Effects of Platelet-Derived Growth Factor on Aqueous Humor Dynamics

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Purpose. It is well known that the small GTPase RhoA modulates actin cytoskeleton and cellular contractility in the trabecular meshwork (TM). Several substances known to contract the TM reduce outflow facility, whereas cellular relaxation is commonly associated with the opposite effect. Inhibitors of the RhoA pathway are under development as antiglaucoma drugs. Here the authors investigate the role of platelet-derived growth factor (PDGF), a known activator of the Rac1 pathway, in cell cytoskeleton, outflow facility, and intraocular pressure (IOP).

Methods. Effects of PDGF on actin cytoskeleton, Rac1, and AKT activation were tested in preconfluent and confluent bovine TM cells in culture. Rac1 and AKT/P-AKT activation were assessed by Western blot analysis. Trabecular outflow facility was measured in bovine perfused anterior segments. Changes in IOP were measured for up to 6 hours after topical application in the cornea of rabbit eyes by means of a contact tonometer.

Results. In TM cells, PDGF (10 ng/mL) activated Rac1 through AKT and induced actin cytoskeleton rearrangement with lamellipodia formation. In this sense, lamellipodia formation in TM cells was prevented by NSC23766, a Rac1 inhibitor, and LY294002, a PI3K inhibitor. In perfused anterior segments, PDGF (100 ng/mL) increased trabecular outflow facility by 26%. In vivo, when topically applied to rabbit corneas, PDGF induced a 20% decrease in IOP (100 ng/mL). This reduction was concentration dependent and presented an EC_{50} value of 2.7 nM.

Conclusions. PDGF, by activating the Rac1 pathway, induces cytoskeletal changes in TM cells that enhance outflow facility. Decreased IOP after PDGF application is likely caused by the facilitation of aqueous humor outflow. Rac1 pathway activation appears to be a positive modulator of outflow facility and an interesting target for decreasing IOP after ocular hypertension. (*Invest Ophthalmol Vis Sci.* 2009;50:3833–3839) DOI:10.1167/iovs.08-2924

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It is widely accepted that elevated intraocular pressure (IOP) is a major risk factor for primary open angle glaucoma. Increased IOP is caused by malfunction of the trabecular meshwork (TM), a tissue located in the anterior chamber angle of the eye that constantly drains aqueous humor out of the eye. Increased outflow resistance in the TM is probably multifactorial and depends on the functionality of TM cells, on extracellular matrix composition, and on several external factors in the aqueous humor that target the tissue. Among the different mechanisms regulating aqueous humor outflow, it is accepted that TM cells are able to modify their shape and contractility by means of an important actin cytoskeleton.

Actin cytoskeleton organization is tightly regulated by the balance among the activation level of different members of small GTPases family (e.g., RhoA, Rac, Cdc42). Although RhoA favors an increase of actin stress fibers and the formation of focal adhesions, Rac activation promotes membrane spreading and cell motility. In the TM many agonists, through the activation of protein kinase C (PKC) or the Rho GTPase pathway, induce actin polymerization and the formation of focal adhesions and adherens junctions, resulting in decreased outflow facility and increased IOP.[1] In contrast, inhibitors of Rho-kinase, myosin light chain kinase, and PKC decrease myosin light chain phosphorylation, inducing cell shape changes and tissue relaxation by means of actin depolymerization, loss of focal adhesions, and adherens junctions. These changes in the TM enhance aqueous humor outflow and support the development of therapeutic compounds to lower IOP.

It has been demonstrated that growth factor–induced membrane ruffling is mediated by Rac1 activation, which promotes the polymerization of short actin filaments at the plasma membrane level to form membrane ruffles and lamellipodial structures.[2] In addition, Rac1 has been implicated in phagocytic processes by activation of NADPH oxidase to generate superoxide.[3] To date, the role of Rac1 in the outflow pathway has not been studied in detail. Interestingly, the presence of several growth factors has been detected in the aqueous humor. In fact, different tissues surrounding the anterior and posterior chambers, such as the ciliary body epithelium, release growth factors (EGF, PDGF, IGF, TGF-α and TGF-β) to the aqueous humor that finally reach the TM.[4] For instance, TM cells express membrane receptors for many growth factors, as detected in tissue extracts from human patients.[5] In particular, TM cells respond to PDGF stimulation in culture by increasing cellular proliferation and metabolic rate.[5] Given that PDGF is a well-known activator of the Rac1 pathway, we studied PDGF effects on the TM cell cytoskeleton and its possible relationship with changes in aqueous humor outflow rate and IOP.

Materials and Methods

Culture of Bovine Trabecular Meshwork Cells

Bovine TM (BTM) cell cultures were performed using eyes from 3- to 6-month-old cows obtained at the local abattoir 0.5 hour to 2 hours after death and kept in PBS at 4°C for not more than 1.5 hours. A slight modification of the technique described by Stamer et al.[6] was used. As

described,[7] TM strips were digested with 2 mg/mL collagenase and 0.5 mg/mL bovine serum albumin (BSA) at 37°C for 2 hours. After trituration with fire-polished glass Pasteur pipettes, the supernatant was collected and centrifuged. The pellet was resuspended and seeded in culture flasks containing Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum, 100 mg/mL L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin-B. Cells reached confluence 12 to 15 days later. Cell passages were performed with the use of trypsin-EDTA. Cells from passages 1 to 3 were used. All products for cell culture were obtained from Sigma (Madrid, Spain).

