H-7 Disrupts the Actin Cytoskeleton and Increases Outflow Facility

Baohe Tian, MD; Paul L. Kaufman, MD; Tova Volberg, MSc; B'Ann T. Gabelt, MS; Benjamin Geiger, PhD

Objectives: To determine the effects of the serine-threonine kinase inhibitor H-7 on (1) cell junctions and the attached actin-based cytoskeleton in cultured bovine aortic endothelial cells, and (2) outflow facility in living monkeys.

Methods: Bovine aortic endothelial cells were cultured by standard techniques. The architecture and distribution of actin filaments, vinculin, and β-catenin in bovine aortic endothelial cells were studied by immunolabeling before and after exposure to H-7 at various concentrations and durations. Outflow facility (perfusion) and intraocular pressure (Goldmann tonometer) were determined before and after the intracameral or topical administration of H-7 or a vehicle.

Results: In bovine aortic endothelial cells, exposure to H-7 produced a reversible time- and concentration-dependent disruption of actin microfilaments and an alteration in the organization of cell-cell and cell-matrix adhesions. In monkeys, intracameral and topical administration of H-7 dose dependently and reversibly doubled facility, and topical H-7 reduced intraocular pressures.

Conclusion: H-7 increases outflow facility in monkeys, probably by inhibiting cell contractility, cytoskeletal support, and cell-cell adhesions in the trabecular meshwork.

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From the Department of Ophthalmology and Visual Sciences, University of Wisconsin Medical School, Madison (Drs Tian and Kaufman and Ms Gabelt), and the Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, Israel (Ms Volberg and Dr Geiger). The University of Wisconsin Medical School and the Weizmann Institute of Science have filed a related patent application; accordingly, Drs Kaufman and Geiger have a proprietary interest.

ADHESIONS between cells, and between cells and extracellular matrix, in the trabecular meshwork (TM) contribute to TM geometry and flow resistance. These adhesions contain complex transmembrane interactions between the external cell surface and the actin-based cytoskeleton, mediated by specific adhesion receptors (integrins and cadherins) and a variety of submembrane anchor proteins that link the microfilaments to the membrane in these sites. In recent years, much information has accumulated on the mode of interaction of the various anchor proteins, such as vinculin[1] and catenins,[2] with the membrane, with the cytoskeleton, or with each other, shedding light on the molecular architecture of the adhesion sites. In addition to "structural proteins," these cell adhesions also contain a variety of signal transduction molecules such as protein kinase C and different tyrosine kinases, which can affect cell function and fate.[3] Cell adhesions are dynamic structures that change in number, location on the cell membrane, configuration, protein composition, and "tightness" in response to various factors.

Agents that interfere with the polymerization of actin monomers into F-actin–containing microfilaments (eg, cytochalasins B and D) alter the shape of human TM cells in culture[4,5] and increase TM outflow facility in living monkey eyes,[6-8] accompanied and perhaps caused by a pulling apart of cells in the juxtacanalicular region and the inner wall of the Schlemm canal, with subsequent inner-wall ruptures and a washout of extracellular material.[9,10] Calcium chelating agents, which primarily affect cell-cell junctions, produce similar cell separation and facility increases in living monkeys.[11,12]

The protein kinase inhibitor H-7 (1-[5-isoquinolinyl-sulfonyl]-2-methylpiperazine) inhibits protein kinases A, G, and C and myosin light chain kinase.[13,14] Cellular contraction is associated with calcium-dependent activation of myosin light chain kinase and the consequent phosphorylation of myosin.[15-18] H-7 disrupts microfilament bundles with accompanying decreases in phosphate-32 incorporation into phosphoproteins,[19] and pretreatment with H-7 inhibits the adenosine triphosphate–induced contraction of actin filaments,[15] indicating that the reduction of actomyosin

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MATERIALS AND METHODS

CELL CULTURE STUDIES

Cell Culture

Bovine aortic endothelial cells were cultured in Dulbecco modified Eagle medium supplemented with 10% bovine calf serum (Hyclone Laboratories Inc, Logan, Utah). Confluent cultures were split by treatment for 2 minutes with 0.1% trypsin and checked for the expression of von Willebrand factor by immunofluorescence microscopy to verify their endothelial nature.

Immunochemical Reagents and Methods

Cells cultured on glass coverslips were fixed and made permeable for 2 minutes with 3% paraformaldehyde and 0.5% Triton X-100 (t-Octylphenoxypoly-ethoxyethanol, Sigma Chemical Co, St Louis, Mo), further fixed for 20 minutes with 3% paraformaldehyde alone, then rinsed and incubated for 45 minutes at room temperature with the relevant primary antibodies, washed 3 times with phosphate-buffered sodium, and incubated for 45 minutes with fluorescein- or rhodamine-labeled secondary antibodies (Jackson Laboratories, Bar Harbor, Me). Stained coverslips were mounted in elvanol (Mowiol 4-88, Hoechst Chemical Co, Frankfurt, Germany) and examined with a microscope (Zeiss Axiophot, Carl Zeiss Inc, Thornwood, NY) using a 100/1.3 Planapochromat (Carl Zeiss Inc, Oberkochen, Germany) objective.

Chemicals and Drug Treatment

Monoclonal anti–human vinculin and anti–human von Willebrand factor were obtained from Sigma Immunochemicals (St Louis, Mo), and anti–β-catenin was obtained from Transduction Laboratories (Lexington, Ky). Actin filaments were visualized with tetramethylrhodamine isothio cyanate-phalloidin (Sigma, St Louis, Mo, or Molecular Probes, Eugene, Ore). Fluorescein- and rhodamine-labeled secondary antibodies (reactive with either rabbit or mouse IgG) were from Jackson Laboratories (Bar Harbor, Me) and cross-adsorbed, when necessary, on the heterologous IgG to diminish interspecies cross-reactivity. H-7 (No. I-7016, Sigma Immunochemicals) was added to growth medium from 30-mmol/L stock solution in distilled water, to a final concentration of 30, 100, or 300 μmol/L, for different incubation times.

LIVE ANIMAL STUDIES

Animals and Anesthesia

Normal cynomolgus monkeys (*Macaca fascicularis*, 2.0-5.2 kg) were studied in accordance with University of Wisconsin Medical School, Madison, and the National Institutes of Health guidelines. For anesthesia for anterior chamber (AC) perfusion, intramuscular ketamine hydrochloride (10 mg/kg), followed by intramuscular pentobarbital sodium (35 mg/kg) or by intravenous pentobarbital sodium (15 mg/kg initially, 5.0-7.5 mg/kg every 45-60 minutes), was administered. For anesthesia for intraocular pressure (IOP) measurement and slit-lamp examination, intramuscular ketamine hydrochloride (10 mg/kg initially, 5 mg/kg every 30-45 minutes) was administered.

