human cells, but the translocation was blocked by U0126.

Conclusions—Activation of PKC α alone by inducing phosphorylation of ERK 1/2 and translocating it to the nucleus is necessary and sufficient to cause conjunctival cell proliferation in rat, and probably human, goblet cells.

Large gel-forming mucins including MUC5AC, -5B, and -2 secreted by goblet cells protect the airways, gastrointestinal tract (GI), and ocular surface from the external environment. The quantity of mucin production is tightly regulated in each of these tissues as mucin overproduction and/or underproduction can cause disease. In the airways, mucin overproduction from an increase in goblet cell number and increase in mucin synthesis occurs in chronic obstructive pulmonary disease, asthma, and bacterial infection.¹ Alterations in the cell number and mucin content occur in the GI tract in Crohn's disease, ulcerative colitis, and colonic neoplasia.^{2–4} On the ocular surface, both a decrease and an increase in conjunctival goblet cell mucin lead to disease. Conjunctival goblet cell mucin is decreased in dry eye disease, herpes keratitis, and anesthetic cornea and increased in allergy, atrophy, and vernal conjunctivitis.^{5,6}

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The amount of mucin secretion in the airways, GI tract, and ocular surface is regulated by controlling the rate of mucin synthesis, the rate of mucin secretion, and the number of goblet cells (cell proliferation). These three processes are differentially controlled in these tissues with allergic, fungal, or viral inflammation causing goblet cell differentiation and mucin synthesis in the airways,¹ enteric nerves, enteroendocrine cells, and resident immune cells stimulating colonic mucin secretion,⁷ and nerves and growth factors stimulating conjunctival mucin secretion and number of goblet cells.^{8–11} One regulatory compound common to many of these processes is EGF. It stimulates goblet cell mucin synthesis and hyperplasia in airway goblet cells,¹² regulates goblet cell differentiation as the cells move from the base to the tops of colonic crypts,¹³ and induces growth in a goblet cell line from human colorectal adenocarcinoma.¹⁴ It also plays a pivotal role in conjunctival mucin production by inducing goblet cell mucin secretion and goblet cell proliferation.^{8,10}

EGF is a critical growth factor for epithelial cells. Its over-expression plays a role in many cancers as well as normal development. EGF can activate multiple signaling pathways including: (1) phospholipase $C\beta$, which increases the intracellular $[Ca^{2+}]$ and activates protein kinase C (PKC); (2) the nonreceptor tyrosine kinases Grb2, Shc, and Sos, which ultimately activate extracellular signal-regulated kinase 1/2 (ERK 1/2 also known as p44/p42 mitogen-activated protein kinase [MAPK]); (3) phosphoinositide-3 kinase and protein kinase B (AKT); (4) p38 MAPK; and (5) c-Jun NH-(2) terminal kinase (JNK).^{15,16}

Not only can EGF activate each of these pathways individually, but these pathways can themselves interact. In particular, activated PKC isoforms can stimulate ERK1/2.¹⁷ The PKC superfamily of lipid regulated serine/threonine kinases includes 10 different isoforms. Specific isoforms play critical roles in the signal transduction pathways that regulate cell proliferation, transformation, differentiation, and secretion.¹⁸ The location, activation, and function of each isoform are tissue specific. The PKC isoforms can be divided into three classes based on structure and cofactor. Classic or conventional PKCs ($cPKC\alpha$, $-\beta_1$, $-\beta_2$, and $-\gamma$) are activated by Ca^{2+} and diacylglycerol. Novel PKCs ($nPKC\delta$, $-\epsilon$, $-\eta$, and $-\theta$) are activated by diacylglycerol, but not Ca^{2+} . Atypical PKCs ($aPKC\zeta$ and $-\iota/\lambda$) are unresponsive to both diacylglycerol and Ca^{2+} . Using Cos-7 cells transfected with Raf, MEK, and p42 MAPK, Schönwasser et al.¹⁷ found that all PKC isoforms activated MEK and p42 MAPK, with classic and novel, but not atypical, PKC isoforms activating Raf. However, the actions of PKC isoforms are cell-type and agonist specific. In lung fibroblasts, PKC ϵ activates ERK, but PKC α inhibits it.¹⁹

