Role of PKC ϵ in PGF $_{2\alpha}$ -Stimulated MMP-2 Secretion from Human Ciliary Muscle Cells

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ABSTRACT

Studies were designed to examine the roles of individual protein kinase C (PKC) isoforms in the prostaglandin F $_{2\alpha}$ (PGF $_{2\alpha}$)-induced matrix metalloproteinase-2 (MMP-2) secretion from human ciliary muscle cells.

Studies utilized primary cultures of human ciliary muscle cells. Individual PKC isoforms were detected by Western blotting, using PKC-isoform-specific antibodies. To evaluate MMP-2 secretion, cells were serum-starved overnight, treated with PGF $_{2\alpha}$ (1 μ mol/L) for 4 h and the media analyzed for MMP-2 by Western blotting. To assess ERK1/2 activation, cells were serum-starved overnight, treated with PGF $_{2\alpha}$ (1 μ mol/L) for 5 min and cell lysates analyzed for ERK1/2 phosphorylation by Western blot analysis. To evaluate the roles of individual PKC isoforms, cells were pretreated with PKC inhibitors or siRNAs prior to the addition of PGF $_{2\alpha}$.

In cultured human ciliary muscle cells, the PKC isoforms exhibiting the highest level of expression were PKC α , ϵ , ι , and λ . The δ and η isoforms exhibited moderate levels of expression and β , γ , and φ were not detected. The administration of PGF $_{2\alpha}$ (1 μ mol/L) primarily induced the translocation of PKC ϵ from cytosol to the membrane fraction, as well as increased MMP-2 secretion and ERK1/2 phosphorylation. The secretion of MMP-2 was inhibited by pretreatment with the broad-range PKC inhibitor, chelerythrine chloride; however, this response was not blocked by Go-6976, an inhibitor of conventional PKC isoforms. The PGF $_{2\alpha}$ -induced secretion of MMP-2 was also blocked by pretreatment with the PKC ϵ -selective peptide translocation inhibitor, EAVSLKPT, or the transfection of siRNA-targeting PKC ϵ . The activation of ERK1/2 was inhibited by chelerythrine and the PKC ϵ translocation inhibitor.

Human ciliary muscle cells express the α , ϵ , ι , and λ PKC isoforms. Stimulation of FP receptors in these cells activates PKC ϵ , resulting in ERK1/2 activation and an eventual increase in MMP-2 secretion. These data support the idea that the activation of FP receptors *in vivo* modulate uveoscleral outflow through the PKC ϵ -dependent secretion of MMPs.

INTRODUCTION

PROSTAGLANDIN (PG) F $_{2\alpha}$ IS ONE OF THE BIOLOGICALLY ACTIVE prostanoids formed from the cyclo-oxygenase-catalyzed metabolism of arachi-

donic acid. PGF $_{2\alpha}$ exerts a broad range of actions by binding to the FP receptor. The FP receptor is a member of the superfamily of G-protein-coupled receptors and has been cloned from a number of species, including human,¹ mouse,² and

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bovine.³ Response to FP receptors are mediated by a variety of second messenger generations, including phosphoinositides, intracellular calcium, protein kinase C (PKC), and MAP kinases.⁴⁻⁶ Physiologically, PGF_{2 α} is known to play key roles in regulating smooth-muscle contraction, DNA synthesis, cell proliferation, and extracellular matrix remodeling.⁷⁻¹⁰

Analogues of PGF_{2 α} , such as travoprost¹¹ and latanoprost,^{12,13} are now considered first-line therapies in treating ocular hypertension. The effects of PGF_{2 α} analogues on intraocular pressure (IOP) reduction principally involved an increase in uveoscleral outflow.¹⁴⁻²⁰ This increase in uveoscleral outflow is thought to involve the secretion and activation of various matrix metalloproteinases (MMPs) and the turnover of extracellular matrix.^{17,18,21-23} In the anterior segment tissues, a number of different ligands, such as growth factors, cytokines, prostaglandins, and phorbol esters, have been shown to regulate MMP secretion.^{21,24-27} Although these studies have provided evidence that multiple receptors regulate the secretion of MMPs from cells within the outflow pathway, the signal-transduction systems coupled to these receptors in ciliary muscle cells is not fully understood.