Chemicals and Drug Preparation

H-7 was obtained from Sigma Immunochemicals and dimethyl sulfoxide (DMSO) from Research Industries Corporation (Salt Lake City, Utah). H-7 solutions for AC exchange perfusion (10-300 μmol/L) were freshly prepared in Bárány solution.[21] Topical H-7 was formulated as 0.3 mg in 10 μL of 10% DMSO (90 mmol/L), 1.1 mg in 20 μL of 25% DMSO (150 mmol/L), 2.9 mg in 20 μL of 25% DMSO (400 mmol/L), or 5.7 mg in 24 μL of 25% DMSO (650 mmol/L).

Outflow Facility Measurement

Total outflow facility was determined by 2-level constant-pressure AC perfusion with Bárány solution, correcting for internal apparatus resistance.[22] Most monkeys had undergone prior perfusions but not within the preceding 5 to 6 weeks; all were free of AC cells and flare. Baseline facility was always determined in both eyes for 35 minutes.

AC Exchange H-7 Perfusion. Following the baseline measurement, the AC was exchanged with 2 mL of H-7 solution

contractility is consequent to the decrease of phosphoproteins. H-7 specifically and rapidly affects the actin cytoskeleton of cultured Swiss 3T3 and PTK2 cells, but a potent protein kinase C inhibitor (sangivamycin) or a specific protein kinase A–protein kinase G inhibitor (HA1004) does not.[20] H-7 and a selective myosin light chain kinase inhibitor (KT5926) inhibit actomyosin-driven contractility in chick lens cells and fibroblasts, leading to a deterioration of the actin microfilament system and a perturbation of its membrane anchorage, but highly potent protein kinase C inhibitors (Ro31-8220 and GF109203X) do not.[15] Still, the specific target kinase for H-7 effects on the actin cytoskeleton and actomyosin contractility remains poorly defined.

We report here the effects of H-7 on cell junctions and the attached actin-based cytoskeleton in bovine aortic endothelial cells (BAECs) and on outflow facility in living monkeys.

RESULTS

CELL CULTURE STUDIES

Actin filaments in BAECs are assembled into large radial and circumferential bundles associated with either cell-cell junctions or focal adhesions to the underlying matrix (**Figure 1**, A). Vinculin in these cells is predominantly associated with focal adhesions and occasionally detected at cell-cell interfaces (Figure 1, B). The intercellular junctions, best visualized with anti–β-catenin antibodies (Figure 1, C), are either patchy or continu-

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at a concentration of 10 to 300 μmol/L or vehicle (opposite eyes) for 10 to 15 minutes. The reservoirs were then immediately filled with the corresponding solutions, closed for 45 minutes, then reopened for 45 to 60 minutes of postdrug facility measurement.

Topical H-7 Perfusion. After the baseline measurement, the reservoirs were closed to minimize resistance washout. H-7 solution (4×5 μL of H-7 at a concentration of 90, 150, or 400 mmol/L, or 4×6 μL of H-7 at a concentration of 650 mmol/L; 0.3, 1.1, 2.9, or 5.7 mg of H-7, respectively, in separate experiments to generate a dose-response curve; the 150-mmol/L, 1.1-mg dosage was chosen to give H-7 at a concentration of 300 μmol/L in the ~100-μL cynomolgus AC,[23] assuming 1% penetration[24-26] and no drug loss from the AC during entry) or vehicle (corresponding concentrations of DMSO) was administered to the superior cornea of opposite eyes at 60-second intervals; the lower eyelid was lifted several times immediately after each drop to ensure good drug-vehicle contact with the cornea. Two hours later, the reservoirs were reopened for 45 minutes of facility determination.

Reversibility

A (Short-term, 90 Minutes), AC Exchange H-7 Perfusion. The baseline facility measurement was followed by AC exchange with 2 mL of H-7 at a concentration of 300 μmol/L or vehicle in opposite eyes. The reservoirs were then filled with drug-free Bárány solution, closed for 45 minutes, reopened for 45 minutes for postdrug facility measurements, closed again for 90 minutes, and reopened for another 45 minutes for facility measurements.

B (Intermediate-term, 6-24 Hours), Topical H-7 Perfusion. Four 5-μL drops of H-7 at a concentration of 400 mmol/L or vehicle were administered topically at 30-second intervals to the central cornea of opposite eyes of the ketamine-anesthetized supine monkey, with blinking prevented between and after the drops. The monkeys were returned to their cages and reanesthetized for a 55-minute facility measurement beginning 6 or 24 hours after treatment.

C (Long-term, 5-12 Weeks), AC Exchange H-7 Perfusion. The baseline facility measurement was followed by AC exchange with 2 mL of H-7 at a concentration of 100 (8 monkeys) or 300 μmol/L (4 monkeys) in 1 eye and vehicle in the opposite eye (data from H-7 exchange perfusion protocol 1 above). A second baseline was obtained from the same monkeys 5 to 12 weeks later. These animals had exhibited a substantial facility response to H-7 on the initial perfusion.

IOP Measurement

The IOP was determined with a minified Goldmann applanation tonometer[27] in prone monkeys. Three readings were averaged as baseline. H-7 (400 mmol/L) or DMSO (25%) was then administered to the central cornea of opposite eyes of the supine monkeys as four 5-μL drops at intervals of 30 seconds, with blinking prevented. Single IOP readings were taken hourly thereafter for 6 hours.

Slitlamp Biomicroscopy

Integrity of the corneal epithelium, the presence or absence of AC flare or cells, and lens clarity were noted 1, 3, and 6 hours after the topical administration of H-7 at a concentration of 400 mmol/L in the IOP experiment and 3, 7, and 14 days after all topical H-7 doses or perfusions were given.

Data Analysis

Data are given as the mean (±SEM) for number of eyes or animals. The values for predrug- or postdrug-treated vs contralateral control eyes; postdrug or postvehicle vs ipsilateral eyes (baseline); and baseline-corrected values for postdrug-treated vs control eyes were compared using the 2-tailed paired *t* test for ratios vs 1.0 or differences vs 0.0. Facility measurements were analyzed using linear regression models with separate intercept and slope parameters for each of 3 periods (the intercept parameter being the expected facility at the start of each period). Monkey-specific estimates of parameters were used to summarize the repeated measurements for each monkey.[28] Estimates and confidence intervals for population parameters were obtained using the mean and variance of these monkey-specific estimates. Separate analyses were conducted for differences between treated eyes, control eyes, and paired treated eyes vs control eyes. Calculations were performed using statistical software (SAS PROC GLM[29] and SAS PROC UNIVARIATE, SAS Institute Inc, Cary, NC).[30]

ous, depending on the cell density and the duration of incubation.

H-7 induced a dramatic time- and concentration-dependent deterioration of actin filaments (**Figure 2**). Bovine aortic endothelial cells treated with H-7 at a concentration of 30 μmol/L lost most of their actin bundles after 60 minutes, whereas H-7 concentrations of 100 and 300 μmol/L induced a deterioration within 10 minutes. Residual actin filaments were usually associated with the cell periphery rather than with focal adhesions. Following a long incubation (16 hours), short actin bundles associated with small focal adhesions were formed. Vinculin staining (**Figure 3**) indicated a dramatic deterioration of focal adhesions, again slower and milder following an H-7 concentration of 30 μmol/L compared with 100 and 300 μmol/L. Vinculin associated with cell-cell adhesions was considerably more resistant than focal adhesion–associated vinculin, and with long treatment, numerous small focal contacts were formed. The effect of H-7 on cell-cell junctions was best demonstrated with anti–β-catenin. Cell-cell junctions were relatively resistant (Figure 3), even at the higher concentrations (**Figure 4**). β-Catenin–rich junctions were frequently partially split, however, forming intercellular gaps. Following overnight incubation with all concentrations of H-7, catenin-rich cell-cell adhesions deteriorated concurrently with the partial reformation of focal contacts.

