ARTICLE
Journal of Cellular Biochemistry 112:600–606 (2011)

Journal of Cellular Biochemistry

Rho GTPase-Mediated Cytoskeletal Organization in Schlemm's Canal Cells Play a Critical Role in the Regulation of Aqueous Humor Outflow Facility

Janardan Kumar[1*] and David L. Epstein[2]

[1]*Department of Natural Sciences, Becker College, Worcester, Massachusetts 01609*

[2]*Department of Ophthalmology, Duke University Medical Center, Box 3802, Durham, North Carolina 27710*

ABSTRACT

The increased intraocular pressure (IOP) has been considered to be an increased resistance of the aqueous humor outflow through the inner wall of Schlemm's canal (SC) and/or the juxtacanalicular tissue (JCT). The Rho GTPase-regulated actomyosin organization appears to be an important mechanistic determinant of aqueous humor outflow facility. Therefore, in this study, we have evaluated the effects of modulating Rho GTPase activity on actomyosin cytoskeletal organization, monolayer permeability/barrier function of human SC cells, and aqueous humor outflow facility in enucleated porcine eyes ex vivo. Human SC cells, isolated from cadaver eyes, were treated with either Rho GTPase activators such as thrombin and lysophosphatidic acid (LPA), or a specific inhibitor (C3-exoenzyme) of Rho GTPases. Treatment of SC cells with thrombin and LPA led to increased formation of stress fibers, focal adhesion, and increased myosin light chain phosphorylation, whereas treatment with C3-exoenzyme showed the opposite effects like H-7 and ECA, known for increasing the outflow facility in porcine eyes. The findings presented here suggest that LPA and thrombin, presumably through activation of Rho GTPase-mediated actomyosin cytoskeletal reorganization in SC cells, cause a decrease in monolayer permeability of SC cells as well as a decrease in outflow facility of porcine eyes in ex vivo. Our results suggest that decrease in aqueous humor outflow may be correlated better with the changes in cytoskeletal organizations of SC, which could be the prime locus of the outflow resistance. J. Cell. Biochem. 112: 600–606, 2011.

KEY WORDS: ENDOTHELIAL CELL PERMEABILITY; RHO GTPase; CYTOSKELETAL ORGANIZATION

Primary open angle glaucoma is commonly associated with increased intraocular pressure (IOP), which is thought to derive from an increased resistance to aqueous humor outflow through the trabecular meshwork and Schlemm's canal (SC) [Epstein, 1997; Johnson and Erickson, 2000]. Very little is currently known regarding the molecular and cellular mechanisms involved in the maintenance of aqueous humor outflow resistance. Considerable evidence from several laboratories suggests that the inner wall of SC and/or the juxtacanalicular tissue (JCT) is the locus of both normal and abnormal aqueous outflow resistance [Maepea and Bill, 1992; Ethier et al., 1995] and that disruption of the inner wall of SC integrity can be correlated with increased outflow facility [Lindenmayer et al., 1983; Hamanaka and Bill, 1987] Both aqueous humor flow through the paracellular pathway of SC cells [Epstein and Rohen, 1991] and/or funneling mechanisms involving secondary effects of SC "porosity" on JCT flow pathways [Johnson et al., 1992] have been hypothesized to be involved in outflow resistance modulation.

Cytoskeleton-interacting agents, including those which depolymerize the actin as well as microtubule networks, have been observed to alter outflow resistance in various in vitro perfusion models and in in vivo systems [Kaufman and Barany, 1977; Epstein et al., 1987, 1999; Tian et al., 1998]. Mechanisms involving cellular contraction and relaxation and the function of the intercellular junctions have been proposed [Johnstone et al., 1980; Erickson-Lammy et al., 1992; Citi et al., 1994; Gills et al., 1998; Pierzchalska et al., 1998]. Such cellular contraction and relaxation phenomena are typically regulated by the actomyosin system and the phosphorylation status of the myosin light chain (MLC) [Kaufman and Barany, 1977; Tian et al., 1998; Epstein et al., 1999]. Interestingly, both microtubule disrupting agents such as ethacrynic acid (ECA) and vinblastine, which elicit cellular contraction, as well

Abbreviations used: TM, trabecular meshwork; SC, Schlemm's canal; MLC, myosin light chain; LPA, lysophosphatidic acid.

Grant sponsor: NIH; Grant number: RO1 EY01894; Grant sponsor: Research to Prevent Blindness Research Fund.

*Correspondence to: Janardan Kumar, Department of Natural Sciences, Becker College, Worcester, MA 01606. E-mail: janardan.kumar@becker.edu

Received 10 October 2010; Accepted 28 October 2010 • DOI 10.1002/jcb.22950 • © 2010 Wiley-Liss, Inc.
Published online 22 November 2010 in Wiley Online Library (wileyonlinelibrary.com).

as F-actin depolymerizing agents, cytochalasins, 1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine (H-7), and latrunculin, which lead to cell relaxation, have been found to increase outflow facility [Kaufman and Barany, 1977; Epstein et al., 1987, 1999; Tian et al., 1998]. These observations underscore the importance of delineating which of these phenomena—cellular contraction or relaxation, or both, play a critical role in the regulation of outflow facility.