In conjunctival goblet cells EGF induces proliferation by activating both ERK1/2 and PKC α and $-\epsilon$.²⁰ In the present study we used constitutively active PKC α (myrPKC α) to determine whether PKC α , which stimulates proliferation by itself, activates ERK1/2 to induce goblet cell proliferation. In both human and rat conjunctival goblet cells myrPKC α phosphorylated ERK1/2 and translocated it to the nucleus, thereby stimulating proliferation.

Materials and Methods

EGF was purchased from PeproTech, Inc. (Rocky Hill, NJ), calphostin C and U0126 from EMD Chemicals (Madison, WI), and the cell proliferation reagent WST-8 from Dojindo Molecular Technologies (Gaithersburg, MD).

Antibodies

Antibody to β -actin was obtained from Sigma-Aldrich (St. Louis, MO). Mouse monoclonal antibodies, used for Western blot experiments, were generated against ERK1 and phosphorylated ERK1 and -2 (pERK, which specifically reacts with Tyr-204 of phosphorylated ERK1/2). They and an antibody to PKC α were from Santa Cruz Biotechnology (Santa Cruz,

CA). A rabbit polyclonal antibody against phosphorylated ERK 1/2 at Thr-202 and Tyr-204 was purchased from Cell Signaling Technology, Inc. (Danvers, MA) and used for immunofluorescence experiments. The secondary antibodies used for immunofluorescence microscopy were Cy2 or -3 conjugated to mouse or rabbit IgG from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Horse-radish peroxidase (HRP)-conjugated secondary antibodies for Western blot analysis were from Santa Cruz Biotechnology.

Animals

Male Sprague-Dawley rats weighing between 125 and 150 g were obtained from Taconic Farms (Germantown, NY). The rats were anesthetized with CO₂ 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 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 in a protocol that adhered to the tenets of the Declaration of Helsinki. The protocol was approved by both 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 and two women, age range, 35–86 years; average age, 68 years) and was removed after informed consent was obtained. The diagnoses for the patients were retinal detachment (four patients), vitrectomy for age-related macular degeneration with subretinal hemorrhage, vitrectomy for aqueous misdirection, and vitrectomy for nonclearing vitreous hemorrhage. The tissue was immediately placed in 1× phosphate buffered saline (PBS) consisting of 3× (300 µg/mL) penicillin-streptomycin.

Goblet cells from rat and human conjunctiva were grown in organ culture, as described previously.^{8,10,11,21} Pieces of minced conjunctiva were placed culture with RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 100 µg/mL penicillin-streptomycin. The tissue plug was removed after nodules of cells were observed. As described previously, cells were identified as goblet cells by the following characteristics: (1) morphology, as visualized by light microscopy; (2) positive staining with the lectin *Ulex europaeus* agglutinin (UEA-1) and antibody to cytokeratin 7 viewed by immunofluorescence microscopy; and (3) negative staining for the stratified squamous cell markers the lectin *Griffonia (Bandeiraea) simplicifolia* Lectin I and antibody to cytokeratin 4 viewed by immunofluorescence microscopy.^{8,11,21} First-passage goblet cells were used in all experiments.

Expression of Constitutively Active PKCα

An adenovirus expressing PKCα with a myristic acid (Ad-myrPKCα) was used as described previously.²² The myristic acid moiety of the PKCα construct targets it to cellular membranes where it is constitutively active. First-passage goblet cells were incubated with Ad constructs for 24 hours at 1 × 10⁷ pfu.

