Latrunculin-A Increases Outflow Facility in the Monkey

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PURPOSE. To determine the effect of Latrunculin (LAT)-A, a macrolide that binds to G-actin, which leads to the disassembly of actin filaments, on shape, junctions, and the cytoskeleton of cultured bovine aortic endothelial cells (BAECs) and on outflow facility in living monkeys.

METHODS. Latrunculin-A dose-time-response relationships in BAECs were determined by immuno-fluorescence and phase contrast light microscopy, facility by two-level constant pressure anterior chamber perfusion.

RESULTS. In BAECs, LAT-A caused dose- and incubation time-dependent destruction of actin bundles, cell separation, and cell loss. Cell-cell adhesions were more sensitive than focal contacts. Recovery was also dose- and time-dependent. In monkeys, exchange intracameral infusion and topical application of LAT-A induced dose- and time-dependent several-fold facility increases. The facility increase was completely reversed within several hours after drug removal. However, for at least 24 hours after a single topical LAT-A dose, perfusion with drug-free solution caused an accelerated increase in facility beyond that attributed to normal resistance washout.

CONCLUSIONS. Pharmacological disorganization of the actin cytoskeleton in the trabecular meshwork by specific actin inhibitors like LAT-A may be a useful antiglaucoma strategy. *(Invest Ophthalmol Vis Sci.* 1999;40:931-941)

In many types of cultured cells, induced deterioration of the cytoplasmic microfilament system leads to alterations in the submembrane junctional plaque and consequent weakening of cell-cell and cell-extracellular matrix adhesions.[1,2] Cytochalasins B and D disrupt the actin cytoskeleton by a complex mechanism,[3,4] altering the shape of human trabecular meshwork (TM) cells in culture[5,6] and increasing TM outflow facility in the living monkey eye.[7-9] This increase may be due to the separation of cells in the juxtacanalicular region of the TM and the lining of the inner wall Schlemm's canal, causing subsequent ruptures in the inner wall and washout of extracellular material.[10,11]

Latrunculin A (LAT-A), a 16-membered macrolide derived from the Red Sea sponge *Latrunculia magnifica*, is a specific actin-disrupting agent that disassembles actin filaments by sequestering monomeric actin.[12,13] To better characterize the role of the microfilament system in maintaining cell-cell and cell-extracellular matrix adhesions, in normal outflow resistance, and as a possible target for therapeutic facility enhancement, we studied the effects of LAT-A on cultured bovine aortic endothelial cells (BAECs) and on outflow facility in the living monkey.

METHODS

LAT-A and Dimethyl Sulfoxide

LAT-A (from Yoel Kashman, Tel-Aviv University) was stored as a 0.2 mM, 0.5 mM, or 20 mM stock solution in dimethyl sulfoxide (DMSO) at $-20°C$ (Rehovot) or 4°C (Madison). The 0.2 mM stock solution (in Madison) was stored in either 1% or 100% DMSO. Dimethyl sulfoxide was obtained from Sigma Chemical (St. Louis, MO).

Cultured BAECs

An established line of BAECs was used to model LAT-A's effects on actin filaments and cell-cell adhesion and its dose-response relationship. BAECs form, in culture, a continuous monolayer with well-developed intercellular junctions, extensive adhesion to the underlying matrix, and a well-developed network of actin microfilaments. The cells were plated at half confluence on glass coverslips and cultured in Dulbecco's modified Eagle's medium, containing 1 g glucose, 9% bovine calf serum, 100 mg penicillin, and 100 mg streptomycin/550 ml, for 2 to 4 days at 37°C, 7% CO_2/93% O_2, with 90% humidity. LAT-A, from 500 μM stock solution in DMSO, was added to growth medium to a final LAT-A concentration of 0.2, 1, 2, or 5 μM for 0.5, 2, or 24 hours, after which the cells were washed with 50 mM MES (2-(N-morpholine) ethanesulfonic acid) buffer, permeabilized

