

Prostaglandin F_{2α} Receptors in the Human Trabecular Meshwork

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PURPOSE. Prostaglandin F_{2α} (PGF_{2α}) and analogs, such as latanoprost, are thought to lower intraocular pressure (IOP), primarily by increasing uveoscleral outflow. However, outflow through the trabecular meshwork may be increased as well. The authors hypothesize that any effect on the trabecular meshwork is mediated by prostanoid FP receptors (receptors for prostaglandin F_{2α}) in this tissue.

METHODS. To test this hypothesis, tissue sections of the human trabecular meshwork and cultures of human trabecular meshwork cells were examined for the presence of FP receptors using immunofluorescence microscopy with affinity-purified antibodies raised against a glutathione-S-transferase (GST)-FP_A receptor fusion protein. The presence of the receptor was confirmed by using reverse transcription-polymerase chain reaction (RT-PCR), functional assays of PGF_{2α}-stimulated inositol phosphate hydrolysis, and intracellular calcium measurements.

RESULTS. Positive FP_A receptor immunolabeling was observed in sections of the human trabecular meshwork and in cultured human trabecular meshwork cells. In both cases, specific labeling could be blocked by preincubation with a GST-FP_A receptor fusion protein. Cross-blocking experiments with other receptor fusion proteins did not block specific labeling in cultured trabecular meshwork cells. PGF_{2α} caused a dose-dependent increase in total inositol phosphate accumulation and intracellular calcium release in human trabecular meshwork cells that was consistent with the presence of FP receptors. Using RT-PCR, message-encoding prostanoid FP_A receptors were found in total RNA isolated from human trabecular meshwork cells.

CONCLUSIONS. Prostanoid FP_A receptors exist in human trabecular meshwork cells, as shown by the presence of mRNA, protein, and a functional response to PGF_{2α}. This study indicates that functional FP receptors are present in the human trabecular meshwork and that they may be involved in mediating some of the IOP-lowering effects of PGF_{2α} in the eye. (*Invest Ophthalmol Vis Sci.* 1998; 39:315-321)

The trabecular meshwork is the major route for aqueous outflow in the anterior chamber of the human eye. As such, the trabecular meshwork has an important role in the regulation of intraocular pressure (IOP) and is frequently a target in the pharmacologic management of glaucoma. Cells of the trabecular meshwork and of the Schlemm's canal are involved with aqueous humor outflow, but the details of their involvement, at least at the molecular level, are not clearly understood. In particular, specific receptors that may be present in these cells and that may mediate some of the IOP-lowering effects of drugs are largely unknown.

Pharmacologic management of glaucoma has included a variety of drugs acting at different receptors and locations in the eye. For example, α₂-adrenergic agonists and β-adrenergic

antagonists lower IOP by acting on their respective receptors in the ciliary epithelium to decrease the secretion of aqueous humor.^{1,2} Muscarinic agonists, however, act on muscarinic acetylcholine receptors in the ciliary smooth muscle to cause contraction and thereby decrease resistance of aqueous outflow through the trabecular meshwork.³ The recently introduced prostaglandin F_{2α} (PGF_{2α}) analog, latanoprost (13,14-dihydro-15R-17-phenyl-18,19,20-trinor-PGF_{2α}-isopropyl ester, PhXA41), is thought to act through prostanoid FP receptors in the ciliary muscle to enhance uveoscleral outflow.⁴⁻⁷

Our knowledge of the FP receptors (receptors for prostaglandin F_{2α}) themselves is limited and prevents a better understanding of the ocular effects of PGF_{2α} and its analogs. Prostanoid FP receptors are members of the seven-transmembrane family of receptors and are coupled to activation of guanine nucleotide-binding proteins (G proteins). cDNA-encoded prostanoid FP receptors were first cloned in 1994,⁸ and recently a novel alternative mRNA splice variant has been identified.⁹ The original FP receptors have been termed FP_A, and the splice variant, which contains a truncated carboxyl terminus relative to the FP_A, has been termed FP_B. Stimulation of both FP receptor isoforms results in the activation of phospholipase C-mediated phosphoinositide turnover.¹⁰ Prostanoid FP receptors are known to be present in the eye, but their specific localization and cellular functions are not well characterized. Recently, however, in situ hybridization and immunohistochemical studies have indicated that FP receptors are present

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Supported in part by grants from the National Institutes of Health (EY09355, EY11291) and by Allergan, Irvine, California.