Members of the Rho GTPase family of small GTP-binding proteins play a critical role in the organization of the actin cytoskeleton, formation of focal adhesions, cell motility, and cellular contraction and relaxation events [Ridley, 1996; Kranenburg et al., 1997; Hall, 1998]. Rho GTPase modulates the organization of actin cytoskeleton via the activation of the Rho kinase/LIM kinase/cofilin pathway [Ridley and Hall, 1992; Maekawa et al., 1999]. Additionally, Rho GTPase has also been shown to influence cellular contraction directly by regulating the state of MLC phosphorylation [Lum and Malik, 1994; Garcia and Schaphorst, 1995]. Previously we have reported the influence of Rho kinase-regulated MLC phosphorylation on human trabecular meshwork (HTM) cells and aqueous humor outflow facility [Mettu et al., 2004]. In this study we sought correlations in SC cells between Rho GTPase-mediated cytoskeletal activation, MLC phosphorylation, and monolayer permeability characteristics, in SC cells. The effects of Rho GTPase activating agents (thrombin and lysophosphatidic acid—LPA) on aqueous outflow facility (the inverse of resistance) were also studied using the enucleated porcine eye perfusion model system.

MATERIALS AND METHODS

MATERIALS

Rabbit polyclonal antibody directed against MLC was a gift of Joe G. Garcia of John Hopkins University (Baltimore, MD). LPA, ECA, H-7 (1-(5-isoquinolinylsulfonyl) 2-methylpiperazine dihydrochloride), rhodamine-phalloidin, anti-paxillin monoclonal antibody, and TRITC-conjugated anti-mouse or rabbit IgG antibodies were purchased from Sigma–Aldrich (St. Louis, MO), C3 (-) exoenzyme and thrombin was purchased from Cytoskeleton, Inc. (Denver, CO) and CalBiochem (San Diego, CA), respectively. The ECL plus Western blotting system was from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). All the reagents were from the highest quality available.

SC CELL CULTURES

SC cells were isolated from human cadaver eyes obtained from the National Disease Research Interchange (Philadelphia, PA), by following the procedures as established in our laboratory [Stamer et al., 1998]. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate (all from GibcoBRL, Gaithersburg, MD) as described earlier [Rao et al., 2001]. Primary cultures of SC cells passaged between three and five times were used in these studies.

STAINING OF ACTIN CYTOSKELETON AND FOCAL ADHESIONS

To record thrombin, LPA, and C3-exoenzyme induced changes in cytoskeletal reorganization, SC cells were grown on 2% gelatin-coated coverslips and maintained in complete media until the population of cells reached confluence. Cells were serum starved for 48 h wherever necessary and treated with thrombin and LPA, prior to being fixed and stained for cytoskeletal proteins [Pierzchalska et al., 1998]. Prior to use of thrombin in particular, it was necessary to make sure if the reagent had no common additives such as EDTA. F-actin was stained with rhodamine–phalloidin and focal adhesions were stained with anti-paxillin antibody and a rhodamine-conjugated secondary antibody as described previously [Rao et al., 2001]. Experiments carried out with C3-exoenzyme pretreatment were maintained in the presence of serum (10%) for 48 h and subsequently, treated either with LPA or thrombin for 1 h.

MYOSIN LIGHT CHAIN PHOSPHORYLATION

Changes in SC cell MLC phosphorylation were assayed qualitatively by following the procedure of Garcia et al. [1995]; using urea-glycerol gel electrophoresis method. Briefly, cells were grown for 8 days in 60 mm Falcon Petri dishes containing complete medium and then treated with different agents for 1 h at 37°C in a CO_2 incubator. Cells were then washed with DMEM medium and total cellular protein precipitated with addition of 10% ice-cold trichloroacetic acid (TCA). Cell pellets were washed and subjected to MLC phosphorylation detection by urea-glycerol gel electrophoresis, followed by Western blot analysis using MLC polyclonal antibody as described previously [Rao et al., 2001].

MEASUREMENT OF INTERCELLULAR BARRIER PROPERTIES

Horseradish peroxidase (HRP) diffusion through SC cell monolayers was determined using transwell cell culture chambers (collagen-coated polycarbonate filters, 3 μM pore size, Becton Dickinson, Bedford, MA). The upper chambers of transwell cell culture chambers were seeded with SC cells in complete culture medium, while the lower chambers were filled with culture medium alone. Cultures were maintained for 10 days and medium replaced once in every 2 days. Treatment of SC cell monolayers with thrombin or LPA was initiated by replacement of the complete medium (500 μl) in the upper chamber with medium containing HRP (0.126 μM; HRP, VI-A type, Sigma) with appropriate agents, whereas the lower chamber being replaced with 600 μl fresh medium. After incubation at 37°C for 1 h, medium from the lower chamber was collected and assayed for HRP enzyme activity. HRP activity was determined colorimetrically as described by Lampugnani et al. [1991]. Results were expressed as percent change in enzyme activity over untreated controls. A paired *t*-test analysis was performed to assess the significance of differences between the treated versus untreated control samples.

OUTFLOW FACILITY MEASUREMENT

Pig eyes were obtained from a commercial abattoir and perfusion of thrombin or LPA was performed by the standard constant-pressure perfusion technique using a Grant stainless steel corneal fitting [Epstein et al., 1997, 1999]. Radial iridectomies were performed to prevent artificial deepening of the anterior chamber. The perfusion medium, Dulbecco's phosphate-buffered salt solution (DPBS; GibcoBRL), 5.5 mM D-glucose was filtered through a 0.2 mm filter, Nalgene Co. (Rochester, NY). The baseline facility was determined

after perfusion of the eye at 15 mmHg at 25°C for 1 h to get a steady state flow value. The corneal Grant fitting was then removed, the anterior chamber solution was replaced using a cellulose sponge; and then refilled with either the perfusion buffer as sham control or the drug containing solution in paired fellow eyes. The perfusion was continued for 5 h and measurements of outflow facility were recorded at hourly intervals. Drug effects were expressed as the percentage change in outflow facility (as compared to baseline values) over 5 h, in drug-treated versus untreated control paired fellow eyes. Values were expressed as means ± SE. Statistical significance was evaluated by a paired two-tailed Student's *t*-test.

