Effects of Rho-Associated Protein Kinase Inhibitor Y-27632 on Intraocular Pressure and Outflow Facility

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Purpose. To elucidate the roles of Rho-associated protein kinase (ROCK) in regulating intraocular pressure (IOP) and outflow facility in the rabbit eye.

Methods. A specific ROCK inhibitor Y-27632 was used. The IOP, the outflow facility, and the pupil diameter were determined before and after the topical, intracameral, or intravitreal administration of Y-27632 in rabbits. Western blot analysis was used to identify specific ROCK isoform in human trabecular meshwork (TM) cells and bovine ciliary muscle (CM) tissues. The cell morphology and distribution of actin filaments and vinculin in TM cells were studied by cell biology techniques. Carbachol (Cch)-induced contraction of isolated bovine CM strips after administration of Y-27632 was measured in a perfusion chamber.

Results. In rabbit eyes, administration of Y-27632 resulted in a significant decrease in IOP in a dose-dependent manner. An increase of the outflow facility and pupil size dilation was also observed in Y-27632–treated eyes. Western blot analysis revealed the presence of p160ROCK in human TM cells and bovine CM tissues. In cultured human TM cells, exposure to Y-27632 caused retraction and rounding of cell bodies as well as disruption of actin bundles and impairment of focal adhesion formation. Y-27632 in addition inhibited Cch-induced contraction of isolated bovine CM strips.

Conclusions. Administration of Y-27632 caused a reduction in IOP and an increase in the outflow facility. The in vitro experiments suggest that the IOP-lowering effects of Y-27632 may be related to the altered cellular behavior of TM cells and relaxation of CM contraction. These studies suggest that ROCK inhibitors may have great potential to be developed for treatment of glaucoma and other ocular diseases. (*Invest Ophthalmol Vis Sci.* 2001;42:137–144)

Rho guanosine triphosphatase (GTPase), a member of the Rho subgroup of the Ras superfamily, participates in signaling pathways that lead to formation of actin stress fibers and focal adhesions.[1–4] Rho is also involved in diverse physiological functions associated with cytoskeletal rearrangements,[5,6] such as cell morphology,[7] cell motility,[8] cytokinesis,[9] and smooth muscle contraction.[10,11] Recently, several putative target molecules of Rho have been identified as Rho effectors, including Rho-associated coiled coil-forming protein kinase, referred to as p160ROCK,[12,13] and its isoform, ROKα/Rho kinase/ROCK II.[14–16] ROCKs play a key role in focal adhesions and stress fiber formation in cultured fibroblasts and epithelial cells,[12,14,16] and in Ca^{2+} sensitization of smooth muscle cells.[17,18] Y-27632 is a recently identified specific inhibitor of the ROCK–ROK family of protein kinases.[19] This compound inhibits agonist-induced smooth muscle contraction both in vitro and in vivo, as well as the stress fibers and focal adhesion formation induced by p160ROCK in cultured cells.[19]

Intraocular pressure (IOP) in the eye is regulated so it remains within the normal range. In abnormal situations such as in glaucoma,[20–22] the IOP is often elevated, causing damage to the optic nerve head that leads to blindness. Because glaucoma is the second leading cause of blindness,[23] extensive efforts have been made to develop antiglaucoma drugs and laser and other surgical procedures to lower IOP.[23] Most of these modalities are intended either to modulate the aqueous outflow facility at sites of the trabecular meshwork (TM) and the ciliary muscle (CM),[21,24–26] or to inhibit aqueous humor production by the ciliary body. Despite the widely recognized functional importance of TM and CM tissues in regulating the aqueous outflow, cellular mechanisms underlying these functions are not well understood. Previous studies have suggested that alteration in activities or behavior of TM cells may change the outflow facility.[21,27,28] Also, modulation of the contractile or relaxant elements in CM may play a direct role in the regulation of aqueous humor outflow.[25] Both TM and CM cells morphologically and electrophysiologically express properties that are typical of smooth muscle cells.[29] Thus, alteration in the contractility of the TM and CM, and modulation of the cellular behavior of TM cells are the likely bases for the development of novel IOP-lowering antiglaucoma drugs. Y-27632 has been shown to induce inhibition of smooth muscle contraction and alter various cellular behavior[19,30] and therefore appears to be a key candidate.

In the present study, we examined whether inhibition of Rho–ROCK signaling by a specific ROCK inhibitor Y-27632 causes an increase in the outflow facility. The data suggest that Y-27632 induced a significant decrease in IOP, possibly through an effect on TM cellular behavior.

Materials and Methods

Animals and Anesthesia

Adult Japanese white rabbits weighing 2 to 2.5 kg were used in this study. All studies were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. For IOP and pupil diameter measurements, the rabbit eyes were anesthetized by topical instillation of 2% lidocaine. For measurements of the outflow facility or the uveoscleral outflow, the rabbits were anes-

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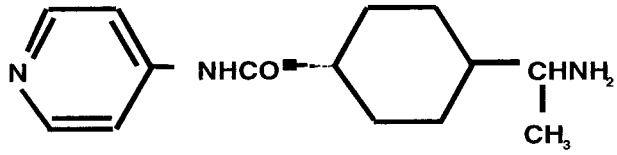

FIGURE 1. The structure of Y-27632.

thetized by gradual administration of a 40% urethane solution (1.0–1.5 ml/kg) into a marginal ear vein. Supplemental doses (0.2 ml/kg) were administered intravenously to the rabbit as needed.