with 0.5% Triton X-100 (Sigma) and fixed with 3% paraformaldehyde, both in MES buffer. The cells were then incubated with rhodamine phalloidin (Sigma) for fluorescence labeling of actin; or with rabbit pan-cadherin primary antibody[14] followed by tetramethyl rhodamine isothiocynate-conjugated goat antirabbit secondary antibody (Jackson Laboratories, Bar Harbor, ME) to visualize cell–cell adherens junctions (AJ); or with monoclonal anti–human vinculin antibody (Sigma) followed by fluorescein isothiocynate–conjugated goat anti-mouse secondary antibody (Jackson) to visualize both AJ and cell–matrix focal contacts (FCs). Vinculin and cadherin are junctional markers used to view the cell–cell junctions. Cells were examined by epifluorescence with an Axiophot microscope (Carl Zeiss, Oberkochen, Germany) equipped with filter sets for rhodamine, fluorescein, and DAPI fluorescence and for phase and Nomarsky optics, using a 100/1.3 Planapochromat objective. To judge reversibility of the LAT-A effect, the medium was changed to an identical one without drug for 0.25, 1, 5, or 22 hours, and the cells were washed, fixed, labeled, and examined as above.

Live Monkeys

Adult cynomolgus (*Macaca fascicularis*) and rhesus (*Macaca mulatta*) monkeys were anesthetized with intramuscular (IM) ketamine (10 mg/kg) followed by IM pentobarbital-Na (35 mg/kg). All eyes were free of anterior chamber (AC) cells and flare by slit-lamp biomicroscopy at the time of experimentation, and all experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Bolus Infusion. Total outflow facility was measured by two-level constant pressure perfusion of the AC with Bárány's mock aqueous humor,[15] correcting for the internal resistance of the perfusion apparatus as appropriate. The AC of both eyes of cynomolgus monkeys was cannulated with a branched 26-gauge needle. One arm of the needle was connected by polyethylene tubing to a continuously weighed reservoir of Bárány's solution, the other to a pressure transducer. Facility measurements were taken for 35 to 45 minutes. With reservoirs open, 10 μl of either 5 μM or 20 μM LAT-A (2 μl DMSO) was injected into one eye via a T-piece connected to the inflow tubing (0.5 μM and 2 μM, respectively, in the 100 μl AC[16]) and vehicle (2% DMSO in AC) into the other, using a micrometer syringe. After 5 minutes of wash-in and 3 minutes of convection mixing of the AC by blowing cold air on the cornea, facility measurements were taken for 90 minutes.

Exchange Infusion. The AC of each eye of normal cynomolgus monkeys was cannulated with a branched needle as above and an unbranched needle with polyethylene tubing attached. This tubing had been filled previously with Bárány's solution and clamped off. Baseline facility measurements were taken for 35 to 45 minutes. The clamped tubing from the unbranched needle was then connected to syringes containing drug (0.2, 0.5, 2, or 5 μM LAT-A) or vehicle (0.01%, 0.25%, 0.01%, or 0.025% DMSO, respectively). Cynomolgus and rhesus monkeys with bilateral surgical aniridia[17] and unilateral ciliary muscle disinsertion[18] received 5 μM LAT-A in both eyes. The syringes were placed in a variable-speed infusion pump, and the tubing previously leading to the reservoir was opened to air, allowing infusion of 2 ml of solution through the AC over 10 to 15 minutes, maintaining intraocular pressure (IOP) at approximately 15 mm Hg. The fluid exchanging through the

eye dripped out of the unconnected tubing. The reservoirs were emptied and filled with the same solution being exchanged through the eye. After the exchange, the unconnected tubing was then reattached to the reservoir, and the tubing leading to the syringes was clamped off. Outflow facility measurements were then immediately taken for 90 minutes.