In the ciliary muscle, we have shown that the activation of PKC plays an important role in the secretion of MMP-2.²⁸ However, the expression pattern and physiologic role of individual PKC isoform in this tissue has not been evaluated. The aim of this study was to elucidate the roles of individual PKC isoform(s) in the PGF_{2 α} -induced increase in MMP-2 secretion from human ciliary muscle cells. In this paper, we provide evidence that PGF_{2 α} primarily activates PKC ϵ in ciliary muscle cells, and this activation of PKC ϵ is an early signaling event regulating the secretion of MMP-2 from these cells.

METHODS

Reagents

Prostaglandin F_{2 α} was obtained from Cayman (Ann Arbor, MI). Monoclonal antibodies to PKC isoforms (α , β , γ , δ , ϵ , η , μ , ι , and λ) were purchased from BD Transduction laboratories (San Diego, CA) and monoclonal MMP-2 antibodies were obtained from Calbiochem (San Diego, CA). Transfection reagent (Silent Fect) was obtained

from Bio-Rad (Hercules, CA) and siRNA duplexes targeting PKC ϵ and Lamin were obtained from Dharmacon RNA Technologies (Dharmacon Inc., Lafayette, CO). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT), and all other cell culture supplies were obtained from Cell Gro (Herndon, VA).

Cell culture

Human eyes (donor ages between 25 and 71 years) without any ocular history were obtained from Life-Point Ocular Tissue Division (Charleston, SC). Human ciliary smooth muscle cells were prepared from normal human eyes by using a procedure previously described.⁴ Briefly, ciliary muscles were dissected with the aid of a dissecting microscope under sterile conditions, cleaned, and cut into 1–2-mm pieces. The explants were placed in Dulbecco's modified Eagle's medium (DMEM) containing 2 mg/mL collagenase type IA, 10% FBS, and 50 μ g/mL of gentamicin and was then incubated for 1–2 h at 37°C with occasional shaking. When a major part of the explant was dispersed into single cells or groups of cells, the cell suspension was centrifuged at 200g for 10 min and resuspended in DMEM199 supplemented with 10% FBS, 100 U/mL of penicillin G, 100 μ g/mL of streptomycin, and 0.25 μ g/mL of amphotericin B and maintained in a 5% CO₂-humidified atmosphere. The confluent cells were subcultured at a split-ratio of 1:4, using 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA). Cultured cells from two to six passages were used in this study. Ciliary muscle cells in culture were identified by their pattern of growth, morphology, and immunocytochemical staining to smooth-muscle actin. These cells formed a confluent monolayer of spindle-shaped cells organized into a "hill and valley" distribution, which is characteristically typical of ciliary smooth muscle cells, as described earlier.⁴

Protein kinase C analysis

PKC isoforms were detected in cytosolic and membrane fractions, using the procedure described earlier,²⁹ by Western blotting. Briefly, cells were lysed in Tris-HCl buffer (pH 7.5) containing 1 mmol/L ethylene glycol tetraacetic acid (EGTA), 2.5 mmol/L of EDTA, 5 mmol/L of DTT, 0.3 mol/L of sucrose, 1 mmol/L Na₃VO₄, 20 mmol/L NaF, and a protease cocktail inhibitor,

using G-20 syringes, followed by centrifugation at 600g for 10 min at 4°C. The supernatant was again centrifuged at 100,000g for 45 min at 4°C. The supernatant was saved as the cytosolic fraction. The pellet was resuspended in lysis buffer containing 0.1% Triton X-100 and served as the membrane fraction. Equal amounts of protein were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes for Western blot analysis, using anti-PKC isoform antibodies (1:1000) and secondary antibodies (horseradish peroxidase [HRP]-conjugated IgG at 1:3000). Rat brain extract, Jurkat, and WI-38 were run as the positive control for the immunodetection of PKC isoforms (as directed by the manufacturer). Band densities were quantified by means of a Bio-Rad Versa Doc Imaging System (Bio-Rad Laboratories).