Western Blot Analysis

The amount of PKCα expressed by cells transduced by Ad-myrPKC was determined by Western blot analysis. Cultured goblet cells were serum starved for 24 hours in RPMI with 0.35% BSA. The cells were scraped and homogenized in RIPA buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, and 1 mM EDTA containing protease inhibitors (phenylmethylsulfonyl fluoride 100 µL/mL, aprotinin 30

$\mu\text{L/mL}$, and sodium orthovanadate 100 nM). Homogenized cells were sonicated and centrifuged at 2000g for 15 minutes at 4°C. Proteins in the supernatant were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis 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, and then incubated with primary antibody for 1 hour at room temperature or overnight at 4°C at a dilution of 1:1000 in TBST followed by incubation with HRP-conjugated secondary antibody. Immunoreactive bands were detected by the enhanced chemiluminescence method. Antibody to β -actin (1:1000 in TBST) was used to control for the amount of cellular protein present in each well. Bands were scanned and analyzed using NIH Image J (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>).

Immunofluorescence Microscopy

The cellular location of pERK1/2 or PKC α 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 10% FBS. Cells were serum starved for 24 hours, Ad-myrPKC α or RPMI was added for an additional 24 hours, and EGF (10^{-7} M) was added for an additional 24 hours. The cells were fixed with ice-cold 4% paraformaldehyde. Fixed cells were incubated for 1.5 hours at room temperature with primary antibody (1:100 dilution in PBS) and 1 hour at room temperature with secondary antibody. Coverslips were mounted on glass slides using PVA mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; DABCO; Sigma-Aldrich), which stains all nuclei. The cells were viewed with a microscope equipped with a digital camera (Eclipse E80i; Nikon, Tokyo, Japan; SPOT camera, Diagnostic Instruments Inc., Sterling Heights, MI). Omission of primary antibody served as the negative control. To quantify the number of cells containing pERK1/2, we counted the total number cells (as determined by DAPI staining) and the number of cells expressing pERK 1/2 in the nucleus. Five fields were counted in a masked fashion.

Measurement of Goblet Cell Proliferation

Human or rat conjunctival goblet cells in primary culture were trypsinized and seeded on 48-well culture plates at a density of 200 cells/well. Cells were grown for approximately 72 hours to subconfluence. After 24 (rat) or 48 (human) hours of serum starvation, the PKC inhibitor calphostin C (10^{-10} – 10^{-7} M), the ERK1/2 inhibitor U0126 (10^{-9} – 10^{-6} M), or nothing was added for 20 minutes followed by RPMI with 0.35% BSA (basal) or EGF (10^{-7} M) and Ad-myrPKC α (1×10^7 pfu), or an adenovirus containing a gene for GFP (Ad-GFP, 1×10^7 pfu) for 24 hours. 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 proliferation and viability. This assay is based on the cleavage of the tetrazolium salt WST-8 by mitochondrial dehydrogenases in viable cells. The absorbance was then read at 465 nm after a 45-minute incubation at 37°C.

Data Presentation and Statistics

Data are expressed as the multiple of change (x -fold increase) over the basal value, which was standardized to 1.0. Results are expressed as the mean $\pm$ SEM. Data were analyzed by Student's t -test. $P < 0.05$ was considered statistically significant.

Results

Stimulation of Rat Conjunctival Goblet Cell Proliferation by PKC α

We previously demonstrated that adenovirus expressing green fluorescent protein transfects virtually all cultured conjunctival goblet cells. In addition, incubation with an adenovirus containing the gene for a dominant negative PKC α significantly decreases EGF-stimulated proliferation, suggesting that EGF stimulates rat conjunctival goblet cell proliferation by activating PKC α .²⁰