The effects of H-7 at a concentration of 300 μmol/L were reversible, with actin filaments reformed following 30 minutes in H-7–free medium (**Figure 5**). Vinculin-containing focal adhesions and cell-cell junctions were also restored, although focal contacts, especially in cells

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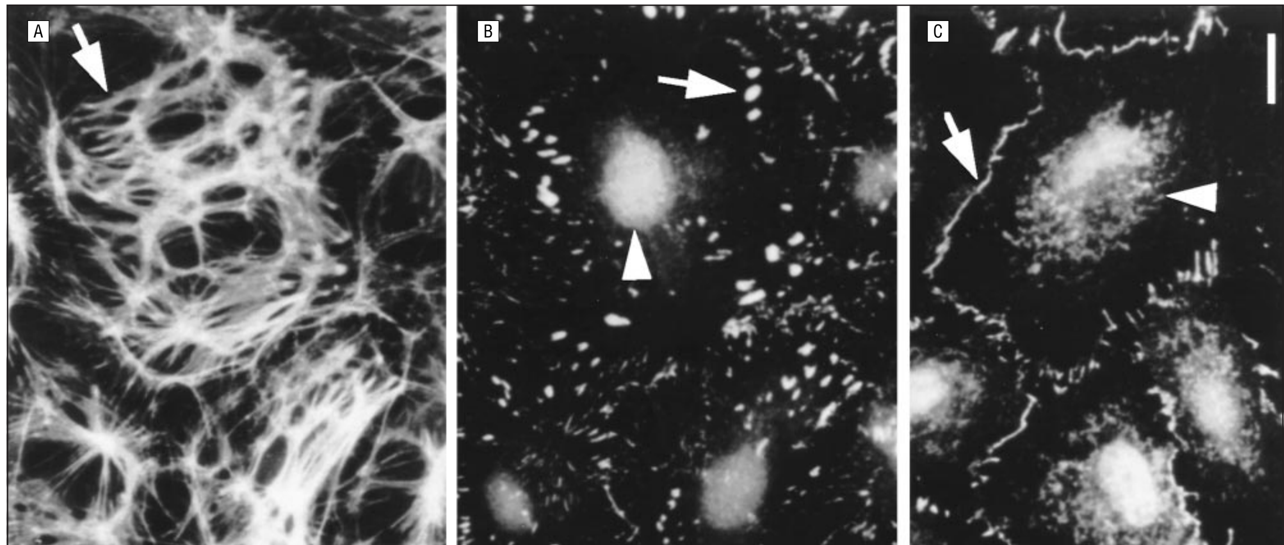

Figure 1. *Localization of adherent junction proteins in normal bovine aortic endothelial cells. Arrows show actin bundles (A), vinculin-containing focal contacts (B), and β-catenin–containing cell-cell adherent junctions (C). Cell nuclei are indicated by arrowheads in B and C. Bar indicates 10 μm.*

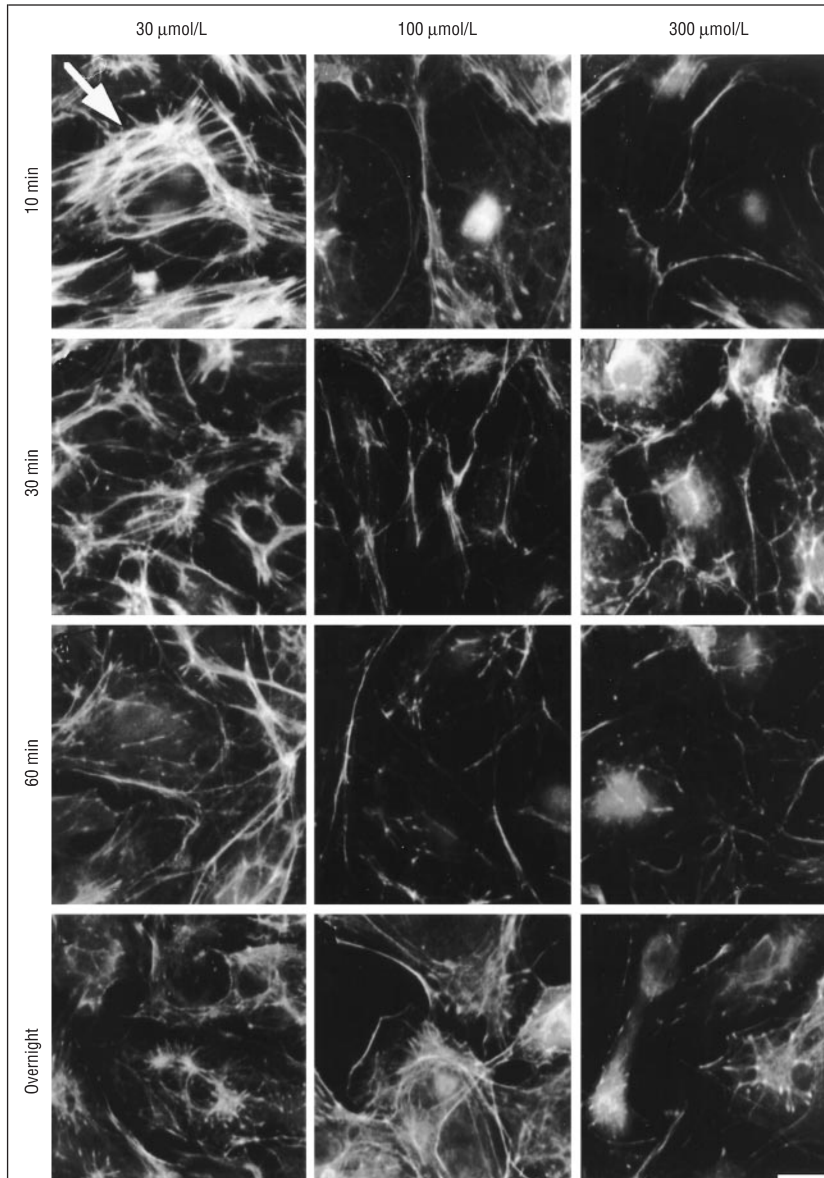

Figure 2. *Distribution of F-actin in bovine aortic endothelial cells treated with H-7 in a concentration of 30, 100, or 300 μmol/L for 10, 30, and 60 minutes and overnight. The arrow, top left, shows actin bundles. See Figure 1, A, for normal appearance. Bar, bottom right, indicates 10 μm.*