In our two-level constant pressure perfusion system, three 5-minute periods (~15 minutes total) at alternating IOP levels are used to obtain a single facility value.[15] However, the weight of the external reservoir is recorded by computer every 5 seconds, so the flow rate from the reservoir into the eye can be calculated with high precision and time resolution. To better define the time of onset of the drug effect, flow data for the first 20 minutes after AC exchange with 5 μM LAT-A were subject to linear regression analysis, searching for the earliest time point when the slopes of the treated and control eyes differed. To distinguish slope changes due to "normal" perfusion-induced resistance washout in the control eyes from LAT-A-induced slope changes above and beyond washout, this analysis ideally assumes that aqueous humor formation (AHF) does not change, or changes identically in the two eyes during the measurement period, and that baseline IOP, both levels of artificially elevated perfusion IOPs, and the various IOP differences are constant and identical in both eyes. An increasing AHF, a lower baseline IOP, or a smaller difference between baseline and elevated perfusion IOPs or between the two perfusion IOPs in the LAT-A-treated eyes compared with the vehicle eyes, would all tend to reduce the difference in flow between the two eyes from the external reservoir after drug administration. This would delay the apparent time of onset of the drug effect estimated by that criterion.

Topical Eye Drops. The ACs of both eyes of normal cynomolgus monkeys were cannulated as for the bolus injections, and baseline facility measurements were taken for 35 to 45 minutes. With reservoirs closed, 2 × 5 μl of 5 mM (21 μg) or 4 × 5 μl of 5 mM (42 μg) LAT-A was injected into one eye, allowing 60 to 90 seconds between drops. The drops were placed at the superior limbus and allowed to flow down the cornea after which the lower lid was lifted two to three times. Vehicle (25% DMSO) was given simultaneously to the opposite eye in a similar manner. If 1% of the applied drug entered the AC[19-21] and no drug was lost from the AC, the resulting AC LAT-A concentrations would be 5 μM and 10 μM, respectively. The system remained closed for 1 hour (21 μg) or 2 hours (21 μg, 42 μg) after which facility measurements were taken for 90 minutes. Biomicroscopy was performed by an ophthalmologist before and 3, 7, and 14 days after drug administration.

Reversibility. Reversibility experiments were performed only in normal cynomolgus monkeys.

AC Exchange. After baseline facility measurements, the ACs were exchanged with 5 μM LAT-A in one eye and vehicle in the other with facility measured for 60 minutes beginning immediately thereafter, followed by a second exchange with vehicle in both eyes and another 60 minutes of facility measurements. For a second protocol, after baseline facility measurements, AC exchange was done with 5 μM LAT-A in one eye, vehicle in the other with facility measured immediately thereafter for 60 minutes. Vehicle was then exchanged bilaterally, after which the system was closed to inflow for 3 hours, then opened to inflow and facility measured for another 60 minutes. During each ex-

change the reservoirs were emptied and filled with the same solution being perfused through the eye.

Topical Eye Drops. Using lid speculums, 4×5 μl of 5 mM LAT-A (42 μg) was administered to one eye of supine ketamine-anesthetized cynomolgus monkeys, vehicle to the opposite eye. Fifteen minutes after the last drop, the monkeys were returned to their cages and allowed to wake up. Approximately 5.5 or 23.5 hours later the monkeys were anesthetized with IM pentobarbital, and both eyes were cannulated with a single branched needle as in the bolus infusion protocol. Facility measurements started 6 or 24 hours after LAT-A administration and continued for 90 minutes. Although baseline facility measurements were not taken, these monkeys were selected for bilateral symmetry of facility measurements at baseline during their most recent perfusion 1 to 3 months earlier.

Preparation of Solutions

The 5 μM LAT-A solution for the 10-μl bolus infusion (0.5 μM in AC) was formulated as 25 μl of 0.2 mM (100% DMSO) stock solution, 175 μl DMSO, and 800 μl Bárány's mock AH.[15] Vehicle contained 200 μl DMSO and 800 μl Bárány's (20% DMSO, 2% in AC). The 20 μM LAT-A solution (2 μM in AC) was formulated as 100 μl of 0.2 mM (100% DMSO) stock solution, 100 μl DMSO, and 800 μl Bárány's. Vehicle contained 200 μl DMSO and 800 μl Bárány's (20% DMSO, 2% in AC). LAT-A solutions and vehicles for the exchange perfusions were formulated as 15, 37.5, 150 μl of 0.2 mM (either 1% or 100% DMSO), 3.75 μl of 20 mM LAT-A stock solution or 1.5, 37.5, 150, or 3.75 μl DMSO (vehicle) for 0.2, 0.5, 2, and 5 μM LAT-A, respectively, and the corresponding amount of Bárány's to bring the total volume to 15 ml LAT-A solution and vehicle for topical applications (5 mM) was formulated as 11.25 μl of 20 mM stock solution or DMSO (25%) and 33.75 μl Bárány's.