Submitted for publication July 14, 1997; revised October 6, 1997; accepted November 11, 1997.

Proprietary interest category: N.

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in the corneal, conjunctival, and iridial epithelia and in the ciliary muscle and ciliary process of the monkey eye.¹¹ Radioligand binding and autoradiography analyses suggest that the FP receptors are localized in the ciliary muscle and the iris sphincter muscle of the human eye.¹²

The present study was initiated to determine whether FP receptors are present in cells of the trabecular meshwork in which their activation may influence aqueous outflow. The results of this study indicate that prostanoid FP_A receptors are present and that they are functionally coupled to the activation of phosphoinositide hydrolysis.

MATERIALS AND METHODS

Human Trabecular Meshwork Cell Culture

Human trabecular meshwork cells were isolated after collagenase digestion of human trabecular meshwork explants exactly as described by Stamer et al.¹³ Human trabecular meshwork cells have been characterized with respect to their growth characteristics, morphology, and presence of a cell-surface receptor for low-density lipoprotein and have been used for the characterization of aquaporin-1¹³ and α_2 -adrenergic receptors,¹⁴ which are present in these cells. Cells were maintained on 10-cm plates with medium (Medium 199; Life Technologies, Gaithersburg, MD) containing 15% fetal bovine serum, 10 μ g/ml endothelial growth supplement (Sigma Chemical, St. Louis, MO), 5 mM HEPES, and 1.7 mM L-glutamine in an incubator at 37°C.

Fluorescence Microscopy

Antibodies to the sheep FP_A receptor were generated in chickens using a recombinant fusion protein consisting of glutathione-S-transferase (GST) and a portion of the carboxyl terminus of the receptor consisting of amino acids 317 through 362. Preparation of the fusion protein and antibody purification were performed as described,¹⁵ except that the sense and antisense primers used for the initial polymerase chain reaction (PCR) reaction were as shown below [the *lowercase italic letters* represent the restriction sites for *Bam*HI (sense) and *Eco*RI (antisense)].

FP_A carboxyl-terminal sense:

5'-GAA Tgg atc cGT GTG TAC CAG ACG CTG T-3'

FP_A carboxyl-terminal antisense:

5'-TAT Tga att CAG TTT GAC TCC TAT AG-3'

The initial characterization of the antibodies was made as previously described¹⁵ using COS-7 cells transfected with plasmids encoding either sheep FP_A or human FP_A. For immunolabeling, cells were cultured for 3 to 4 days on 1% gelatin-coated coverslips; the media were removed, and the cells were washed twice with phosphate-buffered saline. The cells were placed in 4% paraformaldehyde-phosphate-buffered saline for 15 minutes at room temperature, washed once with phosphate-buffered saline, and then placed in 100 mM glycine solution for 20 minutes. Cells were washed again in phosphate-buffered saline and permeabilized with 30 mM sodium chloride-300 mM sodium citrate containing 0.1% Triton X-100 for 1 hour. After an overnight incubation at 4°C with the primary antibody (0.6 μ g/ml), the cells were washed with 30 mM sodium chloride-300 mM sodium citrate and incubated for 2 hours at room temperature with a secondary antibody (rabbit anti-chicken-fluorescein isothiocyanate; Sigma) at a dilution of 1:1000. Coverslips were washed and mounted on glass slides

for viewing. For labeling of human eye tissues, eyes were fixed in 4% paraformaldehyde for 3 to 5 hours, and transverse sections were embedded in paraffin and serially sectioned (10- μ m thickness). Fluorescent immunolabeling was performed as described above with the following addition. After an overnight incubation at 4°C with affinity-purified primary antibody (0.6 μ g/ml), the cells were washed with 30 mM sodium chloride-300 mM sodium citrate and incubated for 1 hour at room temperature with another secondary antibody (rabbit anti-chicken-CY-5; Jackson ImmunoResearch Laboratories, West Grove, PA) at a dilution of 1:750. Eye sections were washed and mounted under glass coverslips for confocal imaging.