RESULTS

THROMBIN AND LPA-INDUCED CYTOSKELETAL CHANGES

To examine whether agents that activate or inhibit Rho GTPase-dependent signaling cause cytoskeletal changes in SC cells, the ability of human thrombin and LPA to elicit formation of actin stress fibers and focal adhesions was assessed. Serum starvation alone decreased filamentous actin and focal adhesion staining as compared to controls maintained in complete medium (Figs. 1 and 2). Treatment of serum-starved SC cells with thrombin (1.0 U/ml) or LPA (20 μM) led to a dramatic induction in the formation of stress fibers and focal adhesions (Fig. 1C,E and D,F) compared to sham-treated controls (Fig. 1A,B). Treatment of SC cells (maintained in complete medium) with 10 μg/ml C3-exoenzyme for 48 h led to decreases in actin stress fibers (Fig. 2C) and focal adhesions (Fig. 2D),

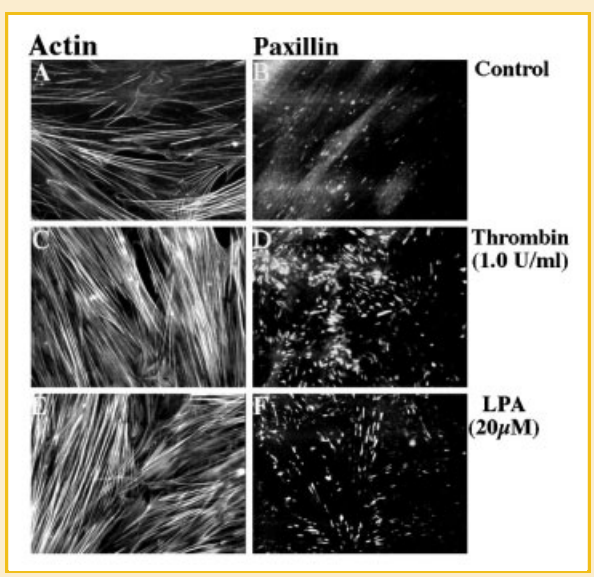

Fig. 1. Thrombin and LPA-induced cytoskeletal changes in SC cells. Serum-starved SC cells (for 48 h) were treated with either thrombin (1.0 U/ml) or with LPA (20 μM) for 1 h in serum-free medium and stained for actin stress fibers and focal adhesions. The panel on the left illustrates changes in actin cytoskeleton: (A) Control cells, (C,E) are cells treated with thrombin and LPA, respectively. The panel on the right shows the changes in focal adhesions. (B) Control cells, (D,F) are cells treated with thrombin and LPA, respectively. Thrombin or LPA treatment increases actin stress fiber and focal adhesion formation in SC cells. Original magnification, ×500.

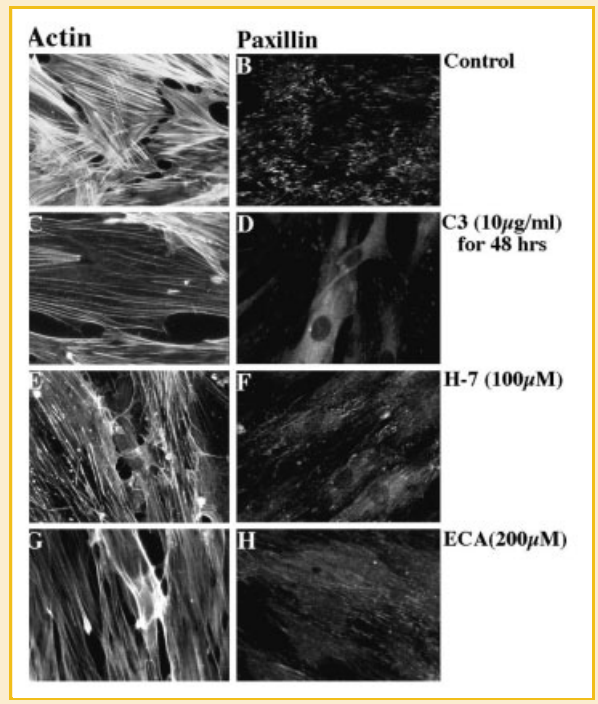

Fig. 2. C3-exoenzyme, H7, and ECA all induce reorganization of actin stress fibers formation and focal adhesions in SC cells. SC cells grown in complete medium were treated with C3-exoenzyme (10 μg/ml for 48 h), H-7 (100 μM for 1 h), or ECA (200 μM for 1 h), in complete medium and stained for F-actin and focal adhesions. The panel on the left shows changes in actin staining; (A) control cells, (C,E,G) are representative profiles from C3, H-7, and ECA-treated cells. The panel on the right shows changes in focal adhesions. (B) Control cells, (D,F,H) are C3-exoenzyme, H-7, and ECA-treated cells, respectively. C3, H-7, and ECA treatment reduce cellular content of actin stress fibers and focal adhesions compared to untreated control cells. Original magnification ×500.

compared to control cells (Fig. 2A,B). Exposure of SC cells to either H-7 (100 μM) or ECA (200 μM) also decreased the content of cellular actin stress fibers (Fig. 2E,G, respectively) and focal adhesions (Fig. 2F,H, respectively), in SC cells. To confirm whether thrombin and LPA-induced changes in actin staining and focal adhesions were mediated through Rho GTPase signaling, confluent cultures of SC cells were pretreated with C3-exoenzyme (10 μg/ml) in the presence of serum for 48 h, then treated with thrombin (1.0 U/ml) or LPA (20 μM) for 1 h as described above. Following this, cells were fixed and stained for actin and focal adhesions. In these experiments, thrombin and LPA failed to induce changes in actin or focal adhesions (data not shown) indicating the requirement of active Rho GTPase for the thrombin and LPA-mediated cytoskeletal changes in SC cells.