Statistical Analysis

Data are presented as means ± SEM for *n* eyes or animals. Pre-drug- or post-drug–treated versus contralateral control, post-drug or post-vehicle versus ipsilateral baseline, and baseline-corrected post-drug–treated versus control comparisons were made using the two-tailed paired *t*-test for ratios versus 1.0. Repeated measurements of fluid in the reservoir (represented by reservoir weight; data collected every 5 seconds) were analyzed for two 20-minute postintervention time periods (immediately and 24 hours after 5 μM LAT-A exchange and 42 μg topical LAT-A, respectively). Each 20-minute time period was divided into 1-minute intervals; for each interval, the average rate of flow from the reservoir to the eye was calculated using linear regression. Differences between the flow rate in treated and control eyes during each interval were calculated for individual monkeys, and the averages evaluated using paired *t*-tests. For analysis of time-dependent effects, repeated facility measurements were collected during three postintervention periods (2, 6, and 24 hours after 42 μg topical LAT-A). The time course of each monkey was summarized by the intercept and slope from a linear regression. Estimates and confidence intervals for the population intercept (expected initial facility) and slope (expected rate of facility change) were obtained from the mean and variance of the monkey-specific values. Differences in time course between postintervention time periods were evaluated using two-sample *t*-tests, allowing for possible nonconstant variance.[22] Separate analyses were conducted for measurements on treated eyes, measurements on control eyes, and paired differences between measurements on treated eyes and measurements on control eyes. Calculations were performed using SAS PROC GLM,[23] SAS PROC *t*-test,[23] and SAS PROC UNIVARIATE.[24]

RESULTS

Cultured BAECs

In untreated cells, the actin cytoskeleton consisted of radial and circumferential microfilament bundles attached to FC and AJ (Fig. 1; top left panel). Vinculin, in the same cells, was associated with radial FC and AJ (Fig. 1; top right panel). The addition of LAT-A resulted in progressive deterioration of the microfilament system and the associated vinculin. At the lowest concentration of LAT-A tested (0.2 μM), the junctional belt of actin had partially deteriorated after 30 to 60 minutes of incubation (Fig. 1; middle two rows), whereas stress fibers and
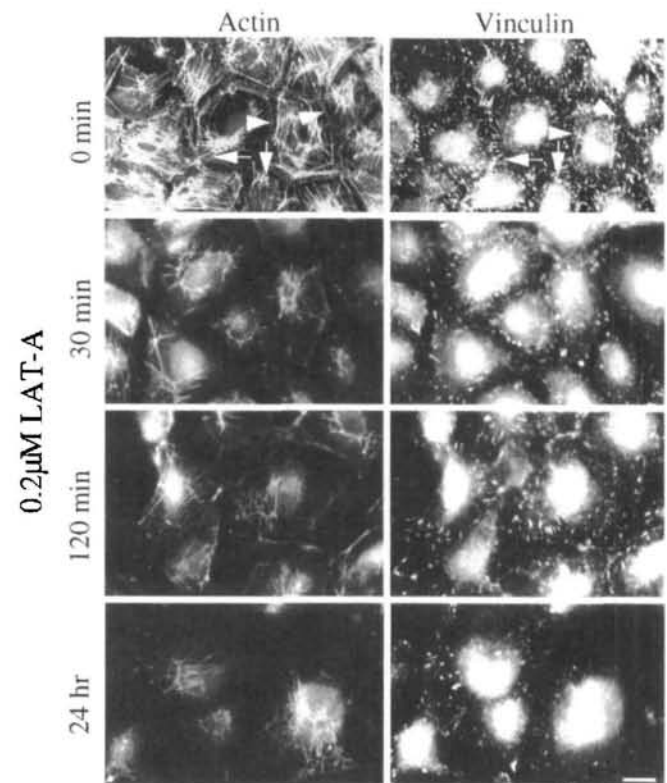