To determine whether PKC α itself can stimulate goblet cell proliferation, cultured goblet cells were incubated with Ad-myr-PKC α , which produces constitutively active PKC α . When cultured cells were assayed by Western blot with an antibody to PKC α , a 24-hour incubation with Ad-myr-PKC α induced the expression of PKC α 3.3-fold compared with nontransfected cells (Fig. 1A). Goblet cells were viewed by immunofluorescence microscopy using an antibody to PKC α . In nontransduced cells PKC α immunoreactivity was detected surrounding the nucleus (Fig. 1B). In cells stimulated with EGF (10^{-7} M) for 24 hours, PKC α immunoreactivity was found predominately in the perinuclear area with some cytosolic staining (Fig. 1B). In cells transfected with Ad-myr-PKC α , PKC α immunoreactivity was similar to that found in cells stimulated with EGF (Fig. 1B). The effect of activated PKC α on proliferation was measured next. EGF (10^{-7} M) stimulated proliferation 2.1 ± 0.2 -fold compared with basal conditions (no additions; Fig. 1C). In cells transfected with Ad-myr-PKC α , goblet cell proliferation was increased 2.5 ± 0.3 -fold, similar to EGF. Addition of both EGF and Ad-myr-PKC α also increased goblet cell proliferation 2.0 ± 0.3 -fold. Incubation with the control virus, Av-GFP, did not increase proliferation (Fig. 1C). Thus, activation of PKC α alone can stimulate rat conjunctival goblet cell proliferation to the same extent as EGF.

To ensure that the effects on proliferation seen with Ad-myr-PKC α were specific to PKC, cultured goblet cells were preincubated with the PKC inhibitor calphostin C (10^{-10} – 10^{-7} M) for 20 minutes before addition of Ad-myr-PKC α for 24 hours. Proliferation was then measured. Calphostin C alone at any concentration did not alter basal goblet cell proliferation (Fig. 2). Ad-myr-PKC α significantly increased goblet cell proliferation 1.8 ± 0.2 -fold above basal (Fig. 2). Pretreatment of these cells with calphostin C significantly decreased Ad-myr-PKC α -stimulated proliferation at 10^{-9} – 10^{-7} M (Fig. 2).

These data indicate that the effect of Ad-myr-PKC α is a result of PKC and is not a nonspecific effect.

Induction of Rat Conjunctival Goblet Cell Proliferation by Constitutively Active PKC α Stimulation of ERK1/2

We previously found that EGF activates ERK1/2 to stimulate goblet cell proliferation.²¹ As PKC is known to activate ERK1/2, we investigated whether myr-PKC α activates ERK1/2. One hallmark of ERK1/2 activation is translocation of phosphorylated (activated) ERK1/2 to the cell nucleus. Cultured goblet cells were incubated with no additions, with Ad-myr-PKC α (1×10^7 pfu) or EGF (10^{-7} M) added for 24 hours. The location of pERK1/2 was determined by immunofluorescence. Under basal conditions pERK1/2 was detected in the cellular cytoplasm of the cultured goblet cells (Fig. 3A). EGF translocated pERK1/2 to the nuclei of most of the cells, although pERK1/2 was additionally detected in the cytoplasm. Myr-PKC α translocated pERK1/2 to the nuclei of the cultured cells as shown by the colocalization with the nuclear marker DAPI. The number of cells with nuclear pERK1/2 was quantified. Under basal conditions $4\% \pm 2\%$ of cells contained pERK1/2 in the nuclei (Fig. 3B). With EGF stimulation, $22\% \pm 5\%$ of cells contained pERK1/2 in the nuclei. After the cells were incubated with Ad-myr-PKC α , pERK1/2 was detected in the nuclei of $64\% \pm 15\%$ of the cells.

The activation of ERK1/2 was also explored by a second method, Western blot analysis using antibodies to pERK1/2 and total (active and inactive) ERK1/2 (Fig. 3C). The amount of pERK1/2 increased with the addition of EGF, Ad-myr-PKC α or the two compounds together.