Reverse Transcription-Polymerase Chain Reaction

Primers were chosen to amplify 1186-nucleotides of the human FP_A receptor. The sense primer (nucleotides 170-193) corresponds to a position 61 nucleotides upstream of the translational start site, and the antisense primer (nucleotides 1333-1356) corresponds to a position 39 nucleotides downstream of the stop codon in the human FP_A sequence. Both PCR primers were 100% homologous with the reported cloned sequence of the human FP_A receptor. The sense and antisense primers were used for reverse transcription (RT)-PCR as previously described with total RNA isolated from primary cultures of human trabecular meshwork cells.¹³ The PCR (final volume, 50 μ l) contained 5 μ l of the RT reaction, 5 μ l of 10 \times PCR buffer (Perkin-Elmer, Norwalk, CT), 5 μ l dimethyl sulfoxide, 2.5 μ l each of the sense primer and antisense primers (20 μ M), and 0.5 μ l taq polymerase (Perkin-Elmer, 2.5 U/ml). The PCR program consisted of an initial step at 95°C for 7 minutes, followed by 36 cycles at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute and a final step at 72°C for 7 minutes. Products were analyzed by electrophoresis in 1% agarose gels.

Inositol Phosphate Assay

Human trabecular meshwork cells were grown in 12-well plates in Medium 199 (Life Technologies) containing 15% fetal bovine serum, 10 μ g/ml endothelial growth supplement (Sigma), 5 mM HEPES, and 1.7 mM L-glutamine and were allowed to grow to 80% confluency at 37°C. Medium was removed 24 hours before assay and replaced with serum-free medium containing 0.2 μ M myo-[³H]inositol. After 24 hours at 37°C, medium was removed and replaced with 1 ml fresh serum-free medium and incubated at 37°C for 1 hour. Lithium chloride (10 mM) was added and allowed to equilibrate for 10 minutes, at which time PGF_{2 α} (endogenous FP receptor ligand) was added and allowed to incubate for 1 hour at 37°C. Plates were placed on ice and immediately aspirated and were washed once with fresh medium. Cold methanol was added (0.5 ml/well), the wells were scraped, and contents were added to 1.5 ml chloroform-H₂O (2:1, vol/vol). The solutions were vortexed and centrifuged at 2100 rpm for 15 minutes. An aliquot (0.9 ml) of the aqueous phase was added to 2 ml H₂O and then applied to a 2.5 ml, anion-exchange column [AG1-x8 (formate form); Bio-Rad, Richmond, CA]. The columns were washed three times with H₂O (5 ml/wash), twice with 5 mM sodium tetraborate (5 ml/wash), and once with 60 mM sodium formate (5 ml). The columns were eluted with 2 ml 0.2 M ammonium formate-0.1 M formic acid, and the radioactivity was determined by liquid scintillation counting.