CHANGES IN MYOSIN LIGHT CHAIN PHOSPHORYLATION

Treatment of SC cells (starved for 48 h) with thrombin (1.0 U/ml) or LPA (20 μM) for 1 h in the absence of serum, stimulated phosphorylation (di-phosphorylated form) of the MLC (Fig. 3A; lanes 2 and 3) compared to control (Fig. 3A; lane 1), while C3-exoenzyme (10 μg/ml) treatment for 48 h in the presence

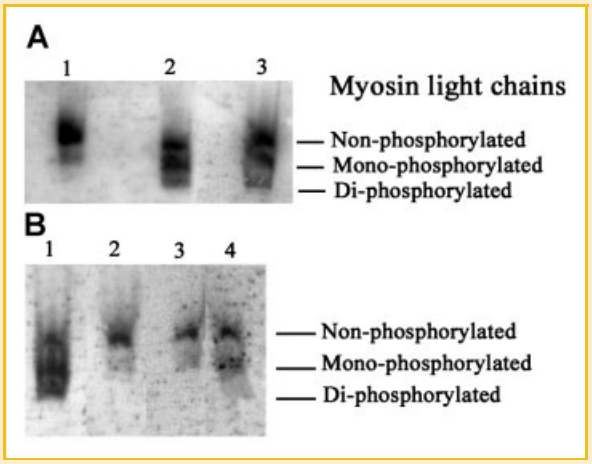

Fig. 3. Changes in myosin light chain phosphorylation in SC cells. Western blot showing the changes in myosin light chain phosphorylation in SC cells treated with thrombin, LPA, and C3-exoenzyme. Panel A: Depicts the effects of thrombin or LPA on SC cell myosin light chain phosphorylation: lane 1, control SC cells (serum starved for 48 h); lane 2, treated with thrombin (1.0 U/ml) for 1 h; lane 3, treated with LPA (20 μM) for 1 h under serum-free condition. Panel B: lane 1, control SC cells; lane 2, treated with C3-exoenzyme (10 μg/ml) for 48 h in complete medium; lanes 3 and 4, pretreatment of C3-exoenzyme for 48 h followed by addition of thrombin (1.0 U/ml for 1 h) or LPA (20 μM for 1 h), respectively. Under serum-free conditions, MLC was present mostly as the dephosphorylated form as shown in lane 1 of panel A. When SC cells were treated with either thrombin or LPA as shown in lanes 2 and 3, phosphorylation of myosin light chains (di-phosphorylated form) was increased. Treatment of SC cells in complete medium with C3-exoenzyme decreased myosin light chain phosphorylation (lane 2 in panel B) and addition of thrombin or LPA to C3-exoenzyme-pretreated SC cells showed no change from the C3 alone induced effects on myosin light chain phosphorylation (lanes 3 and 4 in panel B).

of serum had the exact opposite effect (Fig. 3B; lane 2). In contrast, pretreatment of SC cells with C3-exoenzyme (10 μg/ml for 48 h) failed to induce MLC phosphorylation (di-phosphorylated form) with thrombin (1.0 U/ml) or LPA (20 μM) for 1 h (Fig. 3B; lanes 3 and 4).

CHANGES IN SC CELL MONOLAYER PERMEABILITY CHARACTERISTICS

To explore whether thrombin, LPA, or C3-exoenzyme affect permeability characteristics of SC cell monolayers, we conducted diffusion studies using HRP as a tracer molecule. The results presented in Figure 4 indicate that the Rho-GTPase activators, thrombin (1.0 U/ml), and LPA (20 μM), each caused a decrease in SC monolayer permeability, as measured by HRP diffusion, by $24 \pm 6\%$ ($n = 6$; $P < 0.03$) and $29 \pm 4\%$ ($n = 8$; $P < 0.01$), respectively. C3-exoenzyme, which specifically inactivates $^{Rho-}$GTPase, produced an increase in SC cell permeability of $44 \pm 5\%$ ($n = 5$; $P < 0.013$), compared to untreated controls. Both H-7 (100 μM) and ECA (200 μM) increased SC cell permeability by $57 \pm 5\%$ ($n = 6$; $P = 0.001$) and $96 \pm 7\%$ ($n = 6$; $P = 0.0002$), respectively (Fig. 4). Since well-formed SC cell monolayers are critical for the analysis of change in permeability, serum-free conditions were not performed.

CHANGES IN AQUEOUS OUTFLOW FACILITY

Perfusion of porcine eyes with thrombin (1.0 U/ml) caused an $11 \pm 9\%$ decrease in outflow facility compared to an increase of $7.0 \pm 4\%$ in control eyes after 1 h of perfusion ($n = 8$; $P < 0.04$). Perfusion with LPA (20 μM) similarly decreased outflow facility $14 \pm 4\%$ compared to a $12 \pm 8\%$ increase in the control eyes after 1 h of treatment ($n = 10$; $P < 0.02$). Similar trends were also observed in outflow facility values measured after a 5 h period of perfusion (Fig. 5A,B). The effects of thrombin and LPA were expressed as the percent change in outflow facility from the baseline values in experimental eyes versus percent change in control eyes, as shown in Figure 5. Particularly, the LPA perfusion have shown the similar effect on outflow facility but at a very high concentration of LPA (50 μM) in combination with 0.2% fatty acid-free bovine serum albumin [Mettu et al., 2004]. Thus, in this study the reduced outflow facility with low concentration of LPA (20 μM) perfusion only, eliminates the possibility of vehicle-mediated effect of bovine serum albumin in the outflow pathway cells.