FIGURE 1. The effect of a low dose of LAT-A (0.2 μM) on the distribution of F-actin and vinculin in cultured BAECs. Cells were either untreated or treated with LAT-A for 30 minutes, 2 hours, or 24 hours and double-labeled for actin and vinculin using fluorescein isothiocynate–phalloidin and anti-vinculin, followed by rhodamine-labeled secondary antibody. Note that actin in the untreated cells (*top row*) is organized in dense arrays of bundles, which are associated with vinculin-containing focal adhesions (*matched arrows in top row*) or cell–cell junctions (*matched arrowheads in top row*). LAT-A induces a radical disruption of actin bundles that is already apparent within 30 minutes after addition of LAT-A. Concomitantly, vinculin-containing cell–cell junctions are disrupted, whereas FCs are apparently relaxed, even after 24 hours of treatment. Scale bar, 10 μm.
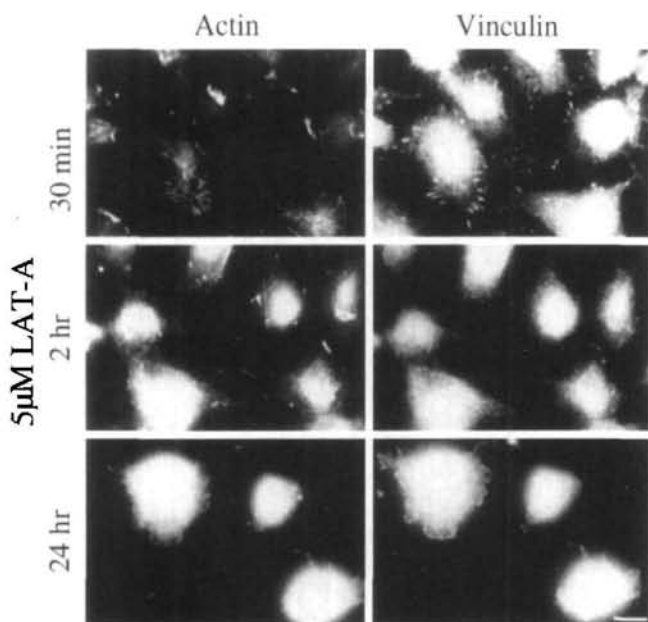
FIGURE 2. The effect of a high dose of LAT-A (5 μM) on the distribution of F-actin and vinculin in cultured BAECs treated with LAT-A for 30 minutes, 2 hours, or 24 hours and double-labeled for actin and vinculin as in Figure 1. Note that actin network is rapidly destroyed after LAT-A treatment. Filament bundles are distorted, and cell–cell junctions, with the associated actin and vinculin, are effectively abolished. Focal adhesions are markedly reduced, even by the short (30-minute) treatment and are essentially abolished on longer treatment. Scale bar, 10 μm.

focal adhesions were still mostly intact. After 24 hours of treatment with 0.2 μM LAT-A, the AJ had completely disappeared, and FC-associated actin was reduced but largely intact (Fig. 1; bottom row). LAT-A at higher concentrations (1 μM, data not shown; or 5 μM (Fig. 2) had a much more vigorous and rapid effect on the microfilament system, manifested by complete disappearance of AJ within 30 minutes of treatment and a progressive destruction of stress fibers. The effect on AJ-associated actin was faster and more prominent than the effect on FC and the attached filaments. Thus, even after long treatment some vinculin-containing FCs were retained (Fig. 2; bottom row). This suggests that LAT-A preferentially affects AJ over FC. In Figures 1 and 2 the sizes of the nuclei in the cells appear to enlarge. Treatment of BAECs and other cell types with LAT-A alters the degree of cell spreading on the substrate, affecting overall cell contour and apparent size of the nucleus (Geiger B, unpublished observations).

To determine directly the effect of LAT-A on AJs, we examined the distribution of cadherin in BAECs after treatment with low (0.2 μM) and high (2 μM) LAT-A concentrations. Within several hours of treatment with 0.2 μM LAT-A, staining at cell–cell borders became irregular and often discontinuous (Fig. 3; middle left panel, compare with top left panel). After longer incubation (24 hours), AJs completely disappeared (Fig. 3; bottom left panel). A major effect on AJ-associated cadherin was already noted within 1 hour (Fig. 3; top right panel) or less of treatment with 2 μM LAT-A. Interestingly, this effect was apparently highly reversible because after further incubation in normal medium AJs showed nearly complete recovery (Fig. 3; bottom right panel). Based on the findings in BAECs, LAT-A doses of 0.2, 0.5, 2, and 5 μM were studied in live monkeys.