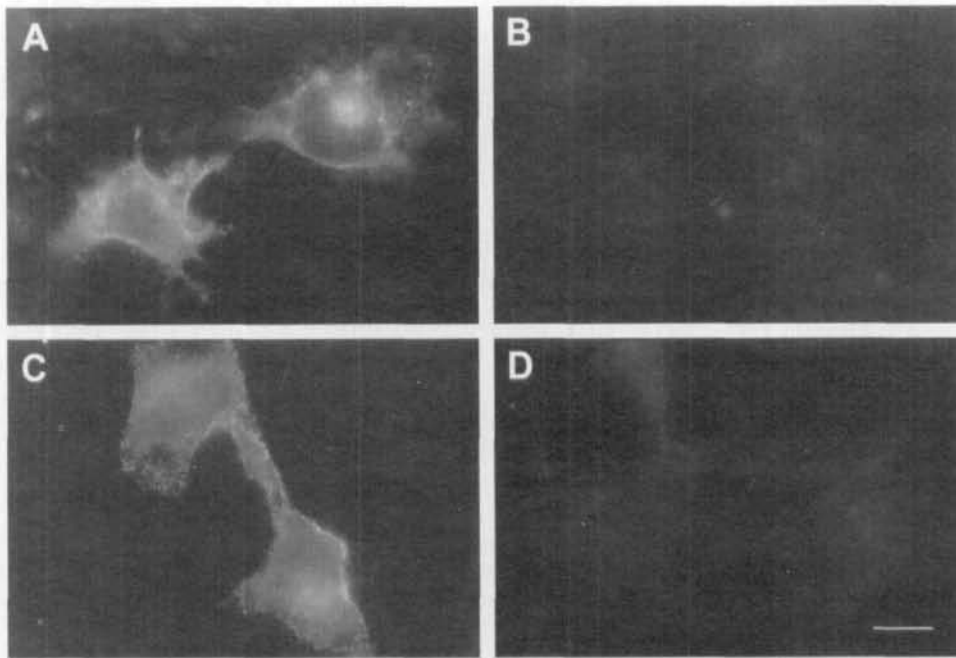


FIGURE 1. Immunofluorescence microscopy of COS-7 cells transiently transfected with plasmids encoding either the sheep or human FP_A receptors and labeled with antibodies to a glutathione-S-transferase (GST)-FP_A fusion protein. Antibody purification and COS cell labeling were performed as described in the Materials and Methods section. Cells transfected with the sheep FP_A receptor were labeled with either primary antibody alone (A) or with antibody that had been preincubated with fusion protein (B). Cells transfected with the human FP_A receptor were labeled with either primary antibody alone (C) or with primary antibody that had been preincubated with fusion protein (D). The secondary antibody was fluorescein isothiocyanate-rabbit anti-chicken immunoglobulin G. Bar = 20 μ m.

Intracellular Calcium Measurements

Human trabecular meshwork cells were grown to confluency in 15-cm culture plates. Cells were trypsinized, counted, and resuspended at a concentration of 3×10^6 cells/ml in medium. Fura2-AM (Sigma; 1 mM, 1 μ l) was added per milliliter of cells. The cells were submerged in a 37°C water bath and incubated with slow shaking for 45 minutes to 1 hour. After the incubation, 40 ml medium was added and cells were centrifuged at 1200 rpm for 5 minutes. The supernatant was aspirated, and the cells were resuspended in medium (2×10^6 cells/ml). Human trabecular meshwork cells were distributed to microcentrifuge tubes in 500- μ l aliquots. Just before use, cells were centrifuged for 1 minute (2000 rpm) in a microcentrifuge. The supernatants were aspirated, and the cells were resuspended in recording buffer (120 mM NaCl, 6 mM KCl, 1.4 mM CaCl₂, 1 mM MgSO₄, 20 mM HEPES, 1 mg/ml glucose, 1 mg/ml sodium pyruvate, pH 7.4). Recording and analysis were performed using a fluorescence spectrometer (LS-50; Perkin-Elmer).¹⁶ The cells were lysed with 0.1% Triton X-100 to obtain maximum fluorescence, and EGTA (final concentration, 6 mM) and NaOH (final concentration, 20 mM) were added successively to obtain minimum fluorescence. The investigation adhered to the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

RESULTS

COS-7 cells were transiently transfected with plasmids encoding either the human or sheep FP_A receptors and were labeled

for immunofluorescence microscopy using antibodies raised against a GST-FP_A carboxyl-terminal fusion protein. Figure 1A shows photomicrographs of cells transfected with the sheep FP_A receptor and examined for fluorescence after incubation with the primary and secondary fluorescein antibodies. Labeling was present across the cell surface, which could be specifically blocked (Fig. 1B) by preincubation of the antibodies with the GST-FP_A carboxyl-terminal fusion protein. Omission of the primary antibody (data not shown) resulted in a low level of background labeling that was comparable to preincubation of the antibody with the fusion protein (Fig. 1B). An analogous series of experiments with COS-7 cells that were transfected with the human FP_A receptor produced essentially identical results (Figs. 1C, 1D), showing that the antibodies could cross-react with the carboxyl terminus of the human FP_A receptor. The homology between the human and sheep FP_A receptors in this region is approximately 70% and is consistent with the observed cross-reactivity. Preincubation of the antibodies with GST alone or with fusion proteins containing portions of other prostanoid receptors did not block labeling (data not shown).

Figure 2 shows the fluorescein fluorescence of human trabecular meshwork cells after labeling with antibodies to the GST-FP_A receptor fusion protein. Similar to the labeling observed with FP_A-transfected COS-7 cells, the human trabecular meshwork cells showed immunoreactivity when they were incubated with the primary and fluorescein isothiocyanate-labeled secondary antibodies (Fig. 2A). In contrast to the labeling of the COS-7 cells, however, all human trabecular mesh-