After confluence, cells were plated in serum-containing media, at a density of 6×10^4 cells, onto 12-mm diameter glass acid-washed coverslips. Cell passages were performed using trypsin-EDTA. Three days after seeding, before the beginning of the experiment, cells were serum starved for 16 hours. Drugs were added to DMEM with or without serum, depending on the experimental protocol.

Immunocytochemistry

After treatment, cells were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 10 minutes, and washed four times with PBS. Blocking and permeabilization were performed with a solution composed of 0.1% TX-100, 2% BSA, and 2% goat serum and incubating the cells for 10 minutes. Next, cells were incubated at room temperature with phalloidin at 2 U/mL (Oregon-green; Molecular Probes, Eugene, OR) to visualize F-actin. Finally, cells were washed five times with PBS and preserved with mounting solution (Mowiol; Polysciences, Warrington, PA) for fluorescence microscopy imaging. Fluorescence images were obtained with an inverted microscope (IX70; Olympus, Tokyo, Japan) using a monochromator (TILL Photonics GmbH, Gräfelfing, Germany) as a source of illumination. Pictures were taken with an attached cooled charge-coupled device camera (Orca II-ER; Hamamatsu Photonics, Hamamatsu, Japan). Photographs were taken from representative fields. Pictures were analyzed with appropriate software (Orca II; Hamamatsu Photonics).

Membrane lamellipodia, stained with fluorescence-conjugated phalloidin, were easily identified as a thin protrusive sheet at the leading edge of the cell membrane, lacking actin stress fibers. We did not distinguish between membrane ruffles and lamellipodia with the use of immunocytochemistry techniques; only cells exhibiting clearly discernible flat lamellipodia were scored as positive. For each condition, an average of 250 to 300 cells from a minimum of three independent experiments were analyzed and quantified. Images were collected in a random fashion and were quantified by an experimenter masked to the treatment applied. Lamellipodia density was estimated as the number of lamellipodia divided by the total number of cells with lamellipodia. Values are expressed as mean ± SEM. Rac1 inhibitor (NSC23766) was purchased from Calbiochem (La Jolla, CA), LY294002 was obtained from Sigma, and RGD peptide was obtained from Genescript (Piscataway, NJ).

AKT and Rac Activation

Cells lysates were collected in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 5 mM NaF, 5 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 5 g/mL leupeptin, and 5 g/mL aprotinin), boiled at 85°C for 3 minutes, sonicated for 5 minutes, and clarified by centrifugation at 17,000*g*. Samples were subjected to SDS-PAGE and Western blot analysis, as described.[8] To quantify Rac activation levels, supernatants were incubated for 1 hour with GST-CRIB (Upstate Biotechnology, Lake Placid, NY) corresponding to the p21-binding domain (residues 67–150 of human PAK-1; expressed in *Escherichia coli* and bound to glutathione agarose). Samples were washed four times with lysis buffer and centrifuged. Pellets were boiled in 2× Laemmli buffer. Samples were separated on 10% SDS-PAGE gels, transferred to nitrocellulose paper, and probed with Rac1 antibody (clone 23A8; Upstate Biotechnology). Supernatants from samples were also run on SDS-PAGE gels and probed for total Rac1. The signal was detected with a chemiluminescence detection system (Pierce, Rockford, IL). Signal detection and density quantification were performed (LAS 3000 Image System; Fujifilm, Tokyo, Japan). All data were normalized to the starvation values. Antibodies used were Rac monoclonal (clone 23A8; reference 05–398 [Upstate Biotechnology]) and polyclonal antibodies against AKT and phospho-AKT (reference 4691 and 9271, respectively [Cell Signaling, Danvers, MA]). Quantification of AKT phosphorylation was normalized to the total amount of AKT. Rac-GTP levels were normalized to the total amount of Rac.

Intracellular Calcium Measurement

Measurement of cytosolic-free Ca^{2+} ($[Ca^{2+}]_i$) was performed as described in detail previously.[9] Briefly, bovine TM cells were plated on 25-mm diameter glass coverslips (VWR Scientific Inc., Philadelphia, PA), then loaded with 5 μM fura-2/AM (Calbiochem, San Diego, CA) for 25 minutes at 37°C in incubation buffer (140 mM NaCl, 4.3 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, at pH 7.4, with NaOH). Coverslips with fura-2 loaded cells were transferred into an open flow chamber (1 mL incubation buffer) mounted on the heated stage of an inverted microscope (IX70; Olympus) using a monochromator (TILL Photonics GmbH) as a source of illumination. Images were taken with an attached cooled charge-coupled device camera (Orca II-ER; Hamamatsu Photonics) and were digitized, stored, and analyzed on a personal computer with appropriate software (Aquacosmos; Hamamatsu Photonics). After a stabilization period of 10 minutes, image pairs were obtained alternately every 4 seconds at excitation wavelengths of 340 (λ1) and 380 nm (λ2; 10-nm bandwidth filters) to excite the Ca^{2+}-bound and Ca^{2+}-free, respectively, forms of this ratiometric dye. Emission wavelength was 510 nm (120-nm bandwidth filter). Typically, 10 to 20 cells were present in a field, and $[Ca^{2+}]_i$ values were calculated and analyzed individually for each single cell from the 340- to 380-nm fluorescence ratios at each time point.[9] Drug responses in each field were homogeneous, and several experiments with cells from different primary cultures were used in all the groups assayed.