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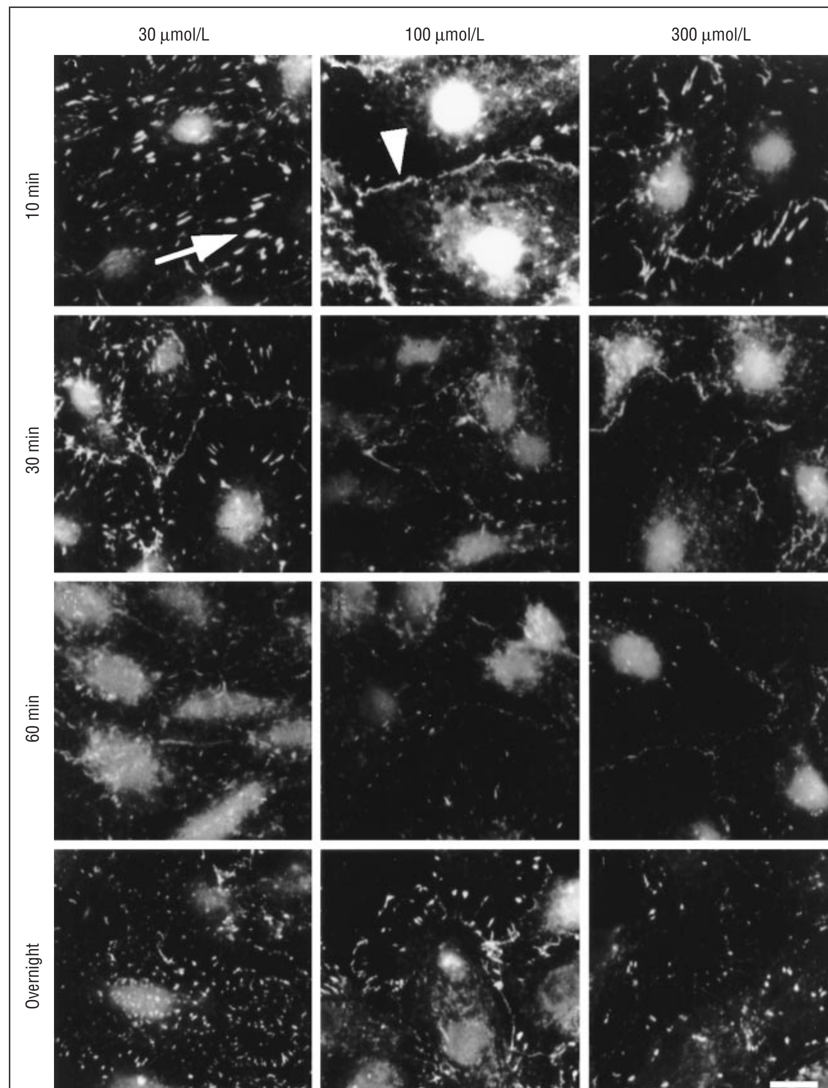

Figure 3. *Distribution of vinculin in bovine aortic endothelial cells treated with H-7 in a concentration of 30, 100, or 300 µmol/L for 10, 30, and 60 minutes and overnight. The arrow, top left, shows vinculin-containing focal contacts. The arrowhead, top center, points to cell-cell adherent junction–associated vinculin. See Figure 1, B, for normal appearance. Bar, bottom right, indicates 10 µm.*

pretreated with H-7 for a longer period, tended to be smaller than those of untreated cells (Figure 5). β-Catenin staining revealed a remarkable recovery of intact junctional structures (Figure 5), although gaps between adjacent cells were frequently noted even after several hours (not shown).

LIVE ANIMAL STUDIES

Outflow Facility

AC Exchange H-7 Perfusion. Anterior chamber exchange with concentrations of H-7 of 10 to 300 µmol/L increased facility dose dependently. Administering H-7 at a concentration of 10 µmol/L had no effect; 100 µmol/L was effective progressively over time, producing facility increases of 17% (±16%) and 53% (±22%) for the first and second half-hours, respectively, after reopening the reservoirs; and 300 µmol/L induced a 143% (±17%) facility increase (**Table 1**), all relative to the baseline and corrected for control-eye washout. When the perfusions were restarted, the initial postdrug facility values for all doses were only slightly, but dose and time dependently, increased relative to control eyes (not shown).

Topical H-7 Perfusion. The topical administration of H-7 at a concentration of 90, 150, 400, or 650 mmol/L increased facility by 21% (±10%), 59% (±14%), 126% (±40%), and 86% (±24%), respectively, adjusted for base line values and contralateral control eyes (**Table 2**). When the perfusions were restarted, the initial postdrug facility values were only slightly increased relative to those for control eyes, but the increase was dose and time dependent (not shown).

Reversibility

A (Short-term, 90 Minutes), AC Exchange H-7 Perfusion. Following AC exchange with H-7 at a concentration of 300 µmol/L or vehicle, the first facility value was only slightly higher in the H-7–treated eyes (**Figure 6**, B). Facility increased significantly with time in the H-7–treated eyes (Figure 6, E) but not in the control eyes (Figure 6, H), so that the treated–control eye difference increased with time (Figure 6, K). The facility increase in the H-7–treated eyes for the 45-minute measurement averaged 86% (±25%), relative to baseline and corrected for the minimal control-eye washout ($P<.01$) (**Table 3**). After another 90 minutes with reservoirs closed, facility

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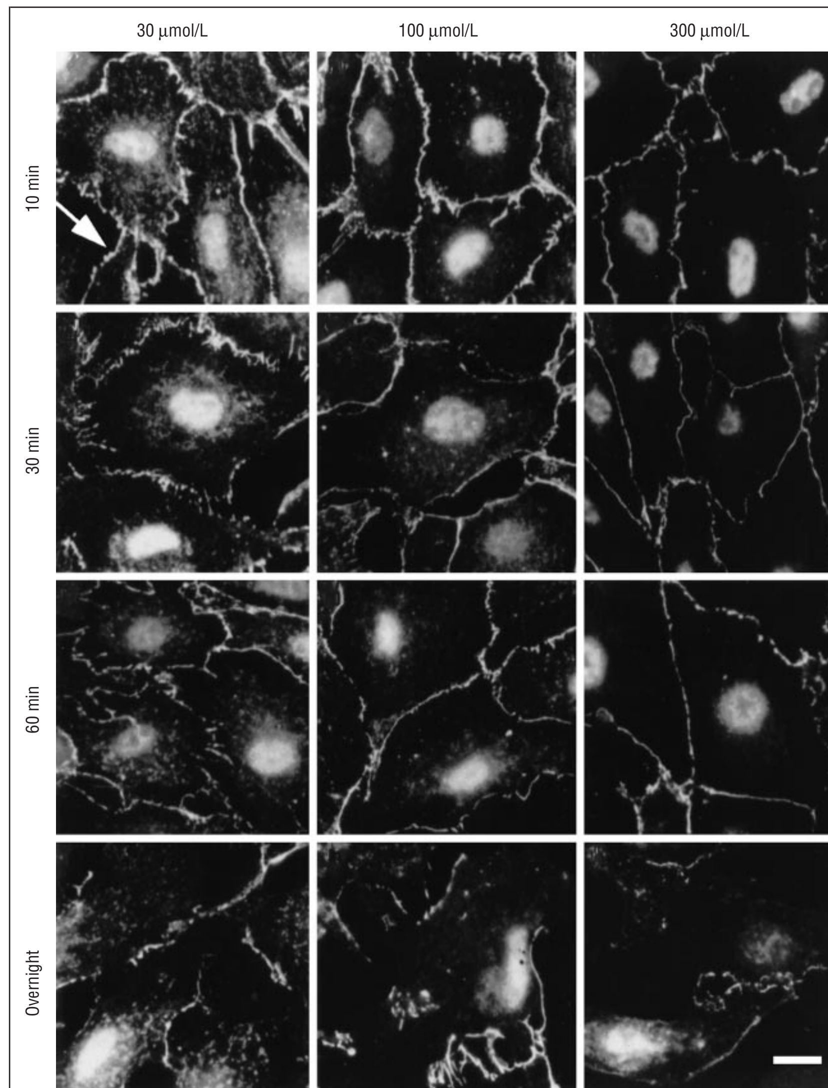