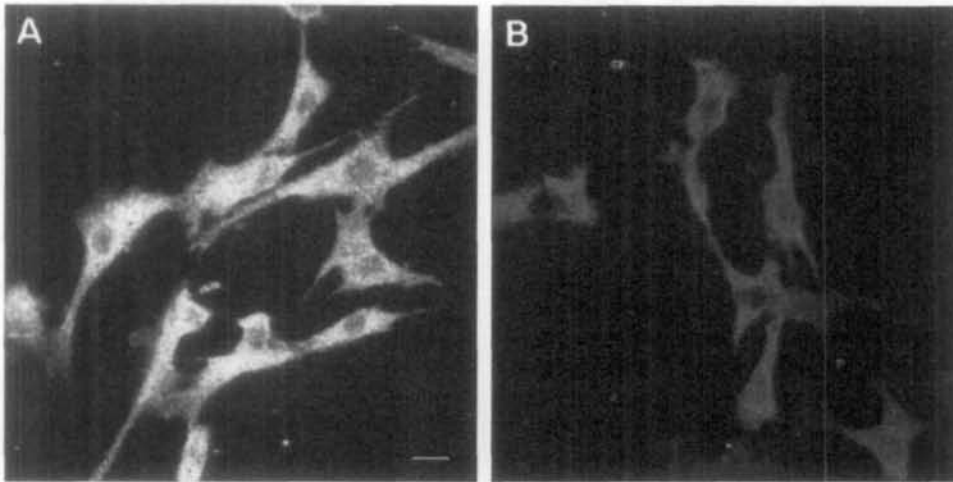


FIGURE 2. Immunofluorescence labeling of human trabecular meshwork cells with antibodies to a glutathione-S-transferase-FP_A fusion protein. Human trabecular meshwork cells were cultured as described in the Materials and Methods section and were labeled with either primary antibody alone (A) or with primary antibody preincubated with fusion protein (B). The secondary antibody was fluorescein isothiocyanate-rabbit anti-chicken immunoglobulin G. Bar = 20 μ m.

work cells showed immunoreactivity consistent with the expression of an endogenous receptor as opposed to the heterologous expression in transfected cells. Preincubation of the primary antibody with the GST-FP_A carboxyl-terminal fusion protein blocked the labeling of the human trabecular meshwork cells (Fig. 2B). Omission of the primary antibody yielded a low level of background fluorescence (data not shown). To see whether FP_A receptor immunoreactivity was present in the trabecular meshwork itself, 10- μ m sections of human eyes ($n = 2$ from separate donors) were labeled and examined by confocal microscopy. As shown in Figure 3, immunoreactivity was observed in the trabecular meshwork (Figs. 3A, 3C) and blocked preincubation of the primary antibody with the GST-FP_A carboxyl-terminal fusion protein (Figs. 3B, 3D).

To confirm that the human trabecular meshwork cells contained mRNA that encodes a prostanoid FP_A receptor, RT-PCR was performed with primers that were predicted to yield an FP_A-specific product of 1186 bp. Figure 4 shows an ethidium-stained agarose gel with the PCR products obtained from cDNA prepared from total RNA isolated from two different donor populations of human trabecular meshwork cells (lanes 1 and 3). To confirm specificity of the PCR products, the products were incubated with the restriction endonuclease *Bam*HI for 1 hour at 37°C. As expected, a single cut was obtained yielding two bands of 825 bp and 361 bp (lanes 2 and 4). A negative control is also shown in which the PCR reaction contained everything except a DNA template (lane 5). As shown, a product of the expected size was obtained with the RNA isolated from both populations of donor cells. Because the primers were chosen to span an intron, the PCR products did not result from the amplification of genomic DNA and are consistent with the presence of mRNA encoding a human prostanoid FP_A receptor.

Stimulation of prostanoid FP receptors by agonists is known to produce intracellular signaling through an increase in phosphoinositide hydrolysis and a release of intracellular calcium.¹⁰ The ability of PGF_{2 α} to stimulate phosphoinositide turnover in human trabecular meshwork cells was examined, and the results are presented in Figure 5. As shown, PGF_{2 α}

produced a dose-dependent increase in [³H]inositol phosphate accumulation with an EC₅₀ level of approximately 100 nM. Using human trabecular meshwork cells prepared from a second donor, a similar dose-response curve was obtained with a maximal stimulation of [³H]inositol phosphate accumulation that was 3-fold higher than basal. In both series of experiments, the stimulation of [³H]inositol phosphate accumulation was statistically significant (ANOVA, $P < 0.01$).