Since C3-exoenzyme cellular permeation is extremely slow, we did not study its effects on outflow facility in these short-term perfusion experiments (for instance, to observe C3-exoenzyme-induced effects in SC cell culture studies in vitro, cells were treated for 48 h). Moreover, previous studies from this laboratory have already shown the effect of H-7, and ECA in increasing the outflow facility of porcine eyes.

DISCUSSION

The results of this study demonstrate the potential involvement of Rho GTPase-dependent signaling pathway(s) in the regulation of aqueous humor outflow facility. Activation of Rho GTPase by thrombin and LPA leads to both decreased outflow facility and SC cell monolayer permeability. We hypothesize that these changes might be associated with Rho GTPase-induced reorganization of the actin cytoskeleton and alteration of MLC phosphorylation status.

Thrombin and LPA are known physiological agents which modulate the organization of the actomyosin cytoskeleton and which are recognized to mediate their effects through Rho GTPase-dependent signaling pathways in various cell types [Lum and Malik, 1994; Garcia and Schaphorst, 1995; Essler et al., 1998; Vouret-Craviari et al., 1998]. Although LPA and thrombin act via distinct receptors, Rho GTPase has been shown to be an important common downstream signaling molecule mediating the cytoskeletal changes and cellular contraction and relaxation responses to these agonists [Essler et al., 1998; Vouret-Craviari et al., 1998].

The involvement of Rho GTPase in cellular contraction and relaxation and cytoskeletal organization has been linked to its effects on the activity of both MLC kinase and phosphatase [Essler et al., 1998; Maekawa et al., 1999]. In our previous study using Y-27632, a specific inhibitor of Rho kinase, we have reported an increase in outflow facility that was associated with decreased MLC phosphorylation in HTM and SC cells [Rao et al., 2001]. To gain additional understanding of the potential relationship between Rho/Rho kinase signaling and changes in aqueous humor outflow facility, we studied the effects of thrombin and LPA on aqueous humor outflow in cadaver porcine eyes.

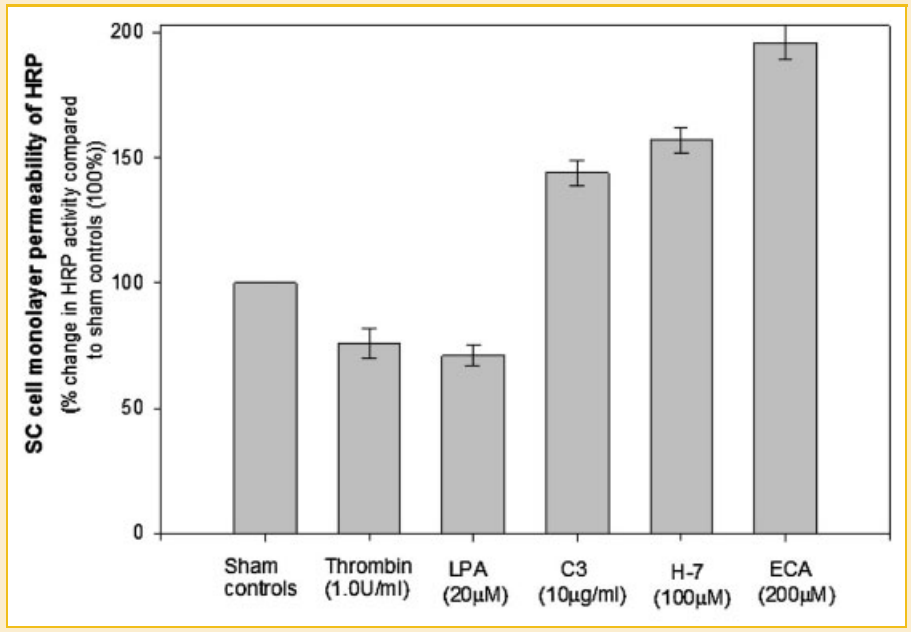

Fig. 4. Changes in SC cell monolayer permeability were determined by measuring clearance of HRP as described in the Materials and Methods Section. The results indicate that the treatment with Rho-GTPase activators, thrombin (1.0 U/ml), or LPA (20 μM) caused a decrease in permeability of 24 ± 6% (n = 6; $P < 0.03$) and 29 ± 4% (n = 8; $P < 0.01$), respectively. Treatment with C3-exoenzyme (10 μg/ml for 48 h), H-7 (100 μM), or ECA (200 μM) for 1 h produced an increase in permeability of 44 ± 5% (n = 5; $P < 0.013$), 57 ± 5% (n = 6; $P < 0.001$), and 96 ± 7% (n = 6; $P < 0.0002$), respectively. Percent change in HRP activity is presented to reflect the changes in SC cell monolayer permeability in response to the various agents employed. Basal value obtained from the sham-treated SC cells was given as 100% permeability. n Represents sample number and values were expressed as % mean ± SE. *P*-value was analyzed by a paired two-tailed Student's *t*-test analysis.