To determine whether activated PKC α uses ERK1/2 to induce cell proliferation, we measured the effect of the MEK inhibitor U0126 on Ad-myr-PKC α -induced cell proliferation. A 24-hour incubation with Ad-myr-PKC α caused ERK1/2 to translocate to the nucleus (Fig. 4A). Preincubation with U0126 prevented this translocation (Fig. 4A). When five independent experiments were performed, Ad-myr-PKC α stimulated goblet cell proliferation 2.7 ± 0.6 -fold compared with that of non-transfected cells (Fig. 4B). Incubation of Ad-myr-PKC α with U0126 blocked proliferation, with a maximum inhibition of $70\% \pm 10\%$ at 10^{-6} M U0126. Addition of U0126 did not significantly alter basal proliferation.

These data suggest that activated PKC α stimulates rat conjunctival goblet cell proliferation by translocating pERK1/2 to the nuclei of cells.

Induction of Human Conjunctival Goblet Cell Proliferation by Constitutively Active PKC α Stimulation of ERK1/2 Activity

Finally, we investigated whether activated PKC α the same effect on proliferation in human compared with rat conjunctival goblet cells. Human goblet cells were incubated for 24 hours with or without Ad-myr-PKC α (1×10^7 pfu). As a positive control, EGF (10^{-7} M for 24 hours) stimulated cellular proliferation 3.1 ± 0.4 -fold compared with nontransfected cells (basal; Fig. 5). Incubation with Ad-myr-PKC α significantly stimulated goblet cell proliferation 2.3 ± 0.3 -fold. When EGF and Ad-myr-PKC α were used together, proliferation was increased 1.3 ± 0.3 -fold, significantly lower than proliferation in the presence of EGF and not significantly different from basal.

The location of pERK1/2 was determined by immunofluorescence. Under basal conditions pERK1/2 was detected in the cellular cytoplasm of cultured goblet cells (Fig. 6A). Similar to rat goblet cells, myr-PKC α (1×10^7 pfu for 24 hours) translocated pERK1/2 to the nuclei of the cultured cells (Fig. 6B). The translocation caused by myr-PKC α was inhibited by U0126 (10^{-6} M, Fig. 6C).

We conclude that activated PKC α stimulates human goblet conjunctival goblet cell proliferation by phosphorylating ERK1/2 and translocating it to the nucleus as in rat conjunctival goblet cells.

Discussion

EGF stimulates cell proliferation by activating multiple signaling pathways including the adaptor proteins PLC γ , which activates PKC and increases intracellular $[Ca^{2+}]$, and Shc and Grb2, which activate ERK1/2. The multiple signaling pathways activated by EGF can each induce proliferation or can interact with components of one pathway stimulating or inhibiting components of other pathways. In conjunctival goblet cells, EGF stimulates proliferation by activating the PLC γ and Shc/Grb2/ERK1/2 pathways.⁸ In preliminary experiments, we found that EGF activates PI-3K and Akt. Activation of other EGF-induced pathways has not been tested. In the present study, we found that activation of the PLC γ pathway and in fact of just 1 PKC isoform, PKC α , of the 10 present in goblet cells, can cause proliferation to the same extent as EGF. We additionally found that constitutive activation of PKC α activates ERK1/2 to cause proliferation. This suggests that PLC γ using PKC α and Shc/Grb2/ERK1/2 form a single pathway to cause goblet cell proliferation as the MEK inhibitor of ERK1/2 (U0126) completely blocked proliferation stimulated with active PKC α . The inhibition of proliferation by U0126 also indicates that PKC α is upstream of ERK1/2.