Perfusion of Anterior Segments

Eyes from 3- to 6-month-old cows were obtained at the local abattoir 0.5 hour to 2 hours after death and were kept in PBS at 4°C for not more than 1.5 hours. Isolation and perfusion of bovine anterior segments were performed as previously described.[9,10] Briefly, bovine anterior segments, placed in a specially designed perfusion chamber, were located with force transducers (Letica, Barcelona, Spain) and the tubing system in an incubator (Selecta, Barcelona, Spain) at 37°C and 5% CO_2. Perfusion was carried out at constant pressure with DMEM. The pressure in the artificial anterior chamber was monitored and recorded throughout the experiment with a pressure transducer (9162-0; Mallinckrodt, Northampton, UK) and was maintained constant at 10 mm Hg by keeping the suspended medium reservoir at the appropriate height. Outflow facility was measured by recording the weight of the reservoir containing the perfusion medium with a force transducer. The decrease in weight of the medium reservoir was proportional to the amount of medium drained out of the anterior chamber through the outflow route. Outflow facility (*C*) was, therefore, calculated as the ratio between the inflow of perfusion medium into the anterior chamber (μL/min) and the perfusion pressure (mm Hg) recorded by a pressure transducer in the artificial anterior chamber. Only anterior segments with baseline outflow facility values between 0.3 and 1.3 μL/min/mm Hg were used. Moreover, anterior segments with baseline facility variability greater than 10% were rejected. Outflow facility was averaged during periods of 15 minutes (mean of 450 data points; sampling rate, 0.5 Hz). After a stabilization period of 30 to 45 minutes, outflow facility was recorded for 90 minutes to obtain the baseline facility (C_0). When drugs were added to the perfusion medium, the tubes and the anterior chamber were flushed and replaced with the new medium. This change was made by rapidly replacing the contents of the artificial anterior chamber by

opening the exit needle until 200% of the volume was exchanged; this exchange was always made at a pressure below 10 mm Hg. The same medium exchange procedure was performed in the control group with medium containing no drugs. Recording of *C* measurements restarted after stabilization of flow.

The perfusion procedure was carried out using a protocol with three periods: an initial perfusion period with control isotonic DMEM (301 ± 1 mOsm/kg) for 90 minutes to establish the C_0, a 150-minute period during which PDGF was introduced to determine changes in outflow facility, and a final period with DMEM to return to baseline conditions.

IOP Measurement in Rabbits

New Zealand White rabbits, each weighing 2 to 2.5 kg, were kept in individual cages with food and water ad libitum. They were submitted to controlled 12-hour light/12-hour dark cycles. All the protocols herein complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were in accordance with the European Communities Council Directive (86/609/EEC).

Formulation and Method of Administration

PDGF was formulated in isotonic saline and tested at the indicated concentrations. The compound was applied unilaterally to the cornea at a fixed volume of 10 μL. The contralateral eye received the same volume of saline (NaCl 0.9%, vehicle). Because the tonometry method could have produced discomfort in the rabbits, corneas were anesthetized by applying 10 μL of 1:10 (vol/vol) oxybuprocaine/tetracaine (4 mg and 1 mg respectively; Alcon-Cusi, Barcelona, Spain). Experiments were performed in a blinded design: no visible indication was given to the experimenter regarding the applied solution (agent or vehicle). IOP measurements were made with a contact tonometer (Tonopen XL; Reichert, Depew, NY) at baseline (pretreatment) and at the indicated times after the instillation of compound. IOP was monitored up to 6 hours to study the time-course of the effect. On any given day, only a single dose was tested on a single animal, which was washed out at least 2 days between doses. After topical application of PDGF to the rabbit ocular surface, no apparent change in corneal aspect, corneal thickness, or corneal transparency was observed. Concerning the general aspect of the eye, neither redness nor edema was detected when the eyes were observed under the biomicroscope before and after the application of PDGF (Supplementary Fig. S1, http://www.iovs.org/cgi/content/full/50/8/3833/DC1).

Statistical Analysis

Data are presented as mean ± SEM and were analyzed with the use of unpaired *t*-tests or two-way ANOVA and appropriate software (Prism 4.0; GraphPad, San Diego, CA). Two-tailed tests were used; statistical significance was set at $P < 0.05$.