Chemicals, Drug Preparation, and Drug Administration

Y-27632 (molecular weight 338.3) was supplied by Welfide (formerly Yoshitomi Pharmaceutical, Saitama, Japan). The structure of Y-27632 is shown in Figure 1. For topical administration, Y-27632 (10 or 100 mM) or vehicle phosphate buffered saline (PBS) was administered to the central cornea as four 3-μl drops at intervals of 30 seconds, with blinking prevented between and after the last drops. The Y-27632 dosage for topical administration was chosen to provide a 10- or 100-μM Y-27632 concentration in the approximately 120-μl rabbit anterior chamber,[31] assuming 1% intracameral penetration and no drug loss from the anterior chamber.[32,33] After topical anesthesia of rabbit eyes, 1.2 μl of 1, 10, or 100 mM of Y-27632 was administered intracamerally (10, 100, and 1000 μM final concentrations, respectively) and 14 μl of 10 or 100 mM Y-27632 was administered intravitreally (100 and 1000 μM, respectively, in the approximately 1.4-ml vitreous space to the rabbit eye).[31] The fellow eyes that were treated with injection of appropriate volumes of vehicle phosphate buffered saline (PBS) served as a control.

Anterior chamber injections were made under an operating microscope, using a microsyringe (Hamilton, Reno, NV) with a 30-gauge needle. The needle was threaded through the corneal stroma for approximately 3 mm, then directed into the anterior chamber so that the wound was self-sealing. For intravitreal injections, the needle was inserted 3 mm through the temporal sclera 2 mm posterior to the limbus.

IOP Measurement

A calibrated pneumotonometer (Alcon, Forth Worth, TX) was used to monitor the IOP. It was measured before the administration of Y-27632 and at 0.5, 1, 3, 6, 9, and 24 hours after administration.

PD Measurement

The pupil diameter (PD) was measured with a millimeter ruler (Digimatic Caliper; Mitutoyo, Tokyo, Japan) under standard laboratory light at the same time points at which the IOP measurements were made.

Total Outflow Facility and Uveoscleral Outflow

Total outflow facility was determined by two-level constant-pressure perfusion (25 and 35 mm Hg) 3 hours after topical administration of 12 μl of 100 mM Y-27632 and vehicle, according to the method of Barany.[34] Briefly, the anterior chambers of rabbits anesthetized with 40% urethane were perfused with mock aqueous humor (BSS plus; Santen Pharmaceutical, Osaka, Japan) by a constant pressure of either 25 or 35 mm Hg, which was alternately applied at 10-minute intervals. During each 10-minute period, fluid flow was measured for 8 minutes beginning 2 minutes after the pressure change was induced.

Uveoscleral outflow was determined with a perfusion technique using fluorescein isothiocyanate-dextran (FITC-dextran, molecular weight 71,200; Sigma, St. Louis, MO)[35,36] 3 hours after the topical administration of 12 μl of 100 mM Y-27632 and vehicle. The rabbits were anesthetized with 40% urethane, and two 23-gauge needles connected to a pair of syringes were inserted into the anterior chamber in each eye of each rabbit. The pair of syringes was controlled by an infusion–withdrawal pump (model 944; Harvard Apparatus, South Natick, MA), and the infusion syringe was filled with 10^{-4} M FITC-dextran. One milliliter of the FITC-dextran solution was washed through the anterior chamber using the syringes at a rate of 0.5 ml/min. The IOP level was then set to 20 mm Hg. The FITC-dextran solution was perfused continuously through the anterior chamber at a rate of 10 μl/min for 30 minutes. The anterior chamber was washed with 2 ml of PBS at a rate of 0.5 ml/min. Each eye was then enucleated and dissected into the following sample groupings: anterior uvea, anterior sclera, posterior sclera plus posterior uvea, and the posterior segment fluid plus vitreous. All samples were homogenized and centrifuged, and the volume of each was measured. The supernatant was measured to determine the FITC-dextran concentration using a fluorophotometer. The uveoscleral outflow (F_u, in microliters per minute) was calculated as follows:

$$F_u = \frac{\sum (a \times b)}{C \times T}$$

where a is the volume of each sample (in milliliters), b is the concentration of FITC-dextran in each sample (in nanograms per milliliter), C (in nanograms per microliter) is the concentration of FITC-dextran in the perfusion fluid (10^{-4} M = 7120 ng/ml), and T (in minutes) is time of perfusion (30 minutes).

Slit Lamp Biomicroscopy

The integrity of the corneal epithelium, the presence or absence of anterior chamber flare or cells, and lens clarity were examined 1, 3, 6, 12, and 24 hours after the administration of Y-27632.

Culture of Human TM Cells

Human eyes from donors were obtained from the Illinois Eye Bank (Chicago, IL). Trabecular tissues excised from eyes were cultured on flasks (Falcon Primaria; Becton Dickinson, Lincoln Park, NJ), as previously described.[37,38] The culture medium included Dulbecco's minimum essential Eagle's medium (DMEM), 10% fetal bovine serum (FBS), and antibiotics. Cells were maintained in a 95% air–5% CO_2 atmosphere at 37°C and passaged using the trypsin-EDTA method. Only well-characterized normal human TM cells from passages three through eight were used for subsequent studies.

Chemicals

Carbachol (Cch), FITC-phalloidin, and mouse monoclonal antibody to vinculin were obtained from Sigma. Rabbit anti-p160ROCK (ROCK I) was prepared as described.[39] Appropriate secondary antibodies were obtained from Chemicon (Temecula, CA) and Amersham (Little Chalfont, UK).