MMP-2 assay

Human ciliary muscle cells were starved in serum-free medium for 16 hours. Cells were treated with vehicle or PGF_{2α} (1 μmol/L) for 4 h, and the media were collected and concentrated tenfold, using Amicon Ultra-10 centrifugal filter concentrators (Millipore, Billerica, MA). Equivalent volumes of media (40 μL) were loaded onto 10% SDS-PAGs followed by transfer to a nitrocellulose membrane. The membranes were then probed with anti-MMP-2 antibodies overnight at 4°C. Bands were visualized by the addition of secondary antibodies (HRP-conjugated at 1:3000) and enhanced chemiluminescence (ECL) reagents. The band intensities were quantified by densitometry by using a Bio-Rad Versa Doc Imaging System (Bio-Rad Laboratories). Purified MMP-2 was run in parallel to confirm the identity of the MMP-2 band.

Extracellular signal-regulated kinase (ERK1/2) assay

Cells were cultured in serum-free medium for 16 h before the addition of any agent. Cells were treated with PGF_{2α} (1 μM) for 5 min. In experiments evaluating chelerythrine chloride or the PKC translocation inhibitor, cells were pretreated prior to the addition of PGF_{2α} for 60 min. At the end of the incubation period, cells were rinsed with ice-cold phosphate-buffered saline (PBS) and lysed by the addition of lysis buffer (50 mM of Tris-HCl buffer, pH 8.0, containing 100 mM

NaCl, 1 mM of EDTA, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholate, 50 mM NaF, 1 mM/L Na₃VO₄, 5 mM of phenylmethylsulfonyl fluoride, 10 μg/mL of leupeptin, and 50 μg/mL of aprotinin) for 20 min on ice. To determine the level of ERK1/2 activation (phosphorylation), equivalent amounts of protein (15 μg) were loaded onto 10% SDS-PAGs, and proteins were separated according to molecular weight by using standard SDS-PAGE protocols and transferred to a nitrocellulose membrane. The membranes were then probed with anti-phospho-ERK1/2 antibodies for 2 h at room temperature. Bands were visualized by the addition of antirabbit HRP-conjugated secondary antibodies (at 1:3000) and ECL reagents. Blots were then stripped by incubation in stripping buffer (62.5 mM of Tris-HCl, pH 6.7, 100 mM of β-mercaptoethanol, and 2% SDS) for 30 min at 50°C, and total extracellular-signal-regulated kinase (ERK) levels (phosphorylated and nonphosphorylated forms) were determined by immunoblot techniques, using polyclonal anti-ERK1/2 antibodies. Band densities were quantified with a Bio-Rad Versa Doc Imaging System (Bio-Rad Laboratories). The level of phosphorylated ERK1/2 isoforms was normalized for differences in loading, using the total ERK protein band intensities.

PKC translocation inhibitor and siRNA treatment

The translocation peptide inhibitor, EAVS-LKPT, selective for PKCε was delivered into human ciliary muscle cells by using Chariot (Active Motif, Carlsbad, CA) as a carrier. Chariot is a reagent capable of efficiently delivering proteins, peptides, and antibodies into cultured mammalian cells.³⁰ A complex of chariot-peptide (50:50) was added to serum-starved human ciliary muscle cells for 60 minutes. Cells were treated with PGF_{2α} (1 μmol/L) for 4 h, and the media were collected and stored at -80°C until analyzed by Western blotting for MMP-2.

For siRNA transfections, cells were grown to 70–80% confluency, and transfection with duplex siRNA was performed by using methods similar to Elbashir and colleagues.³¹ Briefly, human ciliary muscle cells were incubated in serum-free medium containing transfection reagent and siRNA cocktail for 4 h at 37°C in humidified air. After incubation, the serum-free medium was removed and complete medium, containing 10%

FBS, was added and incubation continued for 48 h. Prior to the addition of the agonist, cells were starved overnight in serum-free medium. PKC ϵ and Lamin A/C siRNAs were obtained from Dharmacon RNA Technologies. Twenty-one-nucleotide siRNA for Lamin A/C (Catalogue no. D-001000-02-05) and smart pool upgrade siRNA of PKC ϵ (catalogue no. MU-004653-00-0002) were used in this study. These siRNAs were obtained as annealed, desalted, and in the 2'-hydroxyl form, as prepared by the manufacturer. Preliminary studies utilizing siRNA concentrations ranging from 1 to 100 nmol/L demonstrated that cells exposed to 10 nmol/L produced a maximal knockdown of PKC ϵ with little or no toxicity. All subsequent studies for the knockdown of proteins were performed by using 10 nmol/L of siRNA.