Results

PDGF Effects on TM Cell Morphology

Activation of the Rac1 signaling pathway in fibroblasts and other cell types by growth factors in the serum induces the formation of lamellipodia and membrane ruffles through self-organized polymerization of the actin cytoskeleton.[2] To study the involvement of the Rac1 pathway in TM actin cytoskeleton dynamics and cell morphology, we first investigated the ability of TM cells in culture to generate lamellipodia in response to PDGF, an upstream Rac1 inductor of lamellipodia formation. After incubating TM cells in serum-starvation conditions for 16 hours to favor a quiescent state, the percentage of cells exhibiting lamella was compared in cells treated with PDGF (10 ng/mL), 10% FBS, or starvation conditions (control; Figs. 1A, B). The addition of PDGF rapidly increased the percentage of cells exhibiting lamella, which reached a maximum (approximately 40%) at 5 minutes and slowly declined to basal levels after 120 minutes. FBS, a well-known activator of the Rac1 pathway, induced similar behavior, though the effect was more pronounced (less than 60% of cells showed lamellipodia at 5 minutes). In contrast, starved cells showed only a basal number of lamellipodia (approximately 15%) during the studied time interval. Given that PDGF receptors are downstream linked to phosphatidylinositol-3-kinase (PI3K), we tested whether PI3K

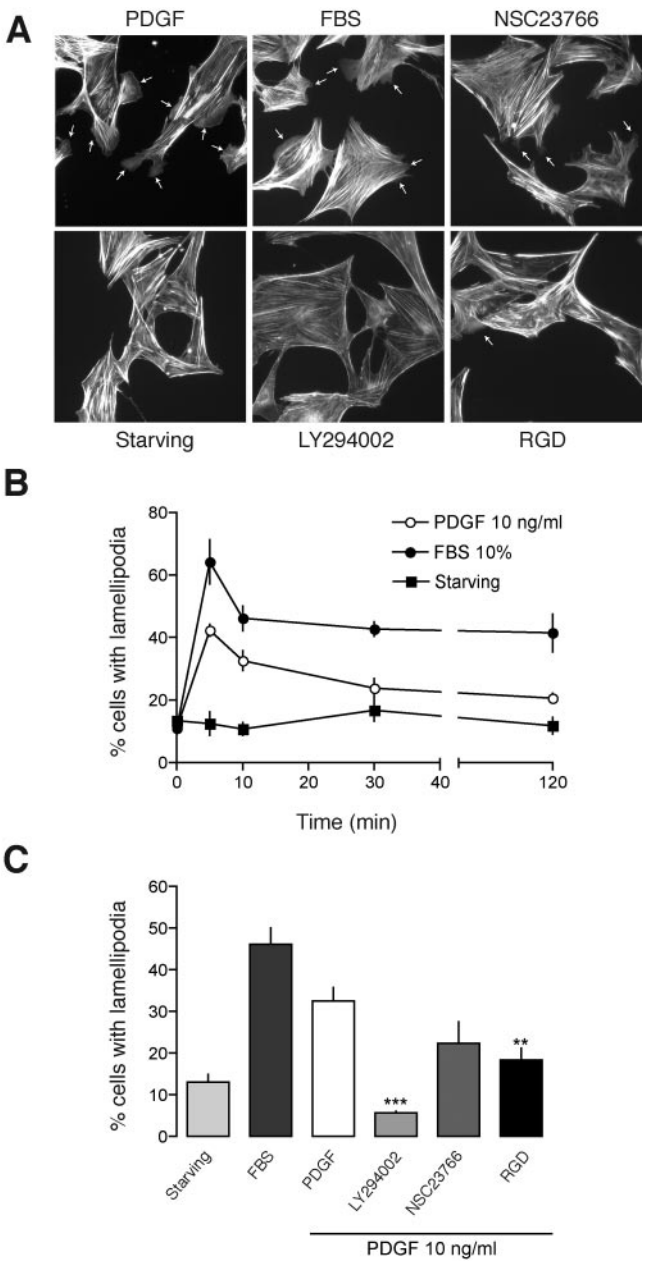

Figure 1. PDGF effects on TM cell actin cytoskeleton. (**A**) Actin-phalloidin staining of bovine TM cells in culture. Induction of lamellipodia formation (*white arrows*) after 5 minutes of treatment with PDGF (10 ng/mL), control starvation conditions, fetal bovine serum (FBS; 10%), cells pretreated with LY294002 (5 μM), NSC23766 (50 μM), or RGD (100 μM) plus PDGF. (**B**) Percentage of cells showing one or more lamellipodial structures over time. Each data point represents the mean ± SEM of four to seven different experiments. In each single experiment, between 250 and 300 cells were counted. (**C**) Quantification of the maximum percentage of cells displaying lamellipodia structures for each experimental group. $^{**}P < 0.01$; $^{***}P < 0.001$ (*t*-tests between PDGF alone and each treatment).

activation is required for lamella induction in TM cells. Treatment with LY294002 (5 μM), an inhibitor of PI3K, markedly reduced the percentage of cells showing lamellipodia compared with PDGF alone ($P < 0.001$; Figs. 1A, C). Despite the lack of good Rac1 inhibitors, we used NSC23766 (50 μM), which was been reported to inhibit Rac1-Tiam1 activation in certain cell types.[11] Our results show a slight reduction of lamella formation after pretreatment with NSC23766, though the effect was not statistically significant (Figs. 1A, C). To further elucidate the involvement of Rac1, and according to studies showing that integrins are necessary to keep Rac1 activated,[12] we treated TM cells with an RGD peptide (100 μM), which impaired integrin-ligand interaction. As expected, preincubation with the RGD peptide significantly reduced lamella formation by PDGF (Figs. 1A, C), implying the involvement of integrins in Rac1-mediated lamellipodia stabilization.[13]