Live Monkeys

Bolus Infusion. Bolus infusion of 5 μM or 20 μM LAT-A (0.5 μM or 2 μM in ACs) had no effect on facility in normal monkeys.

Exchange Infusion. Exchange infusion of 0.5 μM to 5 μM LAT-A induced a dose- and time-dependent facility increase, with 0.2 μM being subthreshold and 5 μM increasing facility approximately fivefold, corrected for baseline differences and control eye washout (Figs. 4A, 4B, 4C, 4C, 4D; Table 1). Based on change in flow rate from the external reservoir into the eyes, with essentially identical IOP in both eyes at baseline and at low and high perfusion pressure, the onset of the LAT-A effect occurred approximately 9 minutes after 5 μM exchange infusion ($P < 0.021$ for all intervals with complete data; Figs. 5A, 5B). One monkey (Fig. 5B) was a late responder, showing that the onset of drug effect was variable between individuals.

As expected,[18] the iridectomized and ciliary muscle–disinserted eyes had significantly lower baseline facilities than the contralateral iridectomized-only control eyes. However, 5 μM LAT-A exchange was equally effective on a proportional basis relative to baseline facility in both eyes, with the ciliary muscle–disinserted and control eyes both showing an approximately 3½-fold increase in facility, using all the post drug data (353% ± 129% [$P < 0.05$] and 328% ± 35% [$P < 0.001$], respectively; proportional difference between the increases, 6% ± 27%, $n = 9$; Figure 4E, Table 2).

Topical Eye Drops. Topical LAT-A eye drops also induced a dose-and time-dependent facility increase. The 21-μg dose, followed by a 1-hour wait before beginning facility mea-
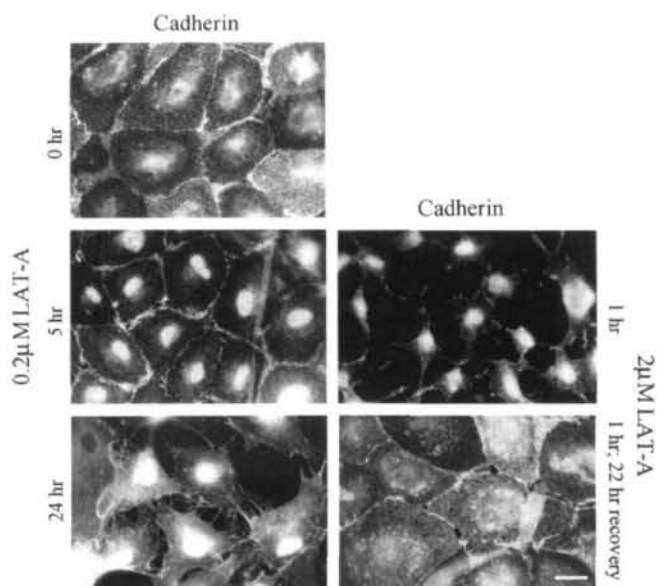