Preparation of Lysates from Whole-Cell and Bovine Tissue and Immunoblot Analysis

To examine the expression levels of p160ROCK, detergent lysates of TM cells and bovine CM tissue were prepared in Laemmli's sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Confluent cultures of human TM cells were kept in FBS-free DMEM overnight to rule out ROCK upregulation. They were placed on ice, washed with ice-cold PBS three times, and then scraped and lysed. Bovine CM tissues were excised from bovine eyes with fine forceps and Vannas scissors under a dissecting microscope. The tissues were lysed, and the total protein content was quantified by a protein assay (DC; Bio-Rad, South Richmond, CA). Equivalent amounts of protein-containing lysates were boiled in sample buffer and subjected to SDS-PAGE using a 5% to 20% gradient gel (Daiichi, Tokyo, Japan). After electrophoresis, the proteins were electrophoretically transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membrane was blocked with 2% bovine serum albumin (BSA) in PBS containing 0.005% Tween 20 (PBST) at 4°C for 16 hours and was

incubated in a 1:1000 dilution of anti-p160ROCK rabbit antibody 20490[39] for 2 hours at room temperature. The membrane was washed with PBST and further incubated with biotin-conjugated anti-rabbit antibody (1:1000; Amersham) and ABC solution (ABC Elite kit; Vector, Burlingame, CA). After extensive washing, the blotted protein bands were visualized with immunostain (HRP-1000; Konica, Tokyo, Japan).

Effects of Y-27632 on the Shape of Human TM Cells

In experiments designed to examine changes in cell shape, sparse cultures (Fig. 4), and semiconfluent cultures (Figs. 5, 6) were incubated with various concentrations of Y-27632 (10–100 μM), with or without serum. The cultures were observed by phase-contrast microscopy and photographed immediately after drug application, and 0.5, 1, 2, and 18 hours later. The drug solution was removed afterward and replaced with plain DMEM containing 10% FBS. In all cases, recovery of normal morphology was documented 24 hours later.

Immunohistochemistry

For immunohistochemistry, TM cells were plated on glass coverslips at a density of 3×10^4 cells per each 6-cm dish in DMEM containing 10% FBS. After culture for 2 days, when cells reached semiconfluence, Y-27632 was added at 10, 100, and 1000 μM and incubated for 10, 30, or 60 minutes. For a control, PBS was added as the vehicle. After the drug exposure, the cells on coverslips were fixed and permeabilized for 2 minutes with 3% paraformaldehyde–PBS and 0.5% Triton X-100 (Sigma) and were further fixed for 20 minutes with 3% paraformaldehyde. The samples were blocked in 2% BSA for 30 minutes. Filamentous actin (F-actin) was labeled with FITC-phalloidin (0.05 mg/ml) for 1 hour at room temperature. For vinculin staining, the coverslips were incubated at room temperature with anti-vinculin antibody (1:400 dilution in blocking solution) for 60 minutes and with Cy-3–conjugated anti-mouse secondary antibody for 30 minutes. Fluorescence was visualized under an epifluorescence microscope (Axioplan; Zeiss, Oberkochen, Germany) and with a confocal laser scanning microscope (Bio-Rad). To determine whether the Y-27632 effects were reversible, the cells were incubated for another 30 minutes in Y-27632-free medium after the various Y-27632 treatments, fixed, and immunostained.

Measurement of Contractility of CM

Enucleated bovine eyes were obtained from a local slaughterhouse and placed on ice. Small CM strips were carefully dissected according to procedures described by Lepple-Wienhues et al.[40] Briefly, after excision of the iris, meridional CM strips were excised perpendicular to the circular ciliary body. The isolated strips were approximately 5 mm long and 1 to 2 mm wide. The CM contractility was measured isometrically with a force-length transducer device. The bovine CM strips were set between a hook and an isometric force transducer connecting an amplifier and a multipen recorder and vertically mounted in a 20-ml Magnus tube (Iwashiyakishimoto, Kyoto, Japan) filled with Krebs-Henseleit solution, which was aerated continuously. The composition of the solution was NaCl, 118.1 mM; KCl, 4.7 mM; $CaCl_2$ 2.5 mM; KH_2PO_4, 1.2 mM; $MgSO_4$, 1.34 mM; $NaHCO_3$, 25.0 mM; and glucose, 11.1 mM; and the temperature was kept at 37°C. At the beginning of each experiment, tissues were subjected to an imposed resting tension and allowed to equilibrate for 120 minutes. To confirm the stability of the preparations, application of Cch and washing were repeated twice. Only strips that showed a stable tone were used for experiments. After reproducible responses to Cch were obtained, bovine CM strips were contracted with Cch that was applied to the bath (10^{-6} M). When Cch-induced contraction became stable (approximately 5 minutes after the application), Y-27632 was added cumulatively to the bath. After the final dose of Y-27632, the strip was perfused with plain Krebs solution (to reestablish baseline tension). Mean data of isometric force measurements were expressed as relative values in comparison with the maximum Cch concentration (10^{-6} M) response. Relaxation responses were expressed as a percentage of the maximum effect (100%) elicited by Cch in each strip.

Statistical Analysis

Data were analyzed by repeated-measures analysis of variance (ANOVA) and Bonferroni as a post hoc test of the time course of IOP. Mann–Whitney test, or Student's *t*-test with the Bonferroni correction was used for aqueous humor dynamics. $P < 0.05$ was considered to be statistically significant.