Further evidence of PI3K-AKT-Rac1 pathway involvement in lamellipodia formation by PDGF was obtained by studying the percentage of Rac activation (Rac-GTP) over total Rac in TM cells. After PDGF treatment of preconfluent TM cells, we found a 50% increase (Fig. 2A; $n = 3$) in the amount of Rac-GTP compared with cells in starvation conditions. In addition, a 22% increase in Rac activation by PDGF was also found in TM cells grown to confluence for 2 weeks to mimic the conditions in the tissue (Fig. 2A; $n = 3$). Similarly, PDGF treatment significantly increased the phosphorylation levels of AKT compared with starvation conditions, both in preconfluent and confluent TM cells (Fig. 2B; $n = 3$). Similar results were described in a previous report after treatment with FBS in the same cell type.[8]

Activation of PDGF receptors has also been linked to sustained increases of intracellular calcium in other cell types,[14,15] which are thought to permit a calcium-dependent activation of several intracellular mediators of PDGF effects. To elucidate whether intracellular calcium is involved in PDGF signaling, we measured intracellular calcium levels in TM cells after treatment with this growth factor and found that 30.6% of cells ($n = 72$) showed a delayed significant increase in intracellular calcium (Figs. 3A, B). After an initial peak, calcium levels returned to basal values over hundreds of seconds, suggesting the activation of a sustained calcium entry (capacitative), as previously described in TM cells for other compounds.[16] In this sense, the application of La^{3+}, a well-known blocker of capacitative calcium entry, rapidly inhibited calcium inflow (Figs. 3A [second panel], B), which supports the hypothesis that PDGF promotes the opening of membrane channels to induce a prolonged calcium entry. Other substances known to mobilize calcium, such as endothelin (ET)-1, bradykinin (BK), and thapsigargin (TG), induced more rapid increases in intracellular calcium in 100% of cells tested ($n = 25$, 20, and 27, respectively), as can be measured by the delay in the response from drug application to the maximum calcium increase (Figs. 3A, C). These results suggest that though ET-1, BK, and TG induce a rapid release of calcium from the intracellular stores followed by a sustained calcium entry,[16] PDGF probably only activates slow-capacitative calcium-entry mechanisms.

PDGF Effects on Outflow Facility and Intraocular Pressure

Previous studies in TM cells have shown that the induction of lamella formation by profilin I was able to increase outflow facility in ocular anterior segments perfused in vitro and to decrease IOP after topical corneal application.[8,17,18] In the present study, we tested whether similar cytoskeletal rearrangements induced by PDGF were also capable of producing similar effects. We perfused bovine anterior segments in vitro, as previously described,[9,19] and challenged them with PGDF. After an initial baseline period, outflow facility started to increase significantly within 15 minutes of the addition of PDGF and over a period of 1 hour and remained elevated for the rest of the experimental time, even after PDGF was washed out from the perfusion medium (100 ng/mL; $n = 6$; Fig. 4A). A maximum increase of 26% over the baseline outflow facility ratio was obtained in the washout period, but similar values were recorded in the presence of PDGF in the perfusion medium. We did not further extend the experiments after 330