Thrombin and LPA stimulated the formation of actin stress fibers, focal adhesions, and enhanced MLC phosphorylation in serum-starved SC cells. While such patterns of cytoskeletal reorganization are a hallmark of Rho GTPase activation, the sensitivity of these events to the Rho GTPase specific inhibitor C3-exoenzyme was studied, in order to confirm Rho GTPase involvement. C3-exoenzyme, which has been used extensively as a potent and specific inhibitor of Rho GTPase, modifies the arginine 41 residue of the Rho GTPase molecule through ADP-ribosylation [Essler et al., 1998]. SC cell cultures treated with C3-exoenzyme alone showed a loss of actin stress fibers and focal adhesions (Fig. 2C,D). C3-exoenzyme-treated SC cells also demonstrated significant decrease in MLC phosphorylation (Fig. 3B; lane 2), in contrast to the increase in phosphorylation noted in response to thrombin and LPA (Fig. 3A; lanes 2 and 3). Additionally, pretreatment of SC cells with C3-exoenzyme suppressed the effects of LPA and thrombin on the actin cytoskeleton and MLC phosphorylation (Fig. 3B; lanes 3 and 4), confirming a requirement for Rho GTPase function in these events. LPA and thrombin both decreased, while C3-exoenzyme increased, SC cell monolayer permeability in HRP diffusion assays (Fig. 4). This observation is consistent with previously reported effects of LPA on endothelial cell permeability [Vouret-Craviari et al., 1998].

Increased MLC phosphorylation is known to cause cellular contraction in many cell types [Lum and Malik, 1994; Essler et al., 1998; Vouret-Craviari et al., 1998]. MLC phosphorylation is regulated primarily through the balance of MLC kinase and phosphatase activities. Rho GTPase regulates MLC phosphorylation through different mechanisms, one of which involves direct MLC phosphorylation, and the other, inactivation of MLC phosphatase through Rho kinase-mediated phosphorylation [Essler et al., 1998]. Thus, the thrombin and LPA-induced changes in SC cell MLC phosphorylation suggest that activation of Rho GTPase very likely initiates cellular contraction in SC cells, while treatment with C3-exoenzyme abolishes Rho GTPase activity, thereby causing cellular relaxation. Thus, at the cellular level, it appears that relaxation, rather than contraction, of SC cells might be associated with increased fluid outflow. This interpretation is consistent with our previous data obtained using the compound Y-27632, which is a potent and specific inhibitor of Rho kinase. Rho kinase is a critical down stream effector in Rho GTPase-mediated signaling. Pretreatment with Y-27632 caused an increase of both outflow facility in porcine eyes and permeability of SC cell monolayers. These changes were also associated with decreased MLC phosphorylation and decreased formation of actin stress fibers [Rao et al., 2001].

It is noteworthy that, in contrast to our findings, other studies have documented the ability of thrombin to increase endothelial cell monolayer permeability, leading investigators to attribute this effect to Rho GTPase-induced cellular contraction via increased MLC phosphorylation [Essler et al., 1998]. Thrombin treatment of human umbilical vein endothelial cells (HUVEC) is also known to produce cell retraction, changes in cell shape and separation of cell–cell junctions [Essler et al., 1998; Vouret-Craviari et al., 1998]. However, we did not observe these changes in SC cells, suggesting that differences in either the extent of Rho GTPase activation or in inherent contractile properties may explain these differences.

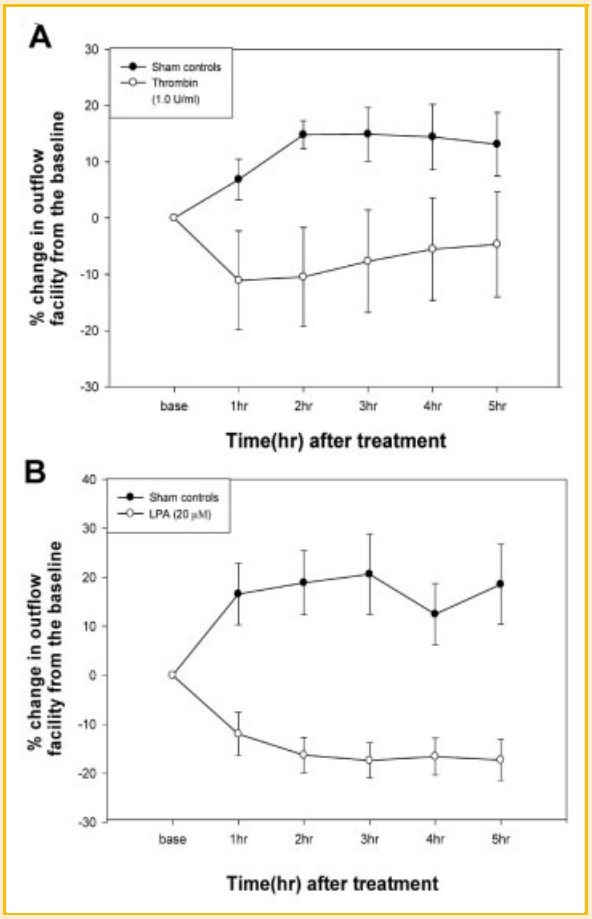

Fig. 5. Thrombin and LPA-induced outflow facility changes in enucleated porcine eyes. Thrombin (1.0 U/ml) and LPA (20 μM), caused a decrease in outflow facility after 1 h of perfusion, of 11 ± 9% (n = 8; $P < 0.04$) and 14 ± 4% (n = 10; $P < 0.02$), respectively, compared to an increase of 7 ± 4% and 12 ± 8%, respectively, in sham-manipulated control eyes. Results are expressed as percent change in outflow facility over the time course of the perfusion experiment: (A,B) depict the effects of thrombin (1.0 U/ml) and LPA (20 μM), respectively. Contralateral-paired eyes were used as sham-manipulated controls. n Represents number of samples and expressed as mean ± SE. Statistical significance (*P*-value) was analyzed by a paired two-tailed Student's *t*-test analysis.