RESULTS

Previous studies from our laboratory have demonstrated that the PGF $_{2\alpha}$ -induced secretion of MMP-2 from human ciliary muscle cells is mediated by the activation of FP receptors.²⁸ To initially investigate the involvement of PKC in PGF $_{2\alpha}$ -induced MMP-2 secretion, cells were treated with the broad-spectrum inhibitor, chelerythrine chloride (1 μ mol/L). As shown in Figure 1, the PGF $_{2\alpha}$ -induced increase in MMP-2 secretion was blocked by the administration of chelerythrine chloride. In contrast, the addition of Go-6976 (1 μ mol/L), a conventional PKC-iso-

form-selective inhibitor, did not significantly alter MMP-2 secretion (Fig. 1). Whereas these studies support the idea that FP receptors in human ciliary muscle are linked to one of the novel or atypical PKC isoforms, they are complicated by the fact that the PKC inhibitor, chelerythrine, has been shown to influence the activities of multiple kinases and receptors.³²⁻³⁵

To investigate the involvement of individual PKC isoform in PGF $_{2\alpha}$ -mediated MMP-2 secretion from human ciliary muscle, we first evaluated the expression pattern of PKC isoforms in these cells. The Western blot analyses of PKC isoforms in cytosolic and membrane fractions are shown in Figure 2. The PKC α isoform exhibited the highest level of immunoreactivity in these cells, when compared to other PKC isoforms. The PKC ϵ , ι , and λ isoforms exhibited moderate levels of expression, whereas PKC η and δ isoforms exhibited very low levels of expression. The PKC β , γ , and φ isoforms were not detected in human ciliary muscle cells. All the isoforms were detected in positive controls (rat brain extract, Jurkat, and WI-38), suggesting that the antibodies had sufficient titer and binding affinity for visualization (data not shown).

To determine if PGF $_{2\alpha}$ activates a single or multiple PKC isoform(s), the translocation of individual PKC isoforms from cytosol to the membrane fraction were measured in the presence or absence of PGF $_{2\alpha}$. These responses were then compared to the translocation induced by PMA, an activator of both conventional and novel PKC isoforms. As shown in Figure 3, the addition of

FIG. 1. Effects of protein kinase C inhibitors on matrix metalloproteinase-2 (MMP-2) secretion from human ciliary muscle cells. Serum-deprived human ciliary muscle cells were pretreated with vehicle, 1.0 μ mol/L of chelerythrine chloride (A), or 1.0 μ mol/L of Go-6976 (B) for 60 min, followed by 1.0 μ mol/L of prostaglandin F $_{2\alpha}$ treatment for 4 h. The media were then collected, concentrated, and analyzed for MMP-2 by Western blotting, using anti-MMP-2 antibodies. Data are the mean $\pm$ standard error of densitometry measurements ($n = 3-7$; * $P < 0.05$ when compared to the control levels).

FIG. 2. Western blot analyses for individual protein kinase C (PKC) isoforms in cytosolic (C) and membrane (M) fractions of nonstimulated human ciliary muscle cells. Equal amounts of protein (15 μ g) from cytosolic or membrane fractions were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes and probed with primary antibodies selective for each PKC isoform, as described in the Methods section.

PGF_{2 α} (1 μ mol/L) increased the association of PKC ϵ with the membrane fraction by $535 \pm 146\%$, when compared to control preparations. The addition of PGF_{2 α} produced a relatively small increase (70%) in the translocation of PKC α isoform, and no significant changes in the distribution of PKC δ , η , ι , and λ isoforms in the membrane fractions (data not shown). In contrast, the addition of PMA, increased the translocation of PKC α and PKC ϵ isoforms to the membrane fraction by $730 \pm 159\%$ and $256 \pm 46\%$, respectively. The addition of PMA did not alter the membrane association of atypical PKC isoforms ι and λ (data not shown).