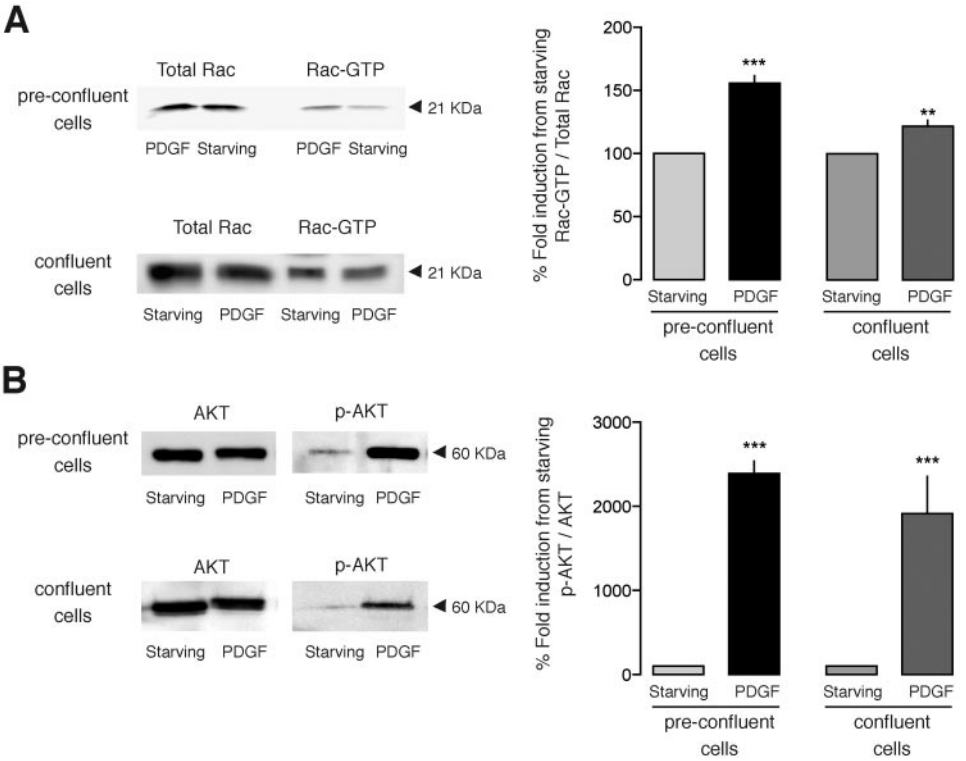

FIGURE 2. Activation of Rac and AKT by PDGF. (**A**) TM cells in culture were treated with PDGF (10 ng/mL) for 10 minutes, and total and GTP-bound forms of Rac protein were detected by Western blot analysis in preconfluent cells and cells grown to confluence for 2 weeks. *Left*: representative membranes showing total Rac and Rac-GTP in cells treated with PDGF or cells in starvation conditions. *Right*: mean of three different experiments showing the percentage fold induction of Rac-GTP compared with total Rac in preconfluent and confluent cells. (**B**) Effect of PDGF treatment on AKT phosphorylation. *Left*: representative membranes showing the phosphorylated form of AKT compared with total AKT in preconfluent and confluent cells. *Right*: mean of three different experiments showing the percentage fold induction of phospho-AKT compared with total AKT in preconfluent and confluent cells. $^{***}P < 0.001$; $^{**}P < 0.01$ (*t*-tests between PDGF treatment and starvation conditions).

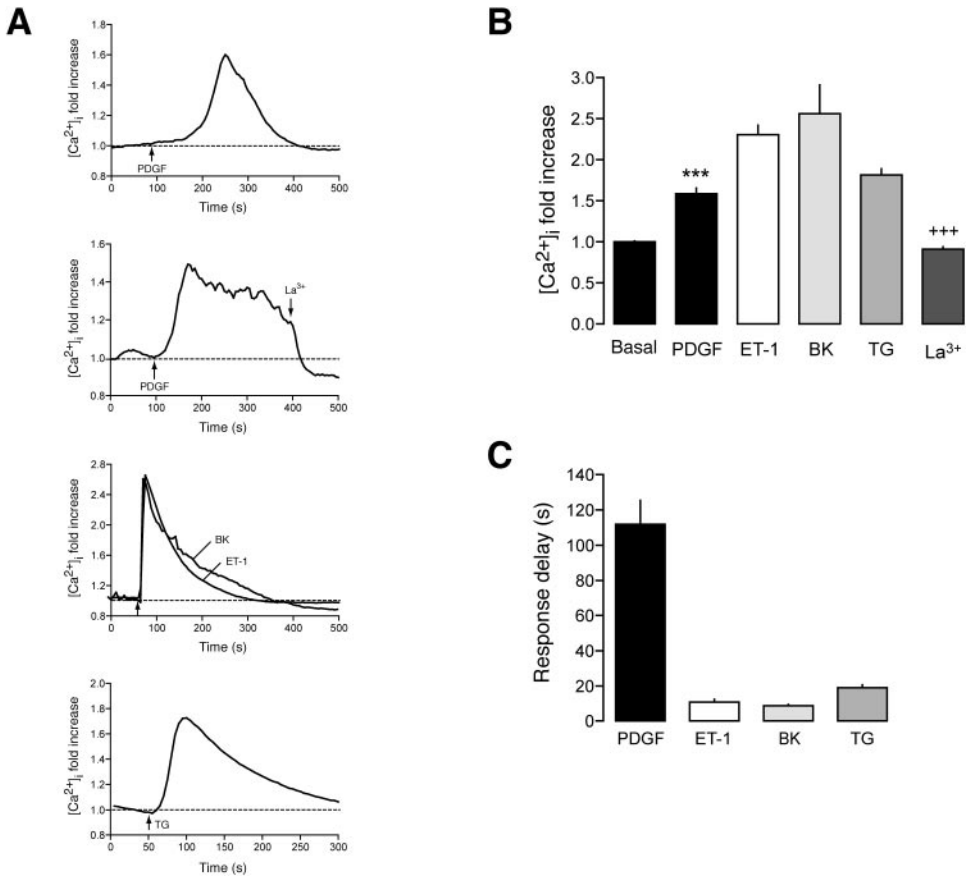

FIGURE 3. Effects of PDGF on intracellular calcium. (**A**) Representative experiments of cells treated with PDGF (10 ng/mL), ET-1 (0.1 μM), bradykinin (BK; 1 μM), or thapsigargin (TG; 1 μM). (**B**) Quantification of maximum fold increase in intracellular Ca^{2+} concentration over baseline after treatment with PDGF, ET-1, BK, and TG. In the second example, after the addition of PDGF, La^{3+} (100 μM) was added to block the sustained Ca^{2+} entry. $^{***}P < 0.001$ (*t*-test PDGF vs. basal calcium); $^{+++}P < 0.001$ (*t*-test PDGF vs. La^{3+} application). (**C**) Mean delay time between drug application and maximum Ca^{2+} increase for each experimental group.

minutes of perfusion to prevent the washout of proteins from the tissue, which would mask the effects of the drug. In comparison, the control group ($n = 10$; Fig. 4A) showed only a slowly developing washout effect that was more evident after 270 minutes of perfusion. Statistically significant differences between both groups were found ($P < 0.01$; two-way ANOVA).