Constitutively active PKC α stimulates conjunctival goblet cell proliferation by activating ERK1/2. ERK1/2 is the terminal kinase in a cascade consisting of (1) phosphorylation of the EGF receptor; (2) tyrosine phosphorylation of the adapter proteins Shc and Grb2; (3) activation of the guanine nucleotide exchange factor SOS, which exchanges GDP for GTP; (4) activation of Ras and translocation to the cell membrane; (5) translocation of the kinase Raf-1 to the membrane where it is activated by phosphorylation on both tyrosine and serine residues (although additional mechanisms of Raf activation exist²³); (6) an increase in MEK1/2 activity by phosphorylating it on both tyrosine and threonine residues; and (7) stimulation of ERK1/2 by tyrosine and threonine residue phosphorylation.²⁰ In conjunctival goblet cells PKC α activated ERK1/2 by stimulating any step in this cascade. In Swiss 3T3 cells, transfection with dominant negative or constitutively active PKC isoforms demonstrated a unique role for the conventional PKC isoforms in phorbol ester-stimulated proliferation. Constitutively active conventional PKC isoforms, with PKC α used as the prototype, stimulated proliferation independent of phorbol esters and activated the ERK1/2 pathway at the level of cRaf1. Schönwasser et al.¹⁷ hypothesized that PKC α activates cRaf1 by targeting it to the membrane where it could be activated by Ras. Substantially similar effects were found by Cai et al.²⁴ Thus, we suspect that PKC α activates the ERK1/2 pathways in conjunctival goblet cells by stimulating Raf.

Schönwasser et al.¹⁷ found that stimulation of cells with constitutively active PKC α prevents the subsequent induction of proliferation by phorbol esters or serum, that is the response was downregulated. Similarly, we found that incubation of lacrimal gland acinar cells with constitutively active PKC α completely blocked cholinergic agonist-stimulated protein secretion, but did not alter stimulation with α_{1D} -adrenergic agonists.²² As cholinergic agonists, but not α_{1D} -adrenergic agonists, activate PKC α to stimulate lacrimal gland protein secretion, we concluded that PKC α was already activated so that cholinergic agonists could not further stimulate it—that is, PKC α was downregulated. This result is in agreement with those of the present study in which constitutively active PKC α stimulation inhibited, but not completely, EGF induction of proliferation of human goblet cells. This inhibition was not as extensive in rat goblet cells. Thus, EGF activates proliferation through a PKC α /ERK1/2 mechanism, but also could use an additional pathway, as the proliferation was not completely inhibited. One possible mechanism is through the novel PKC ϵ that is used by EGF along with PKC α to stimulate goblet cell proliferation.²⁰ We have evidence whereby a dominant negative PKC ϵ inhibits EGF-stimulated goblet cell proliferation by 92% in cultured rat goblet cells. A second possible mechanism is stimulation of PI-3K by EGF, in that EGF activates PI-3K in rat and human conjunctival goblet cells. Our laboratory is currently investigating whether this pathway is separate from the PKC α /ERK1/2 pathway. Finally, it is possible that, if myrPKC α had been added before EGF instead of simultaneously, the EGF response would be completely blocked.

Whether EGF stimulates cell proliferation depends on the strength and duration of EGF stimulation of ERK1/2. For stimulation of proliferation, EGF stimulates immediate early genes that activate transcription factors such as Fos, FosB, Fra-2, and Jun (part of the AP-1 transcription factor), which in turn activates late-response genes.²⁵ If immediate early gene production is stabilized and ERK is retained in the nucleus, proliferation occurs, but if immediate early gene products are degraded, proliferation is inhibited. Phosphorylated ERK1/2 must remain in the nucleus for a sufficient time to induce synthesis of immediate early gene products and to cause their stabilization. Since constitutively active PKC α stimulates conjunctival goblet cell proliferation to the same extent as EGF, we suggest that constitutively active PKC α activates and translocates ERK1/2 to the nucleus in a manner similar to EGF.

Select experiments were performed in human goblet cells cultured from conjunctival pieces removed at the time of retinal surgery. Although these cells were cultured from male and female patients 35 to 86 years of age (mean, 68 years), they responded similarly to goblet cell cultured

from young male rats. In human goblet cells, constitutively active PKC α stimulated proliferation to the same extent as EGF, phosphorylated ERK1/2, and translocated ERK1/2 to the nuclei. Inhibition of ERK1/2 completely blocked constitutively active PKC α -stimulated proliferation. Thus, cultured rat conjunctival goblet cells are an excellent model for the study of human conjunctival goblet cell function.