To investigate if the PGF_{2 α} -induced increase in MMP-2 secretion is mediated through the activation of PKC ϵ , human ciliary muscle cells were pretreated with the PKC ϵ translocation inhibitor, EAVSLKPT, or siRNA targeting PKC ϵ . As shown in Figure 4, the PGF_{2 α} -induced increase in MMP-2 secretion was blocked by a pretreatment with the PKC ϵ translocation inhibitor. However, administration of the PKC ϵ translocation inhibitor alone did not significantly alter basal MMP-2 secretion.

The cellular responses to siRNA targeting of the endogenous PKC ϵ isoform are shown in Figure 5. Transfection of human PKC ϵ siRNA (10 nmol/L) resulted in an over 80% knockdown of PKC ϵ for up to 48 h. However, PKC α expression was not affected by this treatment. Similarly, expressions of other PKC isoforms were not altered by this treatment with siRNA targeting PKC ϵ (data not shown). In cells treated with siRNA targeting PKC ϵ , the PGF_{2 α} -induced secretion of MMP-2 was blocked. However, in cells transfected with siRNA targeting Lamin A/C, no change in the expression of PKC α or ϵ were measured, and the PGF_{2 α} -induced increase in MMP-2 secretion was not significantly altered.

Previous studies have shown that ERK1/2 is the converging pathway for PGF_{2 α} -induced MMP-2 secretion from ciliary muscle cells. To investigate if the activation PKC ϵ is a prerequisite for the PGF_{2 α} -induced ERK1/2 activation, cells were treated with the nonselective PKC inhibitor, chelerythrine chloride, or the PKC ϵ translocation

FIG. 3. Effects of prostaglandin F2-alpha (PGF_{2 α}) on the translocation of protein kinase C (PKC) isoforms in human ciliary muscle cells. Serum-deprived human ciliary muscle cells were treated with vehicle or 1 μ mol/L PGF_{2 α} for 5 min, followed by the isolation of the membrane fraction and Western blot analysis. The upper panel sections are representative immunoblots of PKC α (A) and PKC ϵ (B) translocations to the membrane fraction. Summary data (lower panel sections) are the mean $\pm$ standard error of densitometry (n = 3–7; *P < 0.05).

FIG. 4. Effects of protein kinase C (PKC) ϵ translocation inhibitors on matrix metalloproteinase-2 (MMP-2) secretion from human ciliary muscle cells. Serum-deprived human ciliary muscle cells were treated with vehicle or 200 nmol/L of PKC ϵ translocation inhibitor, EAVSLKPT, for 60 min followed by 1 μ mol/L PGF_{2 α} treatment for 4 h. The media were then collected, concentrated, and analyzed for MMP-2 by Western blotting, using anti-MMP-2 antibodies. The upper panel section shows representative immunoblots of MMP-2. Summary data (lower panel section) are the mean $\pm$ standard error of densitometry measurements ($n = 4$; * $P < 0.05$, compared to control levels).

inhibitor, EAVSLKPT (Fig. 6). Pretreatment with either chelerythrine, or the PKC ϵ translocation inhibitor, blocked the PGF_{2 α} -induced activation of ERK1/2.

DISCUSSION

PKC is a family of serine-threonine kinases that regulate cellular differentiation, growth, tumor promotion, and muscle contraction. The PKC isoforms are activated in response to a large variety of stimuli, such as growth factors, hormones, and neurotransmitters^{36,37}; however, our understanding of the role of PKC in aqueous-humor dynamics remains incomplete. Studies have shown that PKC can regulate trabecular-meshwork contractility and alter cytoskeletal organization.^{38,39} In addition, pharmacologic modulators of PKC have been shown to modulate the outflow facility in perfused porcine eyes.^{38,40} Although prostaglandin FP agonists have been shown to lower IOP by increasing uveoscleral outflow and the activation of PKC is often linked to the activation of prostanoid receptors, a definitive role for PKC in the regulation of uveoscleral outflow remains an open question. Recently, we have