Results

IOP Measurements

Compared with contralateral vehicle-treated control eyes, the IOP in Y-27632–treated rabbit eyes was significantly lowered 0.5 hours after topical administration of 100 mM Y-27632 eye drops ($P < 0.005$). Maximal IOP reduction was observed between 1 and 3 hours with 100 mM of Y-27632 (Fig. 2Aa). After intravitreal administration, significant IOP reductions were noted between 0.5 and 24 hours, and the maximal reductions were seen between 6 and 9 hours with the 1000-mM concentration ($P < 0.005$; Fig. 2Ab). When the drug was administered intracamerally, significant IOP reductions occurred between 0.5 and 6 hours and 1000 mM Y27632 produced the maximal reductions ($P < 0.001$; Fig. 2Ac). No anterior chamber, lens, or fundus abnormalities in rabbit eyes were detected by slit lamp examination after either the topical, intracameral, or intravitreal administration of Y-27632. These experiments demonstrated the potent IOP-lowering effects of Y-27632 in rabbit eyes.

PD Measurement

Compared with contralateral vehicle-treated control eyes, the PD in rabbit eyes after Y-27632 treatment was significantly ($P < 0.005$) dilated from 1 to 9 hours after topical administration of 100 mM of Y-27632 eye drops, and the maximal PD was observed at 3 hours ($P < 0.01$; Fig. 2Ba). After intravitreal administration, significant PD dilation was noted from 1 to 12 hours, and the maximal dilation was seen at 3 hours with the 1000 mM concentration ($P < 0.01$; Fig. 2Bb). When administered intracamerally, significant PD dilation occurred from 1 to 6 hours and 1000 mM of Y27632 produced the maximal dilation at 1 hour ($P < 0.005$; Fig. 2Bc).

Measurements of Outflow Facility

The outflow facility was measured 3 hours after topical administration of Y-27632 when maximal IOP reduction was observed. Results summarized in Table 1 showed that the average outflow facility was approximately two times higher (+100%) in the eyes treated with 100 mM Y-27632 (0.24 ± 0.02 μl/min per millimeter mercury, $P < 0.001$) than that in the contralateral PBS-treated control eyes (0.12 ± 0.01 μl/min per millimeter of mercury). The uveoscleral outflow was also increased by 30% in the treated eyes (0.60 ± 0.05 μl/min) compared with the control eyes (0.46 ± 0.04 μl/min), although the difference was not statistically significant.

Western Blot Analysis for the Identification of ROCK in TM Cells and CM

A series of in vitro experiments was performed to elucidate the mechanisms of the IOP-lowering and outflow facility–enhancing effects of Y-27632 observed in animals. First, immunoblotting was conducted to identify protein expression of ROCK, a target molecule of Y-27632. As shown in Figure 3, Western blot analysis using anti-p160ROCK antibody detected a protein band of approximately 160 kDa in both intact