The ability of PGF_{2 α} to stimulate the release of intracellular calcium was examined, and the results are represented in Figure 6. PGF_{2 α} produced a dose-dependent increase in the overall change in intracellular calcium with an EC₅₀ of approximately 60 nM. Human trabecular meshwork cells for the calcium measurements were obtained from the same human donor who contributed to the phosphoinositide hydrolysis assays. These results are consistent with the activation of an FP receptor that demonstrates a downstream effect from the stimulation of phosphoinositide turnover in the human trabecular meshwork cells.

DISCUSSION

Previous studies using *in situ* hybridization, radioligand binding, and immunohistochemical analyses have identified prostanoid FP receptors in several regions of human and primate eyes. Some of the areas in which FP receptors were present included the corneal, conjunctival, and iridial epithelia, iris sphincter, and ciliary smooth muscles.^{11,12} These studies did not report, however, on the presence of FP receptors in the trabecular meshwork, nor did they establish the nature of the FP receptor isoforms that may be present or the second messenger pathways of these receptors. In the present study, immunohistochemical and PCR analyses were used to identify the presence of the FP_A receptor isoform in cultured cells from the human trabecular meshwork. It was also determined that the activation of these receptors by PGF_{2 α} stimulated phosphoinositide turnover and the mobilization of intracellular calcium.

The physiological and pharmacologic significance of prostanoid FP receptors in the human trabecular meshwork re-

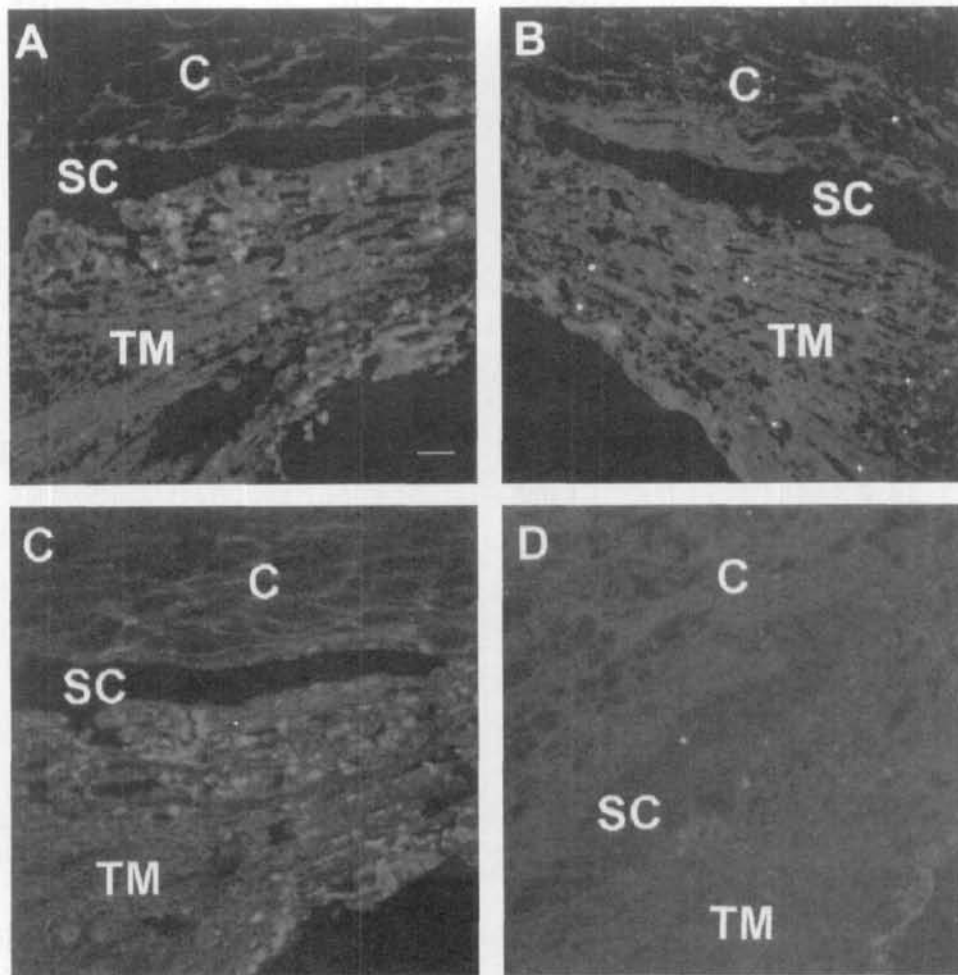


FIGURE 3. Confocal microscopy of human trabecular meshwork after labeling with antibodies to a glutathione-S-transferase-FP_A fusion protein. Fixation and labeling were as described in the Materials and Methods. The figure represents serial sections (10 μm , $n = 2$ donor eyes) of the anterior chamber labeled with either primary antibody alone (A, C) or with primary antibody preincubated with fusion protein (B, D). SC, Schlemm's canal; C, cornea; TM, trabecular meshwork. Bar = 20 μm .