FIG. 5. Inhibition of prostaglandin F₂-alpha (PGF_{2 α})-induced matrix metalloproteinase-2 (MMP-2) secretion by siRNA targeting endogenous protein kinase C (PKC) ϵ . Human ciliary muscle cells were transfected with 10 nmol/L of siRNA targeting PKC ϵ or Lamin A/C for 4 h in serum-free media. Two (2) days after transfection, cells were harvested and immunoblotted with PKC isoform and β -actin antibodies. (A) Representative immunoblots of PKC ϵ and PKC α from cells treated with PKC ϵ siRNA, Lamin A/C siRNA, or transfection reagent alone (control). Beta-actin was used as an internal control to insure that equal amounts of proteins were loaded in each lane. Lamin A/C siRNA was used as a positive control for the silencing of nonessential, abundantly expressed housekeeping genes in these cells. (B) Alteration in MMP-2 secretion induced by PGF_{2 α} (1 μ mol/L) produced by siRNA transfection. The upper panel section is a representative immunoblot of MMP-2 from cells treated with PKC ϵ siRNA, Lamin A/C siRNA, or transfection reagent alone (control). Summary data (lower panel section) are the mean $\pm$ standard error of densitometry measurements ($n = 3-7$; * $P < 0.05$, compared to control levels).

FIG. 6. Effects of protein kinase C (PKC) inhibitors on prostaglandin F₂-alpha (PGF_{2α})-induced ERK1/2 activation. Serum-deprived human ciliary muscle cells were pretreated with vehicle, 1 μmol/L of chelerythrine chloride or the PKCε translocation inhibitor, EAVSLKPT, for 60 min followed by 1 μmol/L of PGF_{2α} treatment for 5 min. The cell lysates were analyzed for ERK1/2 phosphorylation by Western blot, using anti-phospho-ERK1/2 and anti-ERK1/2 antibodies.

shown that PGF_{2α} regulates the secretion of MMP-2 from human ciliary muscle.²⁸ This study investigates the expression of PKC isoforms and their roles in the modulation of MMP-2 secretion by PGF_{2α} in human ciliary muscle cells.

As shown in Figure 1, PGF_{2α}-induced secretion of MMP-2 was completely inhibited by pretreatment with a broad range PKC inhibitor, chelerythrine chloride. In contrast, this response was not blocked by pretreatment with the conventional PKC (α, βI, βII, and γ) isoform inhibitor, Go-6976, indicating that PKC isoforms other than conventional PKC isoforms are involved in this process (Fig. 1). Whereas these studies support the idea that FP receptors in human ciliary muscle are linked to one of the novel or atypical PKC isoforms, they are complicated by the fact that chelerythrine has been shown to influence the activities of multiple kinases and receptors.^{32–35} To confirm that PGF_{2α} induces MMP-2 secretion from these cells by a PKC-dependent pathway, we characterized the expression of individual PKC isoforms, the activation (translocation) of these isoforms by PGF_{2α}, and the use of isoform-specific inhibitors to prevent PGF_{2α}-induced secretion of MMP-2.

Using isoform-specific antibodies, we examined the presence and subcellular distribution of PKC α, δ, ε, ζ, λ, and η isoforms in human ciliary muscle cells. Under basal conditions, we found that PKC α, ε, and ζ isoforms were predominantly located to the cytosolic fraction, PKCδ and η were primarily located to the membrane fraction, and PKCλ was almost equally distributed between cytosol and membrane fractions. We found no evidence that PKC β, γ, or φ were expressed in cultured ciliary muscle cells. Our results for PKCα and ε isoforms are similar to previous reports in human ciliary muscle cells.³⁹ As most inactive PKC isoforms reside within the cytosol and translocate to the membrane (or other subcellular sites) upon stimulation, the cytosolic and membrane-associated levels of each PKC isoform were determined in the presence and absence of PGF_{2α} or the PKC activator, PMA. Treatment of human ciliary muscle cells with PGF_{2α} induced almost a complete translocation of PKCε isoform from the cytosol to the membrane fraction. In contrast, PGF_{2α} produced only a small increase in membrane-associated PKCα and no change in the distribution of other PKC isoforms. In contrast, PMA treatment produced a rapid increase of both PKCα and PKCε association within the membrane fraction. As expected, PMA had no significant effects on atypical PKC isoforms in these cells. Overall, these data support the idea that the activation of FP receptors in these cells is primarily coupled to the activation of PKCε.