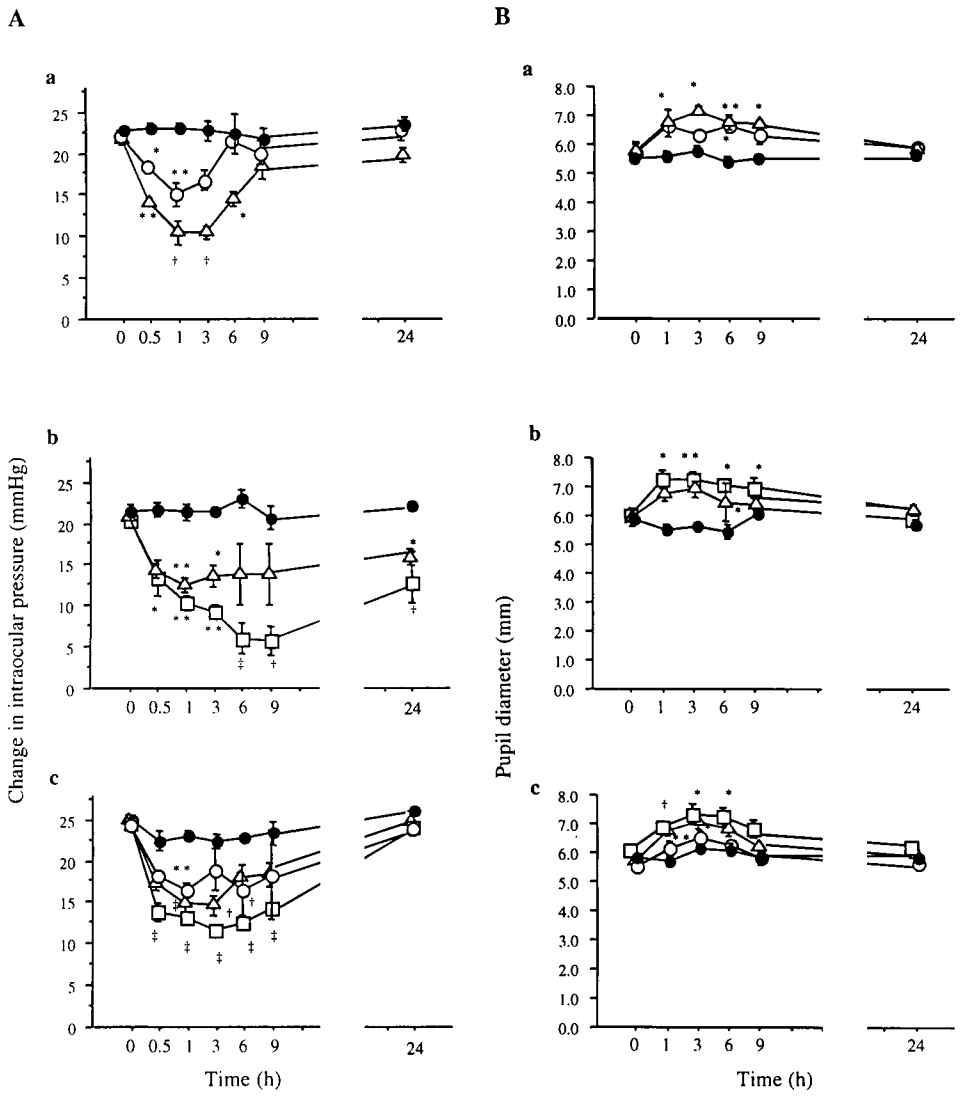

FIGURE 2. Effects of Y-27632 on IOP (**A**) and pupil diameter (**B**). Y-27632 was administered to rabbit eyes topically, intravitreally, and intracamerally. The contralateral eyes were treated with the same volume of vehicle PBS. (a) topical administration, (b) intravitreal administration, and (c) intracameral administration. For (a) (●) vehicle alone; (○) 10 mM; and (△) 100 mM Y-27632. For (b) and (c) (●) vehicle alone; (○) 1 mM; (△) 10 mM; and (□) 100 mM Y-27632. The results are presented as mean ± SEM [$n = 6$ for (a), (b) and (c)]. The significance of the data was evaluated by Student's unpaired *t*-test; $^{*}P < 0.05$ and $^{**}P < 0.01$, †$P <$ 0.005, ‡$P < 0.001$, compared with controls with vehicle alone.

bovine CM tissue (lane 2) and cultured human TM cells (lane 4). This molecular size corresponded to that reported for p160ROCK.[39]

Effects of Y-27632 on Morphology of Cultured Human TM Cells

Next, the morphology of TM cells was examined. By phase-contrast microscopy, treatment with 100 μM of Y-27632 in the presence of serum for 30 minutes induced retraction and rounding of TM cells (Fig. 4A, 4B), similar to the morphologic phenotype observed in Y-27632–treated Swiss 3T3 cells.[41] When semiconfluent cultures were treated with Y-27632, TM cells also retracted and became thinner (Fig. 5; upper two rows). To determine whether such changes were related to the Rho–ROCK pathway by serum stimulation, the cells were also incubated in serum-free medium. Retraction and thinning were seen 30 to 60 minutes later (Fig. 5, bottom row). These results showed that the TM morphology may be influenced by inhibition of the Rho–ROCK signaling system.

TABLE 1. Effects of Y-27632 on Outflow Facility in the Rabbit

	Outflow Facility (μl/min/mmHg)	Uveoscleral Outflow (μl/min)
Y-27632	0.24 ± 0.02	0.60 ± 0.05
Vehicle	0.12 ± 0.01	0.46 ± 0.04
Significance*	$P < 0.001$	NS

Values are means ± SEM for six animals. NS, not significant.
* Mann–Whitney test.

Effects of Y-27632 on Cytoskeleton of Cultured Human TM Cells

To examine whether the actin structure was also affected, 10, 100, or 1000 μM Y-27632 was added to the culture medium. It was found that the distribution of F-actin was altered dramatically in a time- and concentration-dependent manner (Fig. 6). In control cells, actin filaments were assembled into large radial and circumferential bundles in association with focal adhesions (Fig. 6A). After treatment with 100 μM Y-27632 for 30 minutes, the cells lost most of their actin bundles (Fig. 6B). Instead of discrete long filament beams, the phalloidin staining took on a punctate appearance, as residual actin filaments became associated with the cell periphery rather than focal adhesions. The cell borders and processes were outlined distinctly. This change appeared to coincide with the rounding of the cell shape.

Vinculin in control cells was predominantly associated with focal adhesions (Fig. 6A). After the Y-27632 treatment, deteri-

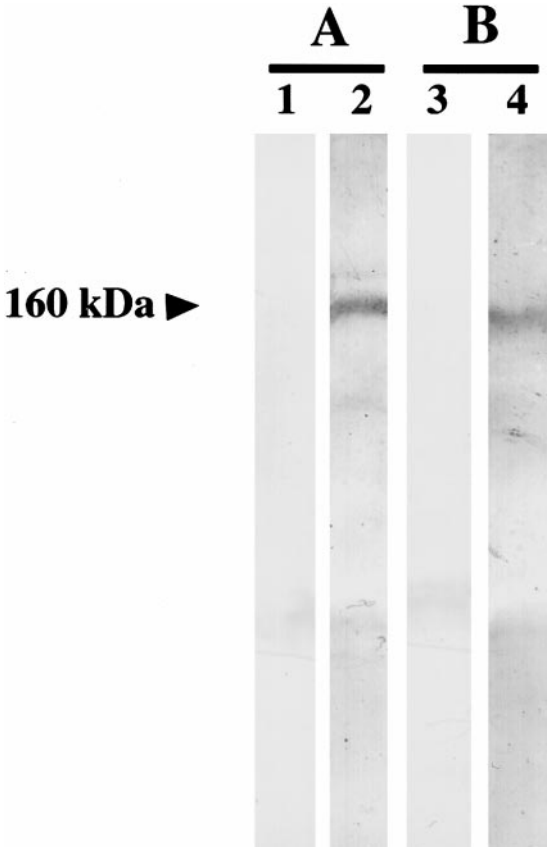

FIGURE 3. Western blot analysis of ROCK in bovine ciliary muscle and cultured human TM cells. Homogenate of bovine ciliary muscle tissue (**A**) and whole-cell lysates from cultured human TM cells (**B**) were run in SDS-PAGE. *Lanes 1* and *3*: control rabbit IgG; *lanes 2* and *4*: p160ROCK.

oration of focal adhesions was evident (Fig. 6B). These cytoskeletal changes were completely reversible within 2 hours and completely recovered after 15 hours. These data suggest that the inhibition of the Rho–ROCK pathway may initiate the rearrangement of cytoskeleton in TM cells.