Figure 4. *Distribution of β-catenin in bovine aortic endothelial cells treated with H-7 in a concentration of 30, 100, or 300 μmol/L for 10, 30, and 60 minutes and overnight. The arrow, top left, shows β-catenin associated with cell-cell adherent junctions. See Figure 1, C, for normal appearance. Bar, bottom right, indicates 10 μm.*

in the control eyes was still essentially unchanged, and facility in the H-7–treated eyes had fallen to essentially the same level (Figure 6, C). On continued perfusion with drug-free solution, facility in both the H-7–treated and control eyes increased significantly with time (Figure 6, C, F, and I), with the treated–control eye difference again increasing progressively but not as rapidly (Figure 6, L). The increase for the entire 45-minute measurement was also less dramatic than in the earlier segment (39% [±20%], corrected for baseline and washout; $P<.01$) (**Table 4**). The time-dependent facility rise at 180 to 225 minutes tended to be less than that at 45 to 90 minutes after H-7 administration (Figure 6), whereas in control eyes, facility tended to rise more rapidly during the later interval (Figure 6), indicating tendencies toward reversibility of the H-7 effect in treated eyes and an acceleration of resistance "washout" in control eyes. The difference between these opposing changes, representing the decrease in the rate of facility rise in H-7–treated eyes after the drug-free period, corrected for the increased resistance washout rate in control eyes, was highly significant (Figure 6).

B (Intermediate-term, 6-24 Hours), Topical H-7 Perfusion. Following the topical administration of H-7 at a concentration of 400 mmol/L (2.9 mg), facility was 44% (±18%) and 23% (±16%) higher than in contralateral vehicle-treated eyes at 6 and 24 hours. For the 400-mmol/L H-7 dose in the topical dose-response protocol, 2-hour posttreatment facility unadjusted for the baseline value was 117% (±39%) higher in H-7–treated eyes than in contralateral eyes (**Table 5**).

C (Long-term, 5-12 Weeks), AC Exchange H-7 Perfusion. Baseline facilities 5 or more weeks after facility-effective AC exchange perfusions with H-7 were 11% (±8%) (100 μmol/L), 43% (±20%) (300 μmol/L), and 21% (±9%) (groups combined) higher than the original baseline facilities, adjusted for control-eye changes (Table 4).

INTRAOCULAR PRESSURE

Significant ipsilateral IOP reduction occurred from hour 1 to 6 after topical H-7 at a concentration of 400 mmol/L

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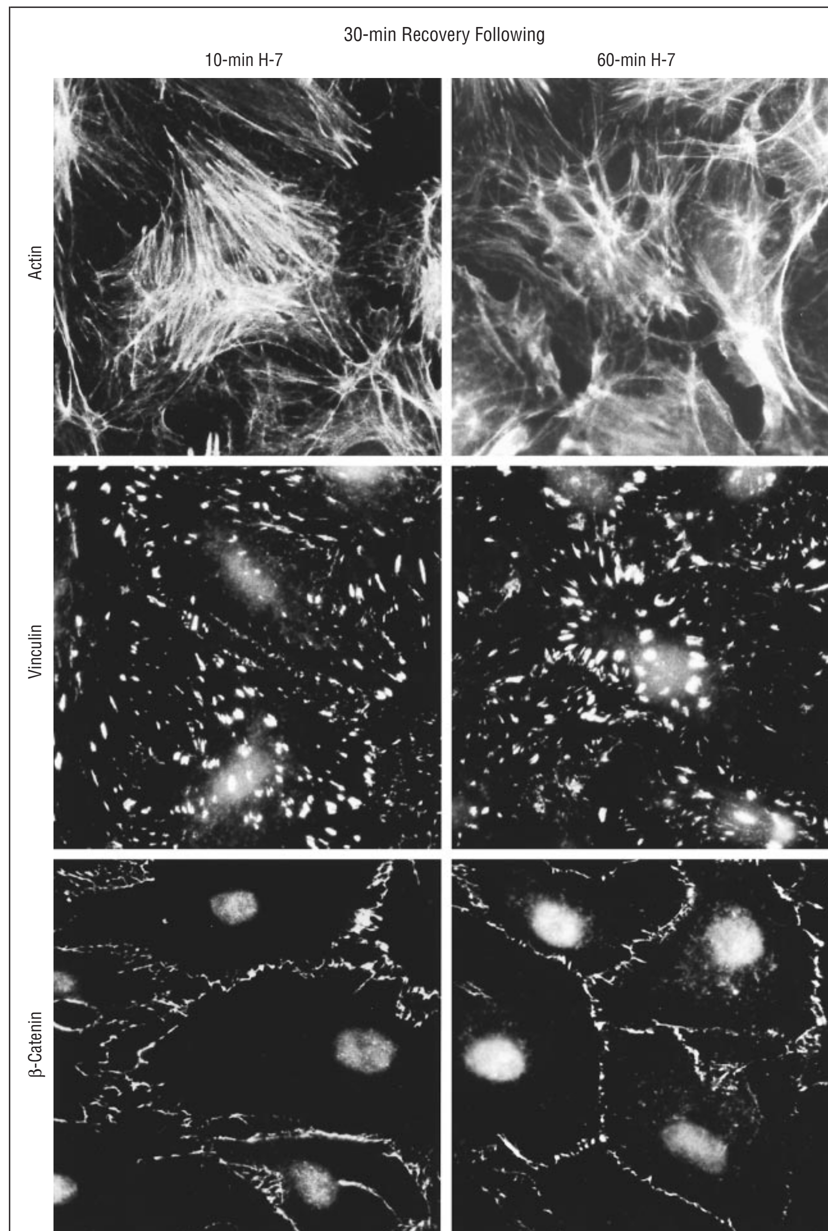

Figure 5. *Recovery of actin, vinculin, and β-catenin in bovine aortic endothelial cells treated with an H-7 concentration of 300 µmol/L for 10 or 60 minutes and allowed to recover for 30 minutes in H-7–free medium. The organization of actin, vinculin, and β-catenin and their associated adherent junction sites recovered well (compare with Figures 2-4, which show the effect of H-7 without a recovery time). Following a longer recovery, the normal organization of the 3 proteins was completely restored (data not shown).*

(2.9 mg) was administered, with a maximal decrease of 3.25 (±0.69) mm Hg at hour 2, compared with that in contralateral control eyes ($P<.01$) (**Figure 7**). The magnitude of IOP reduction was consistent with the facility increase at 2 to 3 and 6 to 7 hours, according to the modified Goldmann equation.[31]

SLITLAMP BIOMICROSCOPY

No AC or lens abnormalities were observed following the topical or intracameral administration of H-7. On the first day after the 400-mmol/L topical dose, the treated eyes exhibited mild punctate corneal epithelial defects and slight epithelial cloudiness compared with control eyes. From 3 to 14 days following all topical doses, no corneal effects were observed.