FIGURE 3. Effect of LAT-A on the distribution of cadherin in cultured BAECs, either untreated (*top left panel*) or treated with 0.2 μM (*left column*) or 2 μM LAT-A (*right column*). Note the progressive separation of the cells after long treatment with 0.2 μM LAT-A (*bottom panel, left column*) and the loss of cell–cell junctions. Within 1 hour, 2 μM LAT-A causes essentially complete destruction of cell–cell adhesions (*top panel, right column*). The basis for the apparent nuclear staining is not clear. The recovery of junctional cadherin labeling after 22 hours' incubation in LAT-A-free medium is shown in the *bottom right panel*. Scale bar, 10 μm.
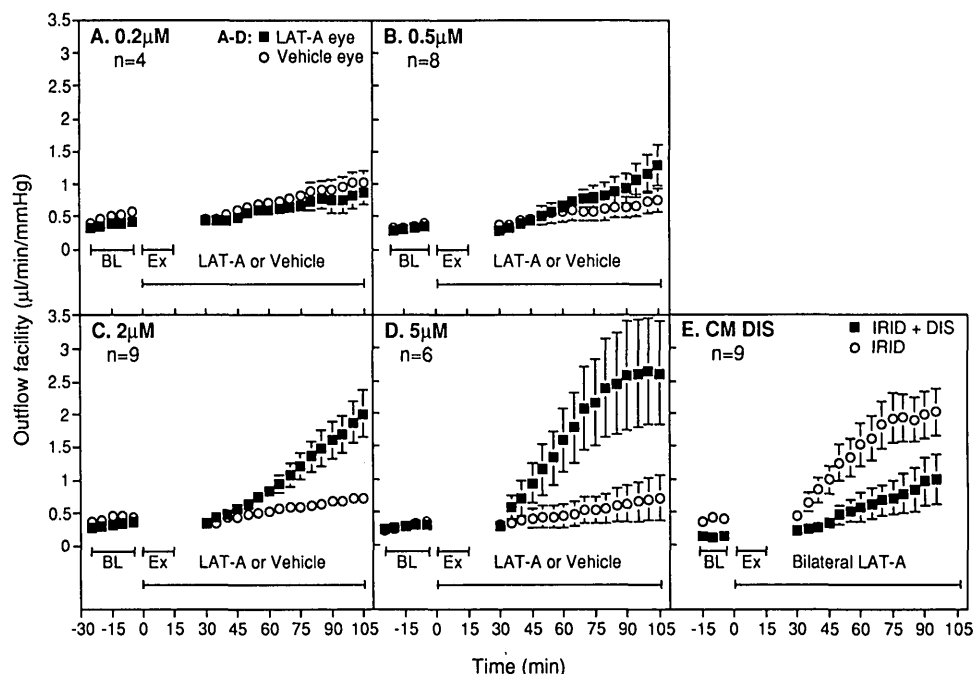
Figure 4. Outflow facilities versus time for LAT-A exchange protocols. Each data point is the mean ± SEM of the facility readings at that time for *n* monkeys. Anterior chamber exchange begins at time 0. BL, baseline; Ex, 2-ml exchange of AC with LAT-A or vehicle; IRID, iridectomized eye; DIS, ciliary muscle-disinserted eye.

surements, induced a mean facility increase of 39% ± 17% (*n* = 8, *P* < 0.1) over 90 minutes of measurements, corrected for baseline differences and control eye washout (Fig. 6A, Table 3). The 21-μg dose with a 2-hour wait induced a mean facility increase of 161% ± 73% (*n* = 7, *P* < 0.1; Fig. 6B, Table 3). The 42-μg dose with a 2-hour wait induced a mean facility increase of 235% ± 29% (*n* = 8, *P* < 0.001; Fig. 6C, Table 3). The initial facility values on restarting the perfusion after the closed reservoir waiting period were only slightly increased relative to the control eyes, but that slight initial increase was both dose- and waiting-time-dependent (Fig. 6).

Biomicroscopy 3 and 7 days after topical drug administration/perfusion revealed only the normal postperfusion AC cells and flare, with no difference between LAT-A-treated and control eyes; by day 14 both eyes were free of AC cells. The corneas in both eyes showed only the normal transient postperfusion localized swelling around the needle tracks, with no differences between the eyes at any time.

Reversibility. Reversibility of the facility increase seen after AC exchange with 5 μM LAT-A followed by vehicle exchange was minimal for the first drug-free hour (Fig. 7A) but was nearly complete after 3 hours with the system closed to inflow after drug removal (Fig. 7B). However, continued perfusion with the drug-free solution caused a progressive facility increase in the previously LAT-A-treated eyes relative to the vehicle-treated eyes, so that after 90 minutes facility was 67% ± 9% (*P* < 0.005; 93% ± 21%, *P* < 0.025 when corrected for baseline) higher in the LAT-A eyes.