To further assess the potential involvement of MLC phosphorylation-associated cellular contraction and relaxation in the regulation of outflow facility, we compared LPA, thrombin, and C3-exoenzyme-induced effects to those of ECA and H-7 in the SC cell monolayer permeability assay (Fig. 4). Similar to the changes induced by C3-exoenzyme, treatment of SC cells with H-7 and ECA in the presence of serum caused a decrease in stress fibers, focal adhesions, and MLC phosphorylation (data not shown), suggesting that these events might be associated with cellular relaxation.

Perfusion of porcine eyes with thrombin and LPA, which activate Rho GTPase signaling, decreased aqueous humor outflow facility (Fig. 5A,B). In contrast, H-7, ECA, and Y-27632, which depolymerize actin stress fibers and decrease MLC phosphorylation have been shown to increase aqueous outflow facility [Tian et al., 1998; Epstein et al., 1999]. Based on the similarity in the cytoskeletal events, and changes in MLC phosphorylation and permeability induced by C3, H-7, and ECA, it is reasonable to speculate that Rho GTPase-mediated cellular contraction via MLC phosphorylation in SC cells does not produce an increase in aqueous humor outflow facility. Rather, cellular relaxation, that results from the inactivation of Rho GTPase and the dephosphorylation of MLC, could produce an increase in outflow facility. This concept is further supported by our recent work with LPA that uses higher concentrations as of this study [Mettu et al., 2004]. It is also reasonable to speculate that H-7 and ECA-induced effects on outflow facility might also involve similar Rho GTPase-mediated cytoskeletal effects and cellular relaxation.

We need to emphasize that in the current investigation we have focused our studies on the SC cell endothelium. As suggested above, the regulation of outflow facility likely involves cellular events both in SC and JCT cells. In fact, in a previous study, we observed that in contrast to effects in SC cells, ECA produced an initial contraction in HTM cells [Gills et al., 1998]. While ECA possibly has multiple mechanisms of action, the results suggest that cellular relaxation and contraction may produce different effects on outflow at different cellular layers in the outflow pathway. Furthermore, Rho GTPase-induced cytoskeletal events in the JCT might also influence the production and organization of the extracellular matrix [Zhang et al., 1997; Yanase et al., 2000], and such effects in turn might also potentially contribute to the observed outflow facility effects noted in response to thrombin and LPA in our studies. It is also important to point out that cellular contraction induced by microtubule depolymerization [Gills et al., 1998] and protein kinase C [Thieme et al., 1999; Tian et al., 2000] activation might not be mechanistically similar to that induced by activation of Rho/Rho kinase.

In conclusion, this study indicates that the thrombin and LPA-induced cytoskeletal effects, that are associated with increased actin polymerization and MLC phosphorylation, decrease both SC cell monolayer permeability and aqueous humor outflow facility. Our results suggest that reduction of aqueous humor outflow facility in porcine eyes using thrombin and LPA at the level of human SC cells in culture may be correlated better with the increased outflow resistance. Since the locus of aqueous outflow resistance has been suggested to be the inner wall of SC and/or JCT in the outflow pathway cells of the eye. Our data also suggest that Rho GTPase-mediated cytoskeletal signaling pathways of SC cells could represent an important link between extracellular cues, which influence the cellular morphology of outflow pathways cells, and the modulation of aqueous humor outflow facility. Thus, we speculate that thrombin and/or thrombin like Rho GTPase activating factors in the aqueous humor might play a critical role in increasing the outflow resistance, and to the progression of certain type of glaucoma.

ACKNOWLEDGMENTS

We are very thankful to L.L. Rowlette for assistance with perfusion studies and providing helpful comments on this manuscript by P.V. Rao. This work was supported by NIH (RO1 EY01894) and Research to Prevent Blindness Research Fund.

REFERENCES

Citi S, Volberg T, Bershadsky AD, Denisenko N, Geiger B. 1994. Cytoskeletal involvement in the modulation of cell-cell junctions by the protein kinase inhibitor H-7. J Cell Sci 107:683–692.

Epstein DL. 1997. Primary open angle glaucoma. In: Epstein DL, Allingham RR, Schuman JS, editors. Chandler and Grant's glaucoma, 4th edition. Media, PA: Williams & Wilkins. pp 194–197.

Epstein DL, Rohen JW. 1991. Morphology of the trabecular meshwork and inner-wall endothelium after cationized ferritin perfusion in the monkey eye. Invest Ophthalmol Vis Sci 32:160–171.

Epstein DL, Freddo TF, Bassett-Chu S, Chung M, Karageuzian L. 1987. Influence of ethacrynic acid on outflow facility in the monkey and calf eye. Invest Ophthalmol Vis Sci 28:2067–2075.

Epstein DL, Roberts BC, Skinner LL. 1997. Non-sulfhydryl reactive phenoxyacetic acids increase aqueous humor outflow facility. Invest Ophthalmol Vis Sci 38:1526–1534.

Epstein DL, Rowlett LL, Roberts BC. 1999. Acto-myosin drug effects and aqueous outflow function. Invest Ophthalmol Vis Sci 40:74–81.

Erickson-Lammy KA, Schroer A, Epstein DL. 1992. Ethacrynic acid induces reversible shape and cytoskeleton changes in cultured cells. Invest Ophthalmol Vis Sci 33:2631–2640.

Essler M, Amano M, Kruse H-J, Kaibuchi K, Weber PC, Aepfelbacher M. 1998. Thrombin inactivates myosin light chain phosphatase via Rho and its target Rho kinase in Human endothelial cells. J Cell Biol 273:21867–21874.

Ethier CR, Coloma FM, Dekater AW, Allingham RR. 1995. Retroperfusion studies of the aqueous outflow system, Part II: Studies in Human Eyes. Invest Ophthalmol Vis Sci 36:2466–2475.