COMMENT

Exposing cultured BAECs to H-7 at concentrations of 30 to 300 µmol/L produced time- and concentration-dependent disruptions of actin microfilaments, primarily affecting stress fibers and cell-extracellular matrix adhesions. Actin filaments in the cell periphery and cell-cell adhesions were less affected, although limited but persistent separation of these junctions occurred. H-7 reduces contractility in cultured chick lens cells and fibroblasts, probably by inhibiting myosin light chain kinase, which plays an indispensable role in actomyosin contraction in smooth muscle and nonmuscle cells.[15] The loss of cellular contractility leads to a deterioration of actin filaments and consequent weakening of cellular adherent junctions, whereas cell contractility may be im-

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Table 1. Effect of Intracameral H-7, Administered by Exchange Perfusion, on Outflow Facility in Monkeys

Dosage Protocol*	Outflow Facility, µL/(min · mm Hg)† H-7	Vehicle	H-7/Vehicle
10 µmol/L (n = 4)			
Baseline	0.28 ± 0.02	0.29 ± 0.05	1.01 ± 0.10
Rx	0.31 ± 0.02	0.31 ± 0.03	1.01 ± 0.08
Rx/baseline	1.15 ± 0.13	1.16 ± 0.17	1.03 ± 0.13
100 µmol/L (n = 8)			
Baseline	0.38 ± 0.05	0.37 ± 0.05	1.05 ± 0.06
Rx	0.43 ± 0.07	0.35 ± 0.06	1.28 ± 0.13‡
Rx_1	0.38 ± 0.06	0.33 ± 0.06	1.19 ± 0.13
Rx_2	0.66 ± 0.12	0.44 ± 0.09	1.58 ± 0.23§
Rx/baseline	1.12 ± 0.10	0.94 ± 0.08	1.25 ± 0.16
Rx_1/baseline	0.97 ± 0.10	0.87 ± 0.07‡	1.17 ± 0.16
Rx_2/baseline	1.68 ± 0.17‖	1.16 ± 0.11	1.53 ± 0.22§
300 µmol/L (n = 10)			
Baseline	0.38 ± 0.06	0.41 ± 0.04	0.97 ± 0.13
Rx	0.88 ± 0.14	0.40 ± 0.05	2.29 ± 0.27‖
Rx/baseline	2.41 ± 0.27‖	1.00 ± 0.09	2.43 ± 0.17‖

*Rx indicates postdrug facility for 45 minutes; Rx_1, postdrug facility for the first 30 minutes; and Rx_2, postdrug facility for the second 30 minutes. Postdrug facility measurement was begun 45 minutes after drug administration.

†Data are mean ± SEM for number of animals, each contributing 1 H-7–treated and 1 vehicle-treated eye.

‡$P<.01$. P values are for ratios different from 1.0 by the 2-tailed paired t test.

§$P<.05$.

‖$P<.001$.

Table 2. Effect of Topical H-7 on Outflow Facility in Monkeys

Dosage Protocol*	Outflow Facility, µL/(min · mm Hg)† H-7	Vehicle	H-7/Vehicle
90 mmol/L (n = 7)			
Baseline	0.40 ± 0.05	0.33 ± 0.07	1.35 ± 0.14‡
Rx	0.55 ± 0.05	0.38 ± 0.07	1.64 ± 0.23‡
Rx/baseline	1.43 ± 0.10§	1.23 ± 0.14	1.21 ± 0.10¶
150 mmol/L (n = 7)			
Baseline	0.29 ± 0.06	0.27 ± 0.06	1.13 ± 0.14
Rx	0.57 ± 0.15	0.33 ± 0.06	1.75 ± 0.22‡
Rx/baseline	1.92 ± 0.15‖	1.24 ± 0.10¶	1.59 ± 0.14§
400 mmol/L (n = 8)			
Baseline	0.36 ± 0.05	0.38 ± 0.04	1.06 ± 0.25
Rx	0.82 ± 0.20	0.37 ± 0.05	2.17 ± 0.39‡
Rx/baseline	2.33 ± 0.55‡	0.99 ± 0.07	2.26 ± 0.40‡
650 mmol/L (n = 6)			
Baseline	0.39 ± 0.08	0.41 ± 0.12	1.13 ± 0.17
Rx	0.93 ± 0.19	0.56 ± 0.19	2.06 ± 0.31‡
Rx/baseline	2.46 ± 0.40‡	1.32 ± 0.08§	1.86 ± 0.24‡

*Rx indicates postdrug facility. Postdrug data encompass 45 minutes, beginning 2 hours after drug administration.

†Data are mean ± SEM for number of animals, each contributing 1 H-7–treated and 1 vehicle-treated eye. P values are for ratios different from 1.0 by the 2-tailed paired t test.

‡$P<.05$.

§$P<.01$.

‖$P<.001$.

¶$P<.10$.

portant in the assembly of microfilament bundles. Thus, the small G-protein rho A stimulates stress fiber assembly, possibly through the induction of myosin light chain phosphorylation,[32] while, in cultured 3T3 cells, the disruption of microtubules, which leads to cell contraction, induces a rapid and H-7–sensitive assembly of microfilaments.[33]

H-7 increased the outflow facility dose dependently and lowered the IOP in monkeys. The facility-effective dose range for AC exchange (100-300 µmol/L) in monkeys was identical to that for actin filament and adherent junction alterations in cultured BAECs, suggesting that H-7 increased facility by a cytoskeletal–cell junctional mechanism. Topical H-7 was also effective dose dependently, with a maximum increase at 2 to 3 hours of 126% occurring at 400 mmol/L (2.9 mg), comparable to the 143% achieved 1 to 2 hours after AC exchange at 300 µmol/L, and lowering the IOP by an amount consistent with the facility increase.[31]

The cytoskeletal effects of H-7 in cultured BAECs and the facility increase in living monkeys were both partly reversible within hours after drug removal, again suggesting the latter's association with actin filament deterioration. Reversibility also indicates that H-7 effects represent transient alterations in cytoskeletal organization and cell adhesions rather than irreversible toxic effects or a major loss of resistance-relevant extracellular matrix (which would require many hours for resynthesis). Baseline facility in previously treated eyes, however, remained modestly (21%) but significantly increased 5 to 12 weeks after a facility-effective H-7 dose, suggesting a long-lasting effect. Analogously, in BAECs following 16 hours' incubation with H-7 at concentrations of 100 or 300 µmol/L, the actin bundles and focal contacts were only partially reformed, appearing shorter or smaller than normal, and the catenin-rich cell-cell adhesions were still deteriorated.