To study a possible correlation between the increase in outflow facility observed and any effects on IOP, we tested whether topical application of different concentrations of PDGF to the cornea were able to modify this parameter. Although saline buffer application was unable to significantly modify IOP in rabbit eyes (Fig. 4B, solid dots; $n = 6$), PDGF application (100 ng/mL) reduced IOP by approximately 20% after 2 hours (Fig. 4B, open dots; $n = 6$). IOP values remained lower than baseline over a period of 4 to 5 hours and recovered basal values within 6 hours of application. To further characterize PDGF activation effects over IOP, we constructed a dose-response curve. Fitting of the experimental data showed an EC_{50} of 2.7 ng/mL (Fig. 4C).

DISCUSSION

It is commonly accepted that the structure of the TM and the functionality of its cells are critical for maintaining a physiological outflow rate.[20,21] In this sense, TM cells present an important cytoskeleton that maintains the correct cytoarchitecture of the tissue, allowing the passage of aqueous humor through the TM to reach the Schlemm canal. Changes in the structure of the TM impair a correct outflow rate, leading to an increased IOP and often to the development of glaucoma. Many extracellular signals, through specific membrane receptors, activate downstream effectors to modulate the actin cytoskeleton and, in consequence, cell morphology. In the TM, the small GTPase RhoA plays a prominent role in the remodeling of the actin cytoskeleton, inducing stress fibers, forming focal adhesions and adherens junctions, and decreasing aqueous humor outflow rate.[1] On the contrary, inhibitors of PKC, Rho-kinase, or myosin light chain kinase produce opposite effects, enhancing aqueous humor outflow, and are interesting candidates for use as antiglaucomatous drugs (Rao PV, et al. *IOVS* 2007;48:ARVO E-Abstract 1144). Less attention has been paid to another small GTPase, Rac1, which is also involved in actin cytoskeleton remodeling. Among other substances, several growth factors, such as PDGF, are well-known activators of the Rac1 pathway. In fact, several growth factors are present in the human aqueous humor, including PDGF (0.3 ng/mL).[22] Receptors for these substances are expressed in TM tissue extracts from healthy and glaucomatous donors and in TM cells in culture.[5] Moreover, it has been reported that PDGF induces the proliferation of TM cells in culture and the acidification of extracellular medium,[5] and it enhances cellular phagocytic activity.[23] Here, we have found that PDGF induces a remodeling of actin that accompanies an extension of lamellipodia through the activation of PI3K and Rac1 (Figs. 1, 2). These results are in agreement with the thread-like patterns, membrane ruffles, and actin ribbons previously described after PDGF exposure of TM cells.[23] In addition, the observed induction of lamellipodial structures by PDGF and their well-known involvement in cellular movement supports the reported effects of PDGF and aqueous humor samples on cell migration.[24]

In addition to PI3K and Rac1 activation, we found that PDGF induces intracellular Ca^{2+} mobilization through the opening of capacitative calcium channels, as suggested by La^{3+} experiments (Fig. 3). Many compounds (ET-1, adenosine triphosphate [ATP]) induce the fast release of calcium from

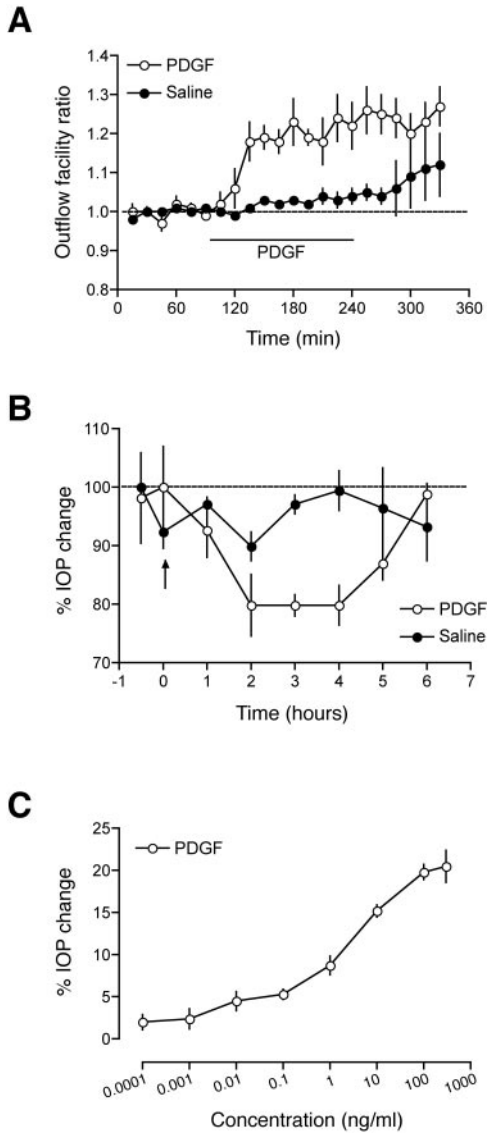

FIGURE 4. Effects of PDGF on outflow facility and IOP. (**A**) Outflow facility ratio (normalized with baseline outflow facility) is plotted against time in perfused bovine anterior segments. Outflow facility increased significantly after 15 minutes of PDGF addition to the perfusion medium (100 ng/mL; $n = 6$) and remained elevated for the remaining experimental time. In contrast, control anterior segments perfused with DMEM showed only moderated washout effect after several minutes of perfusion ($n = 10$). (**B**) A single instillation of PDGF (100 ng/mL) into rabbit eyes produced a sustained decrease on IOP compared with control eyes treated with saline. IOP values remained below control values for 4 to 5 hours, gradually recovering to baseline levels. (**C**) Dose response of PDGF-induced decreases in IOP in the rabbit eye. Each data point represents the mean ± SEM of six different eyes treated with a single concentration of PDGF.