mains to be established. Because it concerns the treatment of glaucoma, it is generally acknowledged that the IOP-lowering effects of PGF_{2 α} and its analogs occur by way of an increase in uveoscleral outflow. However, these agents may have actions in other regions of the eye that influence these effects on IOP.¹⁷ For example, in a recent clinical study of the interaction between PhXA41 and pilocarpine, it was determined that a reduction in IOP could not be explained solely by increased uveoscleral outflow.¹⁸ Among the possibilities suggested was enhanced outflow through the conventional trabecular pathway. The proper function of the trabecular meshwork is critical in the regulation of IOP. Substances produced endogenously or carried to the trabecular meshwork by the aqueous humor act on receptors within the trabecular meshwork. In addition, the effect of PGF_{2 α} on IOP may differ under normal physiological conditions compared with pathophysiological conditions. Several changes in trabecular meshwork cells have been implicated in the pathogenesis of glaucoma.^{19,20} Altered levels of ligands or receptor function could be involved in the generation of glaucoma; therefore, the potential effect of PGF_{2 α} on the trabecular meshwork must be understood.

The role of inositol phosphates, particularly inositol 1,4,5-trisphosphate, in initiating the release of intracellular calcium is well established.²¹ In this study, we have found that PGF_{2 α} can stimulate the turnover of inositol phosphates (EC₅₀, 100 nM) in human trabecular meshwork cells. The EC₅₀ value reported for the stimulation of inositol phosphates in the human trabecular meshwork was within the range of values reported by others for PGF_{2 α} stimulation.²²⁻²⁴ We hypothesize that this would result in a release of intracellular calcium. We found, in the same population of human trabecular meshwork cells examined, that stimulation with PGF_{2 α} resulted in a dose-dependent increase in intracellular calcium release (EC₅₀, 60 nM). The reported EC₅₀ value in this study was within the range of values reported using PGF_{2 α} as the agonist.^{25,26} Taking these data together, we conclude that activation of the prostanoid FP receptors in the human trabecular meshwork cells results in an accumulation of inositol phosphate and causes the release of intracellular calcium. It has been found that trabecular meshwork cells contain contractile filaments similar to those found in smooth muscle.²⁷ In addition, human trabecular meshwork cells have been shown, in electrophysi-

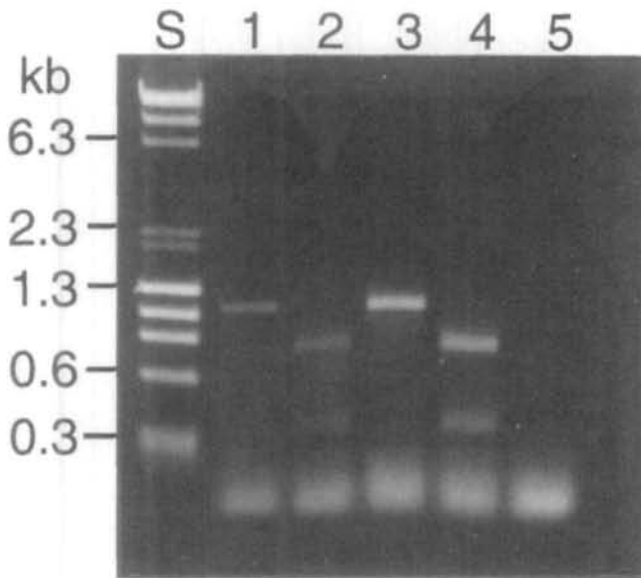


FIGURE 4. Reverse transcription-polymerase chain reaction (RT-PCR) of total RNA isolated from human trabecular meshwork cells and amplified with specific primers for the human prostanoid FP_A receptor. Lanes 1 and 3, products obtained after RT-PCR using total RNA isolated from two populations of donor human trabecular meshwork cells, respectively. Lanes 2 and 4, expected products (825 and 361 bp) obtained after restriction digestion with *Bam*HI. Lane 5 is a negative control without template. Standards (lane 5) are λ -phage and ϕ X174 DNA cut with *Hind*III and *Hae*III, respectively, and are shown in kilobase pair units. The predicted size of the uncut human FP_A receptor PCR product (1186 bp) was obtained from both populations of donor human trabecular meshwork cells.