A bolus infusion of 10 µL of H-7, 0.1 or 1 mmol/L (10 or 100 µmol/L initially in the AC), was ineffective, but 10 µL of H-7, 5 mmol/L (500 µmol/L initially in the AC), was the threshold for increasing facility (B.T. and P.L.K, unpublished data, 1997), analogous to an AC exchange infusion dose of H-7 of about 100 µmol/L. The dose ratio of 5:1 between bolus and exchange infusion holds also for pilocarpine hydrochloride[34,35] and may be consequent to an incomplete mixing and a rapidly decreasing AC drug concentration following a bolus infusion. Thus, the ineffectiveness of our bolus infusion doses was presumably due to an adequate drug concentration not being maintained for an adequate time.

A perturbation of cell contractility and consequent cytoskeletal alterations and weakening of cell adhesions in the TM might lead to increased outflow facility if fluid flow across a TM structure "loosened" by H-7 further loosened and separated TM layers and cells, enhancing the intercellular flow pathways and washout of resistance-relevant extracellular matrix. Our facility data support this hypothesis. Following AC exchange infusion or the topical application of H-7, initial facility values were only slightly but dose dependently elevated. Substantial facility elevation required continued perfusion. Because the reservoirs were closed immediately after H-7 administration and aqueous production under pentobarbital anesthesia is about 1 µL/min,[23,36,37] the H-7 concentration

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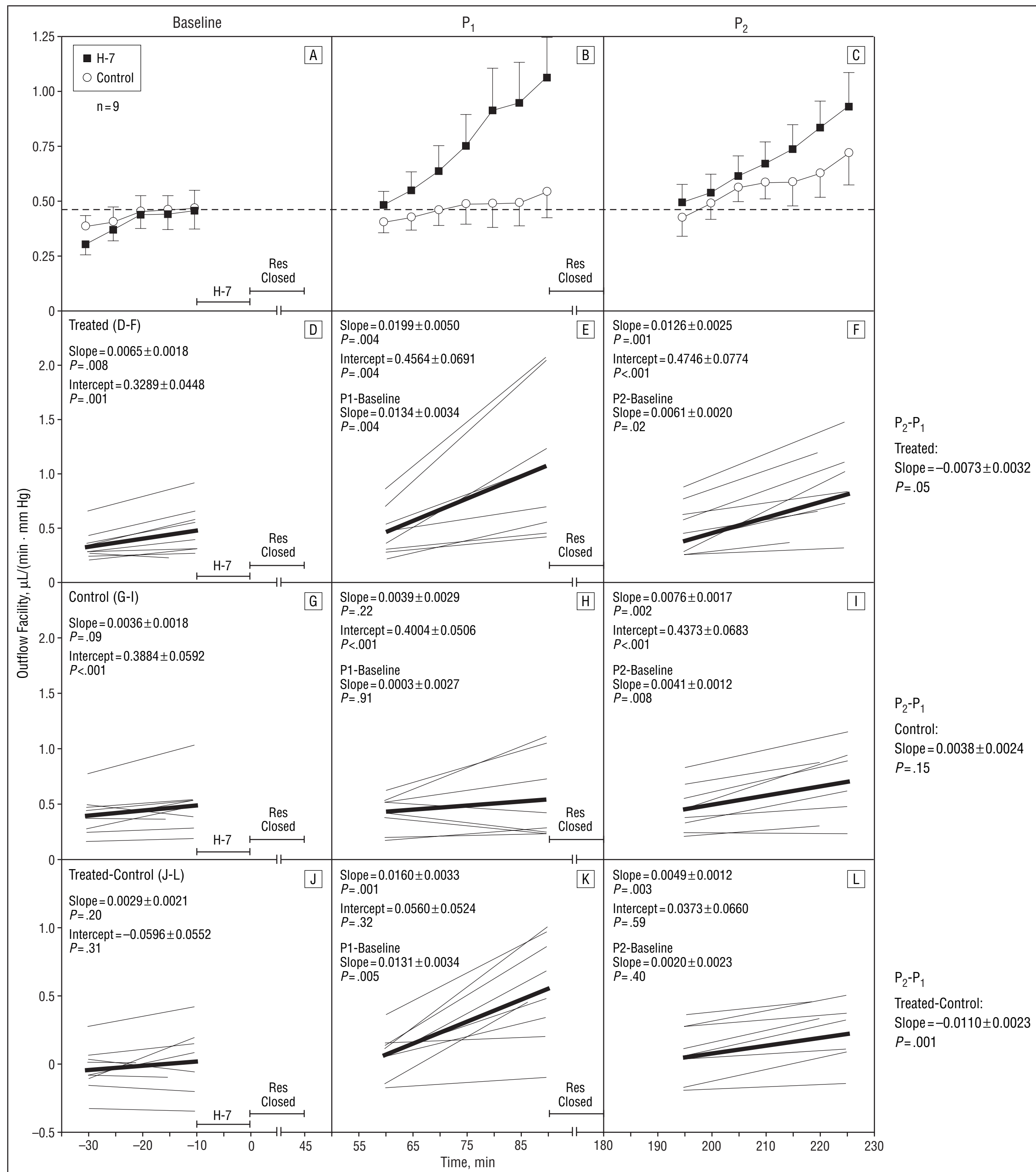

Figure 6. *Short-term reversibility of the facility increase after intracameral infusion of H-7 in a concentration of 300 µmol/L in monkeys. A, D, G, and J show the baseline facility; B, E, H, and K show postdrug facility 45 to 90 minutes after H-7 infusion (P1); and C, F, I, and L show postdrug facility 180 to 225 minutes after H-7 infusion (P2). In A to C, the data are given as mean (±SEM) microliters per minute per millimeters of mercury for number of animals, each contributing 1 H-7– and 1 vehicle–treated eye; the dashed line is the level of the last baseline value in treated eyes. In D to L, thin lines (from individual eye values obtained every 5 minutes) and thick dark lines (from mean values) represent the regression of facility on time. Res indicates reservoir. Slope and intercepts (±SE) reported are derived from the mean values, with* P *indicating the probability that the slope is 0.*

in the AC would have decreased by about 36% (45-minute wait) or 70% (2-hour wait). Therefore, H-7 in a concentration of about 190 or 240 µmol/L would be left in the 100-µL AC 45 minutes after AC exchange with an H-7 concentration of 300 µmol/L or 2 hours after topical administration of 20 µL of an H-7 concentration of 400 mmol/L, respectively. The last facility value 110 minutes after AC exchange of an H-7 concentration of 100 µmol/L is much larger than the first value 45 minutes after AC exchange of an H-7 concentration of 300 µmol/L or 2 hours after topical H-7 in a concentration of 400 mmol/L is administered, indicating that the absence of a