To determine whether the PGF_{2α}-induced secretion of MMP-2 requires the activation of PKCε, human ciliary muscle cells were pretreated with the PKCε translocation inhibitor or siRNAs targeting PKCε prior to the addition of PGF_{2α}. Pretreatment with PKCε translocation inhibitor blocked the secretion of MMP-2 induced by PGF_{2α}. Previous studies in cardiomyocytes have shown that the PKCε translocation inhibitor peptide selectively inhibited the translocation of PKCε without affecting other PKC isoforms.^{41,42} To confirm these results, we utilized siRNA duplexes to the knockdown endogenous expression of PKCε isoform. Our results demonstrate that siRNA technologies can selectively reduce the expression of individual PKC isoforms in primary human ciliary muscle cells. In cells treated with siRNA-targeting PKCε, the PGF_{2α}-induced increase in MMP-2 secretion was completely eliminated. In contrast, treatment of cells with Lamin siRNA did not block the PGF_{2α}-induced increase

in MMP-2 secretion from human ciliary muscle cells. Together, these results demonstrate that the activation PKC ϵ is a required step in PGF $_{2\alpha}$ -induced increase in MMP-2 secretion from human ciliary muscle cells.

Previous studies have shown that PGF $_{2\alpha}$ is an effective activator of the ERK1/2 pathway in ciliary muscle cells, and this activation is also required for PGF $_{2\alpha}$ to induce MMP-2 secretion.²⁸ To begin to understand the relationship between PKC ϵ and ERK1/2 in ciliary muscle, cells were pretreated with chelerythrine or the PKC ϵ translocation inhibitor, and PGF $_{2\alpha}$ -induced ERK1/2 activation was evaluated. These studies demonstrate that both chelerythrine and the PKC ϵ translocation inhibitor blocked ERK1/2 activation. These results, along with earlier studies,²⁸ provide the initial evidence that in ciliary muscle cells, the PGF $_{2\alpha}$ -induced secretion of MMP-2 requires the sequential activation of PKC ϵ > MEK1/2 > ERK1/2.

Several reports have shown the involvement of PKC in agonist-induced MMP secretion from nonocular cells.^{43,44} Further, studies have also shown that TNF α -induced MMP secretion⁴⁵ and interleukin-1 α stimulated upregulation of MMP-3 in trabecular meshwork cells,⁴⁵ are both mediated through PKC-dependent pathways. The FP agonist and latanoprost-induced TIMP-1 secretion from human ciliary muscle cells have been shown to be mediated through PKC activation.⁴⁷ However, the role of individual PKC isoforms in the PGF $_{2\alpha}$ -induced increase in MMP secretion from human ciliary muscle cells has remained unexplored. This study presents the first evidence directly linking PKC ϵ to MMP-2 secretion from human ciliary muscle cells. The cellular mechanism of ocular PG hypotensive action is believed to involve MMP secretion from the ciliary muscle to promote uveoscleral outflow.⁴⁸ Our results demonstrate that FP-receptor activation leads to the acute secretion of MMP-2, and that this functional response is mediated by PKC ϵ - and ERK1/2-signaling pathways.

CONCLUSIONS

In summary, human ciliary muscle cells express PKC α , δ , ϵ , η , ι , and λ isoforms. From the results presented in this paper, we conclude that the activation of FP receptors leads to a rapid secretion of MMP-2, which requires the sequential

activation of PKC ϵ and ERK1/2. Hence, FP agonists *in vivo* appear to modulate uveoscleral outflow and IOP, in part, through the PKC ϵ -dependent secretion of MMP-2.

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