Garcia JG, Schaphorst KL. 1995. Regulation of endothelial cell gap formation and paracellular permeability. J Invest Med 43:117–126.

Garcia JGN, Davi HW, Patterson CE. 1995. Regulation of endothelial cell gap formation and barrier dysfunction: Role of myosin light chain phosphorylation. J Cell Physiol 163:510–522.

Gills PJ III, Roberts BC, Epstein DL. 1998. Microtubule disruption leads to cellular contraction in human trabecular meshwork cells. Invest Ophthalmol Vis Sci 39:653–658.

Hall A. 1998. Rho GTPase and the actin cytoskeleton. Science 279:509–514.

Hamanaka T, Bill A. 1987. Morphological and functional effects of sodium EDTA on the outflow routes for aqueous humor in monkeys. Exp Eye Res 44:171–190.

Johnson M, Erickson K. 2000. Mechanism and routes of aqueous humor drainage. Albert DM, Jakobiec FA, Schuman WB, editors. Principles and practice of ophthalmology. Volume 4: Glaucoma, Chapter 193B. Philadelphia: Saunders Co. pp 2577–2595.

Johnson M, Shapiro A, Ethier CR, Kamm RD. 1992. The modulation of outflow resistance by the pores of the inner wall endothelium. Invest Ophthalmol Vis Sci 33:1670–1675.

Johnstone M, Tanner D, Chau B, Kopecky K. 1980. Concentration-dependent morphologic effects of cytochalasin B in the aqueous outflow system. Invest Ophthalmol Vis Sci 19:835–841.

Kaufman PL, Barany EH, 1977. Cytochalasin B reversibly increases outflow facility in the eye of the cynomolgus monkey. Invest Ophthalmol Vis Sci 16:47–53.

Kranenburg O, Poland M, Gebbink M, Oomen L, Moolenaar WH. 1997. Dissociation of LPA-induced cytoskeletal contraction from stress fiber formation by differential localization of Rho A. J Cell Sci 110:2417–2427.

Lampugnani MG, Resnati M, Dejana E, Marchisio PC. 1991. The role of integrins in the maintenance of endothelial monolayer integrity. J Cell Biol 112:479–490.

Lindenmayer JM, Kahn MG, Hertzmark E, Epstein DL. 1983. Morphology and function of the aqueous outflow system in monkey eyes perfused with sulfhydryl reagents. Invest Ophthalmol Vis Sci 24:710–717.

Lum H, Malik AB. 1994. Regulation of vascular endothelial barrier function. Am J Physiol 267:L223–L241.

Maekawa M, Ishizaki T, Boku S, Watanabe N, Fujita A, Iwamatsu A, Obinata T, Ohashi K, Mizuno K, Narumiya S. 1999. Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and Lim-kinase. Science 285:895–898.

Maepea O, Bill A. 1992. Pressures in the juxtacanalicular tissue and Schlemm's canal in monkeys. Exp Eye Res 54:879–883.

Mettu PS, Deng PF, Misra UK, Gawdt G, Epstein DL, Rao PV. 2004. Role of lysophospholipid growth factors in the modulation of aqueous humour outflow facility. Invest Ophthalmol Vis Sci 45:2263–2271.

Pierzchalska M, Michalik M, Stepien E, Korohoda W. 1998. Changes in morphology of human skin fibroblasts induced by local anaesthetics: Role of actomyosin contraction. Eur J Pharmacol 358:235–244.

Rao PV, Deng PF, Kumar J, Epstein DL. 2001. Modulation of aqueous humor outflow facility by the Rho Kinase-specific inhibitor Y-27632. Invest Ophthalmol Vis Sci 42:1029–1037.

Ridley AJ. 1996. Rho: Theme and variations. Curr Biol 6:1256–1264.

Ridley AJ, Hall A. 1992. The small GTP-binding protein Rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 70:389–399.

Stamer WD, Roberts BC, Howell DN, Epstein DL. 1998. Isolation, culture and characterization of endothelial cells from Schlemm's canal. Invest Ophthalmol Vis Sci 39:1804–1812.

Thieme H, Nass JU, Nuskovski M, Bechraskis NE, Stumpff F, Strauss O, Wiederholt M. 1999. The effects of protein kinase C on trabecular meshwork and ciliary muscle contractility. Invest Ophthalmol Vis Sci 40:3254–3261.

Tian B, Kaufman PL, Volberg T, Gabelt BT, Geiger B. 1998. H-7 disrupts the actin cytoskeleton and increases outflow facility. Arch Ophthalmol 116:633–643.

Tian B, Brumback LC, Kaufman PL. 2000. ML-7, chelerythrine and phorbol ester increase outflow facility in the monkey eyes. Exp Eye Res 71:551–566.

Vouret-Craviari V, Boquet P, Pouyssegur J, Obberghen-Schilling EV. 1998. Regulation of the actin cytoskeleton by thrombin in human endothelial cells: Role of Rho proteins in endothelial barrier function. Mol Biol Cell 9:2639–2653.

Yanase M, Ikeda H, Matsui A, Maekawa H, Noiri E, Tomiya T, Arai M, Yano T, Shibata M, Ikebe M, Fujiwara K, Rojkind M, Ogata I. 2000. Lysophosphatidic acid enhances collagen gel contraction by hepatic stellate cells: Association with Rho-kinase. Biochem Biophys Res Commun 277:72–78.

Zhang Q, Magnusson MK, Mosher DF. 1997. Lysophosphatidic acid and microtubule-destabilizing agents stimulate fibronectin matrix assembly through Rho-dependent actin-stress fiber formation and cell contraction. Mol Biol Cell 8:1415–1425.