Measurement of Contractility of Isolated CM

After adjustment of baseline tension, Cch at a concentration of 10^{-6} M was used to induce contraction in isolated CM strips, as described previously.[42,43] Figure 7 shows a typical recording of the relaxation effects induced by cumulatively added Y-27632. Superfusion with Cch resulted in an immediate, steep-force development, reaching maximum after 3 minutes. Y-27632 led to relaxation of the Cch precontracted CM strips, in a dose-dependent manner. The maximal effect was found in experiments using 10^{-3} M Y-27632, which almost completely abolished (by 97%) the response to Cch. Figure 8 summarizes the data obtained with increasing concentrations of Y-27632. At 10^{-5} to 10^{-3} M of concentrations, the Y-27632–induced relaxation of the Cch-precontracted CM strips was significant. The average relaxation for Y-27632-treated CM strips was 12.2% ± 4.9%, 25.6% ± 7.7%, 43.2% ± 5.0%, 60.9% ± 3.1%, and 96.7% ± 0.6%, for 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} M Y-27632, with $P = 0.6332$, 0.1297, 0.0032, 0.0006, and $<$ 0.0001, respectively, compared with the time-matched control samples.

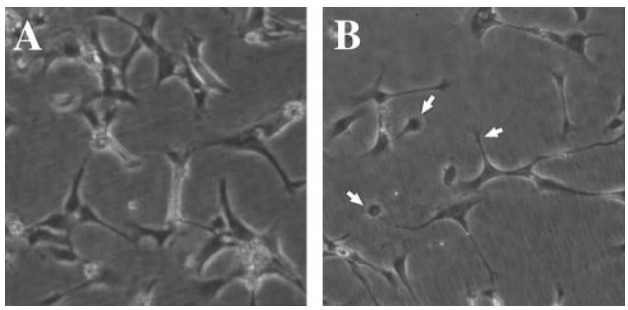

FIGURE 4. Effect of Y-27632 on TM cell characteristics. Effects of Y-27632 on cell morphology. Human TM cells from sparse culture (**A**) before and (**B**) after a 30-minute incubation with 100 μM Y-27632. Marked cell rounding is shown. Original magnification, ×100.

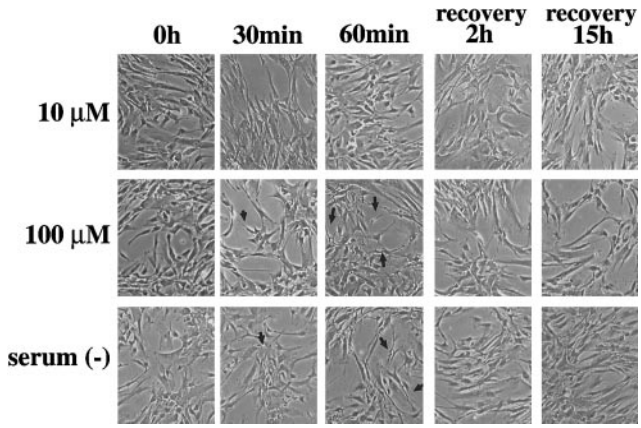

FIGURE 5. Effects of Y-27632 on morphology of cultured human TM cells. Phase-contrast microscopic observation of semiconfluent culture of TM cells. Treatments with Y-27632 in concentrations of 10 and 100 μM for 30 and 60 minutes in the presence of serum resulted in retraction and thinning of the cells (*black arrows*). Serum starvation also resulted in a similar cell shape change with retraction and thinning of the cells (*black arrows*). The drug solution or DMEM without serum were removed afterward and replaced with DMEM containing 10% FBS. Recovery of normal morphology was observed 15 hours later. Original magnification, ×60.

DISCUSSION

The present study demonstrated that a specific inhibitor of the ROCK–ROK family of protein kinases, Y-27632, when administered topically, intracamerally, or intravitreally, induces a significant decrease in IOP in rabbit eyes. In glaucomatous eyes, elevation of the IOP is believed to be a major factor that causes axonal damage in the optic nerve and the subsequent retinal ganglion cell death, leading to blindness.[22,24] To manage and control the IOP for patients, a number of drugs such as pilocarpine, β-adrenergic receptor antagonists, epinephrine and its derivatives, prostaglandin-related compounds, and carbonic anhydrase inhibitors[23] have been applied. These drugs in general exhibit their IOP-lowering effects either through inhibition of aqueous humor production and/or modulation of the outflow facility. The results presented in the present study suggest that Y-27632 elicits its pharmacologic modulation, at least in part, through regulation of the outflow facility.

Immunoblot analysis showed that a specific isoform p160ROCK is present in cultured human TM cells and bovine CM tissues. The p160ROCK isoform, a Rho effector and a target molecule of Y-27632, has been reported to play an important role in smooth muscle contractility and actin cytoskeletal integrity. It appears that TM and CM are both target tissues of Y-27632.