We conclude that activation of PKC α alone, by inducing phosphorylation of ERK1/2 and translocating it to the nucleus where it induces genes that initiate and sustain the cell cycle, is necessary and sufficient to cause rat, and probably human, goblet cell proliferation.

Acknowledgments

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Figure 1.

Effect of constitutively active PKC α on location and proliferation in cultured rat conjunctival goblet cells. Cultured rat conjunctival goblet cells were transduced with adenoviruses containing GFP (1×10^7), myristoylated (active) PKC α (myr-PKC α , 1×10^7 pfu), or with EGF (10^{-7} M) for 24 hours. Western blot analysis was performed with an antibody to PKC α and β -actin (**A**). The blot is representative of two independent experiments. The location of PKC α under basal conditions, incubated with EGF or transduced with Ad-myr-PKC α was determined by immunofluorescence microscopy (**B**). The amount of cell proliferation was also determined (**C**). Data are the mean $\pm$ SEM from two (GFP) or five (EGF and myr-PKC α) independent experiments. *Statistically significant difference from basal (B).

Figure 2.

Effect of inhibition of PKC on constitutively active PKC α -stimulated cultured rat conjunctival goblet cell proliferation. Cultured rat conjunctival goblet cells were pretreated for 20 minutes with the PKC inhibitor calphostin C (10^{-10} – 10^{-7} M) before stimulation with Ad-myr-PKC α (1×10^7 pfu) for 24 hours, and cell proliferation was determined. Data are the mean $\pm$ SEM of results in three independent experiments. Significant differences from *basal and #Myr-PKC α with no calphostin C.

Figure 3.

Effect of constitutively active PKC α on location of ERK 1/2 in cultured rat conjunctival goblet cells. The location of ERK 1/2 under basal conditions, incubated with EGF (10^{-7} M), or transduced with Ad-myr-PKC α (1×10^7 pfu) for 24 hours was determined by immunofluorescence microscopy (**A**). *Green*: pERK; *blue*: colocalization of pERK and DAPI. The total number of cells (as determined by the presence of the nuclear marker DAPI) and the number of cells expressing ERK 1/2 in the nucleus were counted (**B**). Data are the mean $\pm$ SEM of results in three independent experiments. *Significant difference from basal. The amount of pERK and total ERK was determined by Western blot analysis (**C**). The blot is representative of two independent experiments.

A **B** **Figure 4.**

Effect of inhibition of MEK on constitutively active PKC α -stimulated cultured rat conjunctival goblet cell proliferation. Cultured rat conjunctival goblet cells were pre-treated for 20 minutes with the MEK inhibitor U0126 (10^{-9} – 10^{-6} M) before stimulation with Ad-myr-PKC α (1×10^7 pfu) for 24 hours. The location of PKC α under basal conditions, transduced with Ad-myr-PKC α , or U0126 (10^{-6} M) and Ad-myr-PKC α was determined by immunofluorescence microscopy (A). Cell proliferation was determined (B). Data are the mean $\pm$ SEM of results in five independent experiments. *Significant difference from basal.

Figure 5.

Effect of constitutively active PKC α on proliferation of cultured human conjunctival goblet cells. Cultured human conjunctival goblet cells were stimulated with EGF (10^{-7} M), or transduced with Ad-myr-PKC α (1×10^7 pfu) for 24 hours, or both. The amount of cell proliferation was determined. Data are the mean $\pm$ SEM of results in three independent experiments. Significant difference from *basal (B) and #EGF alone and Ad-myr-PKC α alone.

Figure 6.

Effect of constitutively active PKC α on location of ERK 1/2 in cultured human conjunctival goblet cells. The location of ERK 1/2 in cultured human conjunctival goblet cells under basal conditions (A) or transduced with Ad-myr-PKC α (1×10^7 pfu) for 24 hours (B) or U0126 (10^{-6} M) plus Ad-myr-PKC α (C) was determined by immunofluorescence microscopy. Images are representative of two independent experiments.