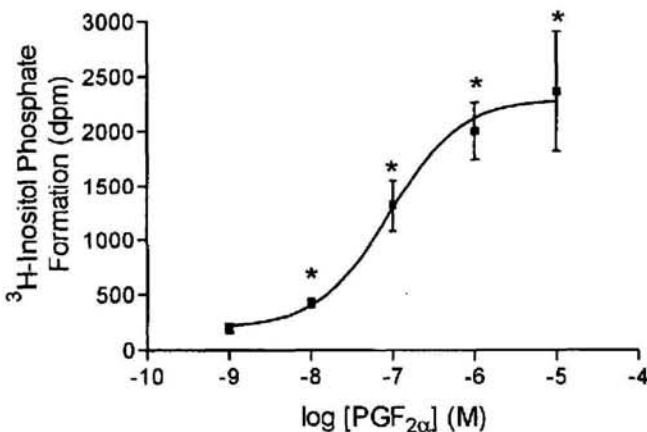


FIGURE 5. Stimulation of [³H]inositol phosphate formation by $PGF_{2\alpha}$ in cultured human trabecular meshwork cells. Incubation conditions and assay of inositol phosphates were as described in the Materials and Methods. Final concentrations of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) are indicated. Data represent the means of two independent experiments, each performed in duplicate using cells from one donor. The experiment was repeated two more times in duplicate with human trabecular meshwork cells from a second donor. The asterisks indicate significant differences from basal (ANOVA, followed by multiple range testing; $P < 0.01$).

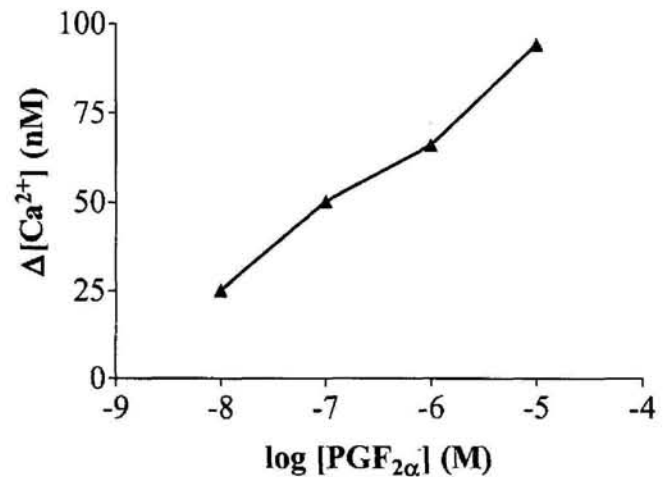


FIGURE 6. Stimulation of intracellular calcium by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in cultured human trabecular meshwork cells. Conditions of the assay were as described in the Materials and Methods section. Final concentrations of $PGF_{2\alpha}$ are indicated. Data represent the values from a population of trabecular meshwork cells obtained from a single donor eye.

ological studies, to exhibit the nifedipine-sensitive electrical behavior characteristics of smooth muscle cells.²⁸ Based on our results, it is possible that the activation of FP receptors in the trabecular meshwork, resulting in the release of intracellular calcium, could modify the shape, size, or both, of the cells through a smooth muscle-like contractile event. This, in turn, may affect resistance and thereby influence aqueous outflow. Obviously, more work is needed to establish this possibility, but at least some of the elements are in place. Furthermore, regardless of whether a contractile event occurs, $PGF_{2\alpha}$ does appear to have an effect on the human trabecular meshwork, and it is reasonable to predict that $PGF_{2\alpha}$ and its analogs mediate their effects through FP_A receptors on human trabecular meshwork cells.

Acknowledgments

The authors thank David Woodward (Allergan, Irvine, CA) for his expert advice and technical assistance with the calcium measurements, Sue Waite for her technical help and suggestions with the inositol phosphate assay, and Richard Seftor (Department of Anatomy, University of Iowa) for providing the human trabecular meshwork cells.

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