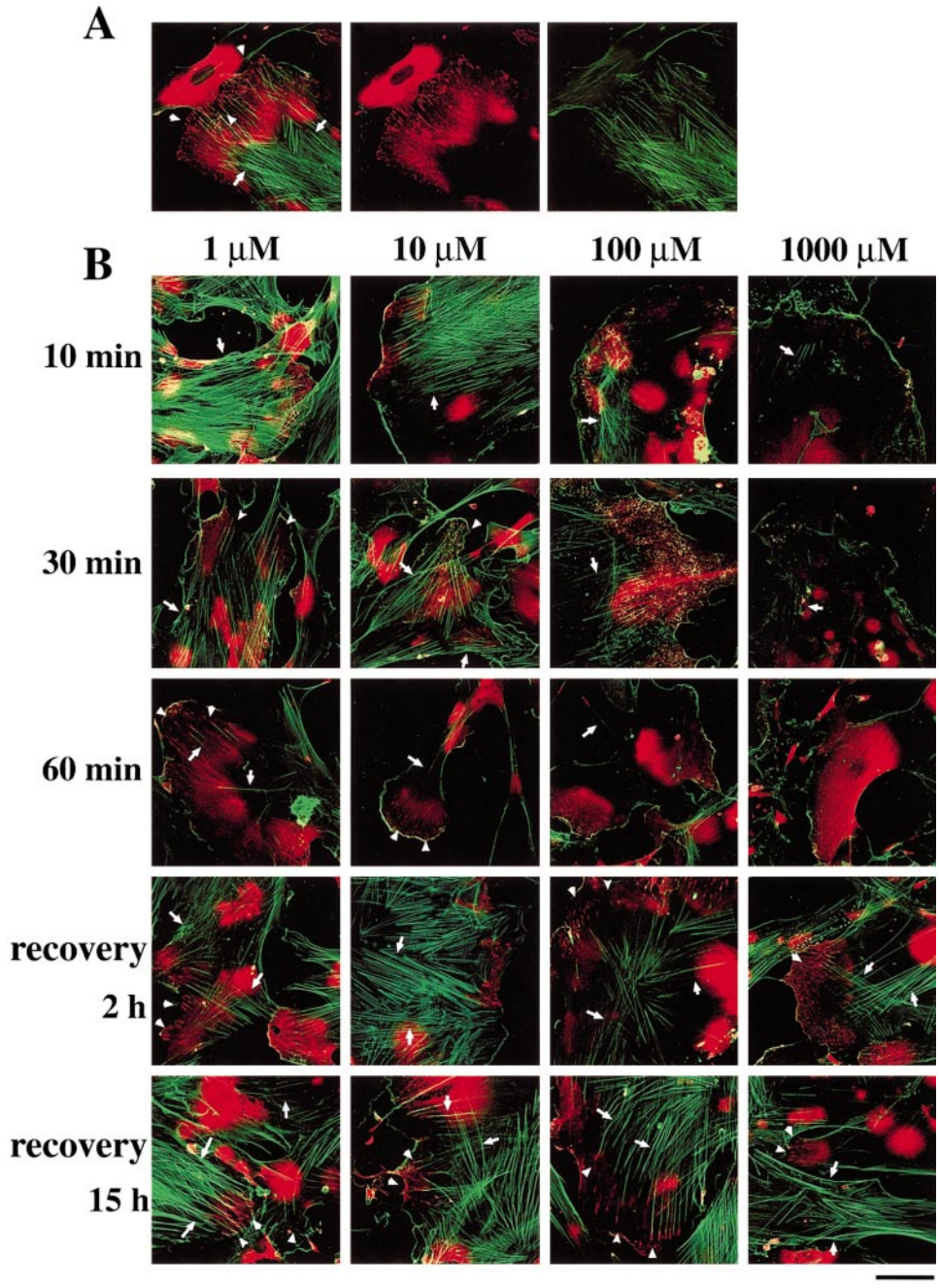

FIGURE 6. Distribution of F-actin and vinculin in human TM cells treated with Y-27632. (**A**) Distribution of F-actin (*green*) and vinculin (*red*) in normal human TM cells. *Arrows*: F-actin bundles; *arrowheads*: focal adhesions containing vinculin. *Left*: Confocal images; *middle*: cells stained with antibody to vinculin; *right*: cells stained with FITC-phalloidin to visualize F-actin. (**B**) Distribution of F-actin and vinculin in human TM cells treated with Y-27632 in concentrations of 10, 100, and 1000 μM for 10, 30, and 60 minutes. The drug solution was removed afterward and replaced with DMEM containing 10% FBS. Recovery of normal morphology was observed 2 hours and 15 hours later. *Arrows*: F-actin bundles, which disappeared with Y-27632 treatment and recovered after drug removal; *arrowheads*: vinculin-containing focal contacts, which were decreased with Y-27632 treatment and recovered by replacement with DMEM. Bar, 10 μm.

There are two routes of aqueous humor outflow: conventional (trabecular) and unconventional (uveoscleral) pathways.[21] In human and primate eyes, conventional outflow is regarded as the main route[37,38,44] and is believed to be regulated by the cellular behavior and contractility of both TM and CM cells.[45] Our outflow facility data suggest that Y-27632 may affect the conventional outflow. The presence of p160ROCK in cultured TM cells and CM tissues further support this conjecture.