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Table 3. Reversibility of Outflow Facility Effect of 300 µmol/L Intracameral H-7, Administered by Exchange Perfusion, in Monkeys

Protocol*	Outflow Facility, µL/(min · mm Hg)† H-7	Vehicle	H-7/Vehicle
Baseline	0.39 ± 0.06	0.42 ± 0.07	1.01 ± 0.13
Rx_1	0.76 ± 0.14	0.46 ± 0.08	1.73 ± 0.18‡
Rx_2	0.65 ± 0.10	0.55 ± 0.08	1.32 ± 0.19
Rx_1/baseline	1.87 ± 0.15§	1.10 ± 0.12	1.86 ± 0.25‡
Rx_2/baseline	1.69 ± 0.17‡	1.33 ± 0.15‖	1.39 ± 0.20‖

*Rx_1 indicates 45 minutes' postdrug facility 45 minutes after H-7 is given; Rx_2, 45 minutes' postdrug facility 180 minutes after H-7 is given.
†Data are mean ± SEM for 9 animals, each contributing 1 H-7–treated and 1 vehicle-treated eye. P values are for ratios different from 1.0 by the 2-tailed paired t test.
‡$P<.01$.
§$P<.001$.
‖$P<.10$.

Table 4. Outflow Facility 5 to 12 Weeks After H-7 Administration by Exchange Perfusion in Monkeys

Dosage Protocol*	Outflow Facility, µL/(min · mm Hg)† H-7	Vehicle	H-7/Vehicle
100 µmol/L (n = 8)			
Baseline	0.38 ± 0.05	0.37 ± 0.05	1.05 ± 0.06
Rx	0.39 ± 0.07	0.34 ± 0.04	1.16 ± 0.11
Rx/baseline	1.02 ± 0.09	0.93 ± 0.08	1.11 ± 0.08
300 µmol/L (n = 4)			
Baseline	0.36 ± 0.05	0.43 ± 0.10	0.98 ± 0.21
Rx	0.44 ± 0.07	0.36 ± 0.06	1.32 ± 0.22
Rx/baseline	1.22 ± 0.12	0.88 ± 0.10	1.43 ± 0.20
100/300 µmol/L (n = 12)			
Baseline	0.37 ± 0.04	0.39 ± 0.04	1.03 ± 0.08
Rx	0.41 ± 0.05	0.34 ± 0.03	1.22 ± 0.10‡
Rx/baseline	1.08 ± 0.07	0.92 ± 0.06	1.21 ± 0.09§

*Rx indicates postdrug facility 5 to 12 weeks after H-7 exchange.
†Data are mean ± SEM for number of animals, each contributing 1 H-7–treated and 1 vehicle-treated eye. P value are for ratios different from 1.0 by the 2-tailed paired t test.
‡$P<.10$.
§$P<.05$.

large initial facility increase is not related to the decay of the H-7 concentration in the AC. Collectively, this suggests that the deterioration of the actin filament network destabilizes cell junctions within the TM and produces slight resistance washout at low transtrabecular pressure gradients and flow rates (spontaneous IOP [ie, perfusion reservoirs closed] under pentobarbital anesthesia is typically <10 mm Hg or 30%-50% lower than that in conscious or ketamine-anesthetized monkeys[23,38,39]; the aqueous humor flow rate under pentobarbital anesthesia is ~30% lower than that under ketamine anesthesia[35]). At higher pressure gradients or flow rates, however (during perfusion from the open reservoir, the IOP averages ~7 mm Hg higher than the spontaneous IOP and flow averages 4-fold to 6-fold higher than spontaneous aqueous humor flow rate [B.T., B.T.G., J. A. Peterson, BS, J. A. Kiland, BS, and P.L.K., unpublished data, 1997][40]), the destabilized TM

Table 5. Reversibility of 400-mmol/L Topical H-7–Induced Increase in Outflow Facility in Monkeys

Protocol*	Outflow Facility, µL/(min · mm Hg)† H-7	Vehicle	H-7/Vehicle
Rx_{2h} (n = 8)	0.82 ± 0.20	0.37 ± 0.05	2.17 ± 0.39‡
Rx_{6h} (n = 6)	0.63 ± 0.11	0.43 ± 0.05	1.44 ± 0.18‡
Rx_{24h} (n = 10)	0.48 ± 0.05	0.44 ± 0.05	1.23 ± 0.16

*Rx_{2h}, Rx_{6h}, or Rx_{24h} indicates 2, 6, or 24 hours after H-7 administration.
†Data are mean ± SEM for number of animals, each contributing 1 H-7–treated and 1 vehicle-treated eye.
‡$P<.05$ for ratios different from 1.0 by the 2-tailed paired t test.

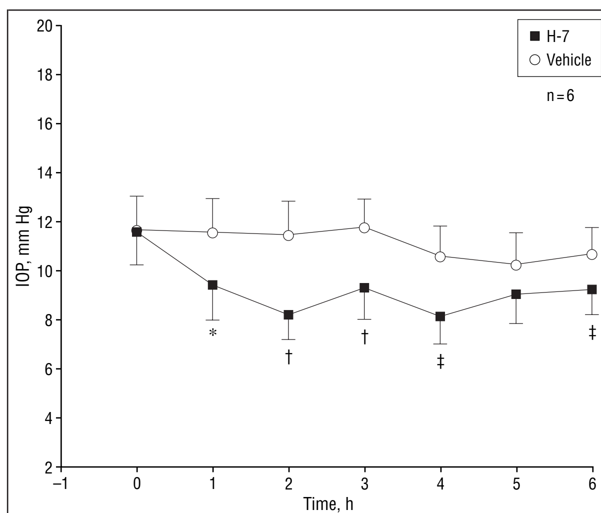

Figure 7. *Effect of 20 µL of the topical administration of H-7 in a concentration of 400 mmol/L (2.9 mg) on the intraocular pressure (IOP) in monkeys. Data are given as mean (±SEM) millimeters of mercury for number of animals, each contributing 1 H-7– and 1 vehicle–treated eye. The difference between eyes does not equal 0.0 by the 2-tailed paired t test. Asterisk indicates P<.05; dagger, P<.01; and double dagger, P<.001.*

architecture is disrupted sufficiently to substantially reduce flow resistance. Under this scenario, H-7 may be effective in glaucoma patients with elevated IOPs, especially if they are not receiving secretory suppressants, and a digital ocular pressure may enhance the effect. Furthermore, cell adhesions, although loosened, are retained and cells are not lost at the H-7 concentrations and durations studied, important considerations for TM function in living eyes. Studies are needed to confirm that the facility-increasing effect is consequent to direct drug action on the TM and to assess other structural and functional effects in living eyes.

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Reprints: Paul L. Kaufman, MD, Department of Ophthalmology and Visual Sciences, University of Wisconsin Medical School , F4/328 Clinical Science Center, 600 Highland Ave, Madison, WI 53792-3220.

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