intracellular stores, followed by activation of membrane channels that permit extracellular calcium entry (also known as capacitative calcium entry).[9,16] PDGF does not release calcium from intracellular stores, but it activates extracellular calcium entry. Similar results have been described in liver myofibroblasts, in which PDGF induces slower and sustained Ca^{2+} mobilization compared with the effects of ET-1 and ATP.[25] Ca^{2+} increases induced by PDGF have a role in cell migration and also in cell proliferation signaling to the nucleus by means of STAT kinases.[26,27] A possible correlation between increases in intracellular Ca^{2+} and lamellipodia induction remains to be studied because a similar percentage of cells (approximately 30%) increased intracellular Ca^{2+} and presented lamellipodia structures after PDGF treatment.

Our data showed that the addition of PDGF to ocular anterior segments perfused in vitro produced a marked increase in aqueous humor outflow rate (Fig. 4). Similar effects were found after the transduction of profilin I, an actin-binding protein that induces important lamellipodia formation in cultured TM cells and increases outflow facility in perfused anterior segments.[8,17] Profilin effects on cell morphology are similar to those of PDGF, but profilin appears to act downstream of Rac1 activation by binding directly to actin because a mutated form of the protein in the actin-binding domain abolishes its effects on lamellipodia formation.[8] In contrast, PDGF appears to activate a complete intracellular signaling cascade from the membrane receptor to the effectors linked to modulation of the actin cytoskeleton (Figs. 1, 2). In a similar manner, caldesmon, a calmodulin-binding protein that inhibits myosin II interaction with actin, produces a remarkable reorganization of the cytoskeleton in TM cells after viral transgene expression.[28] In control conditions, most TM cells present abundant actin stress fibers and a minor proportion of lamellipodial structures, but caldesmon overexpression decreases actin stress fibers and increases the percentage of cells displaying lamellipodia and membrane ruffles.[28] The observed cytoskeletal reorganization produces a significant increase in outflow facility in human and monkey anterior segments after caldesmon overexpression,[29] similar to the effects encountered after the transduction of profilin or by PDGF treatment. All these effects may result from changes in cell morphology that favor cellular relaxation and alteration of the geometry of the outflow pathway to enhance aqueous humor passage between trabecular beams and through the inner wall of the Schlemm canal. Other pharmacologic strategies have led to similar effects on outflow facility after impairing RhoA signaling by inhibiting protein geranylgeranyl transferase type I with GGTI-DU40, which induces changes in TM cell morphology by decreasing actin stress fibers, focal adhesions, and adherens junctions.[30] Cholesterol-lowering statins also inhibit geranylgeranylation and impair RhoA signaling. In endothelial cells, simvastatin produces a decrease on myosin light chain phosphorylation but increases activated Rac1 levels; both effects contribute to diminish the endothelial barrier permeability to thrombin treatment.[31] In consequence, it is possible that the inhibition of RhoA signaling and the activation of Rac1 contribute to the enhanced outflow facility reported after treatment with statins in pig eyes.[32]

Apparently, RhoA and Rac1 pathways are mutually antagonistic in TM cells, as proposed in other cell models.[33] Compounds activating RhoA kinase induce actin polymerization and the formation of focal adhesions and adherens junctions and decrease outflow facility.[1] In contrast, factors activating the Rac1 pathway induce changes in cell shape; promote cell relaxation, loss of focal adhesions, and lamellipodia formation; and increase outflow facility. In this sense, PDGF, together with other growth factors or substances in the aqueous humor, may have a role in the maintenance of a physiological outflow rate through activation of the Rac1 pathway. These effects would counteract the action of other compounds, such as ET-1 and muscarinic agents that induce opposite effects by activating RhoA kinase.

In addition to the effects on the outflow pathway, PDGF application into the rabbit eye reduces IOP for several hours (Fig. 4). The effect is dose dependent and is likely to be mediated, at least in part, by an increase in outflow facility. Again, similar results were previously described after the transduction of profilin, indicating that the enhancement of aqueous outflow is important enough to decrease IOP. Although the

effect of PDGF on IOP was clear when it was applied topically to the eye, inflammation and histologic changes were not observed after the application of this growth factor (Supplementary Fig. S1, http://www.iovs.org/cgi/content/full/50/8/3833/DC1). We wondered, therefore, how PDGF acts within the eye and whether it can pass through the cornea. Further research will be necessary to fully investigate this point.

In conclusion, our data indicate that pharmacologic compounds activating this route may be used alone or in combination with other available therapies to decrease IOP and to prevent further optic nerve damage in patients with glaucoma.

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