In rabbit eyes, the anterior chamber has no true trabecula, and the vascular anatomy of the outflow pathways and orbit differs from that of the primate.[31] The rabbit has a venous plexus in intimate association with the chamber angle tissues and a large orbital venous sinus; there is no Schlemm's canal or collector channel arrangement as in primates. The significant IOP-lowering effect of Y-27632 found in rabbit eyes thus may be related not only to alterations in the trabecular facility, but

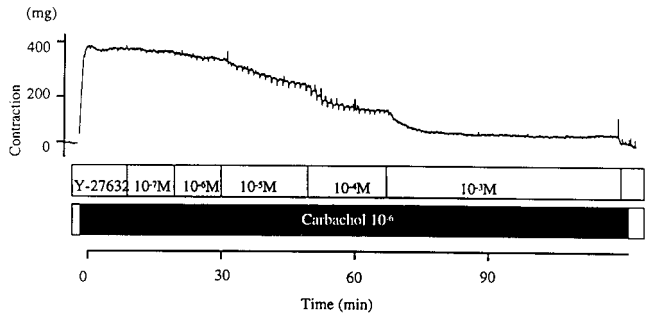

FIGURE 7. Representative recordings of isometric force developed in isolated bovine CM strips. After a Cch-induced (10^{-6} M) contraction, ROCK inhibitor Y-27632 led to relaxation in the isolated ciliary muscle. When the Cch response became stable, Y-27632 was cumulatively added to the strip.

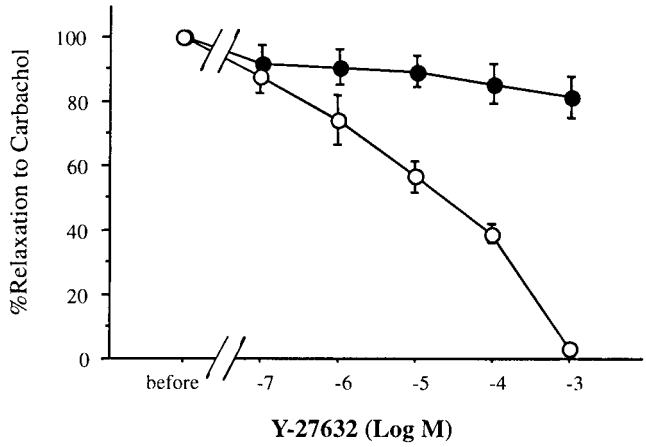

FIGURE 8. Dose-dependent relaxation of isolated ciliary muscle strips by Y-27632. Cch was applied to isolated CM strips ($n = 4$). The percentage of maximum carbachol response is shown with increasing concentrations of Y-27632 plus 10^{-6} M Cch. Data are means ± SEM; the four ciliary muscle strips are from four bovine eyes. (●) Vehicle alone; (○) Y-27632.

also to changes in the permeability of the chamber angle venous plexus and/or the iris vasculature. The two-level constant pressure perfusion system used in our experiments for the outflow facility measurements monitors changes in flow from an elevated external reservoir into the anterior chamber that represent total facility—i.e., the sum of conventional and unconventional outflow facility. Y-27632 induced a twofold increase in the total outflow facility. Our measurements of unconventional outflow revealed a modest increase in the uveoscleral outflow in rabbit eyes by Y-27632, but its effects were not statistically significant. These results suggest that IOP-lowering effects of Y-27632 are largely mediated by the altered conventional facility.

Exposure of the cultured TM cells to 10 μM of Y-27632-induced retraction and rounding of cell bodies, as well as disruption of actin microfilament bundles and impairment of focal adhesion formation. These morphologic and cytoskeletal events were similar to those reported previously in other cell types such as N1E-115 and Swiss 3T3 cells.[41] The kinetics of the alterations in the cultured TM cells paralleled that of the observed IOP and outflow facility changes in animal eyes after administration of 10 to 1000 μM Y-27632.

It has been shown previously that the Rho–ROCK signaling pathways acts, at least in part, by controlling the phosphorylation of myosin light chain (MLC) and ultimately the organization of the actin cytoskeleton through the actomyosin system.[46] This mechanism of action contrasts with that of cytochalasin and latrunculins, which are effective IOP-reducing agents as well, but with disassembly of the cytoskeletal actin as the primary event.[28] Other compounds such as protein kinase inhibitor staurosporine, and serine-threonine kinase inhibitor H-7, which can decrease outflow resistance,[47,48] also cause cytoskeletal perturbation as their common feature. Similar to the ROCK inhibitor, however, the actin effect only occurs secondarily through interference of the actomyosin interaction.[49] Between the different compounds even in similar categories, there are still differences in effects and mechanisms.

Y-27632 also reduces contractility in cultured neuronal cells, nonneuronal cells, and fibroblasts.[43] The contraction of the actomyosin system in smooth muscle and nonmuscle cells is thought to be regulated by two mechanisms: the increase in free Ca^{2+} ion in the cell mediated by MLC kinase and a Ca^{2+} sensitization mechanism. Several lines of evidence now indicate a role for the ROCK-ROK family in the latter mechanism.[16,17,19] In our experiments, Y-27632 led to relaxation of bovine CM strips in a dose-dependent manner. Many investigators have reported that relaxation of CM would not increase trabecular outflow. Thus, at present, we cannot conclude that Y-27632-induced changes in CM contribute to hypotensive effects of this drug.

In summary, the present study shows that Y-27632, a selective ROCK inhibitor, reduces IOP and increases the outflow facility. Such effects may be related to altered cellular behavior of TM cells and relaxation of CM tissues. Selective inhibition of the Rho–ROCK signaling pathway may be developed into a novel neuroprotective strategy for the treatment of glaucoma and retinal ischemic diseases.

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