To allow comparison with the 6- and 24-hour data during which the eyes were not cannulated until just before the facility measurements were done, the facility data obtained 2 hours after topical 42 μg LAT-A administration (Fig. 6C) were analyzed without correcting for baseline differences between

paired eyes. Hence, 2, 6, and 24 hours after topical 42 μg LAT-A, mean facility measured over 90 minutes exceeded that in contralateral vehicle-treated eyes by 272% ± 80% (*n* = 8, *P* < 0.02; Figs. 6C and 8A, Table 3), 135% ± 30% (*n* = 5, *P* < 0.02, Fig. 8B, Table 3), and 34% ± 10% (*n* = 4, *P* < 0.05, Fig. 8C; Table 3), respectively. Regression analysis showed that the facility increase induced by 42 μg of LAT-A (measured over 90 minutes) was significant compared with contralateral control eyes at all three times, but the slope of the differences in facility between LAT-A-treated and the vehicle-treated eyes declined progressively (0.022 ± 0.015; *P* < 0.005; probability that slope = 0.0), 0.012 ± 0.009 (*P* < 0.043), and 0.002 ± 0.0003 (*P* < 0.002) μl/min per mm Hg at 2, 6, and 24 hours, respectively (Figs. 8D, 8E, 8F). The facility increase induced by LAT-A at 2 compared with 24 hours differed significantly (*P* < 0.008), whereas 2 versus 6 hours (*P* < 0.15) and 6 versus 24 hours (*P* < 0.07) were not significantly different (Figs. 8D, 8E, 8F). The intercept parameter of the regression analysis is the expected facility at the start of each time period; for the LAT-A-treated eye this value tends to decline nonsignificantly but remain fairly constant in the control eye (Figs. 8A, 8B, 8C). Thus, the regression analysis shows that the LAT-A-induced facility increase was significant and reversible. Linear regression analysis of flow rate from the reservoir into the eyes 24 hours after topical LAT-A or vehicle indicated the onset of increased flow in the LAT-A-treated eyes compared with control eyes by the end of the 18th minute after starting perfusion (*P* < 0.007 for intervals with complete data; analysis as for Fig. 5; data not shown), another indication that even 24 hours after topical LAT-A there was still a significant effect on facility.

Baseline facility 4 to 14 weeks after a facility-effective intracameral (2 μM or 5 μM exchange) or topical (21 μg or 42 μg) LAT-A dose was not significantly higher (12% ± 15%, *n* =

TABLE 1. Intracameral LAT-A Exchange and Facilities by 30-Minute Intervals

| LAT-A Dose | Facility (μl/min per mm Hg) | | | |
	LAT-A	Veh	LAT-A/Veh	(LAT-A/BL$_L$)/(Veh/BL$_V$)
0.2 μM (n = 4)				
BL	0.38 ± 0.04	0.49 ± 0.09	0.80 ± 0.09	
0-30 min	0.50 ± 0.05	0.59 ± 0.11	0.90 ± 0.12	1.13 ± 0.12
30-60 min	0.65 ± 0.11	0.79 ± 0.12	0.85 ± 0.13	1.06 ± 0.14
60-90 min	0.79 ± 0.19	0.96 ± 0.16	0.85 ± 0.18	1.06 ± 0.20
0.5 μM (n = 8)				
BL	0.32 ± 0.05	0.36 ± 0.09	1.02 ± 0.13	
0-30 min	0.45 ± 0.08	0.47 ± 0.13	1.21 ± 0.19	1.17 ± 0.08
30-60 min	0.76 ± 0.15	0.59 ± 0.16	1.51 ± 0.23	1.53 ± 0.18‡
60-90 min	1.07 ± 0.26	0.69 ± 0.17	1.71 ± 0.29†	1.77 ± 0.26‡
2 μM (n = 9)				
BL	0.33 ± 0.03	0.42 ± 0.06	0.85 ± 0.09	
0-30 min	0.58 ± 0.09	0.45 ± 0.05	1.33 ± 0.13†	1.61 ± 0.15¶
30-60 min	1.09 ± 0.17	0.58 ± 0.08	1.91 ± 0.19¶	2.33 ± 0.22#
60-90 min	1.74 ± 0.30	0.69 ± 0.11	2.56 ± 0.29#	3.12 ± 0.36#
5 μM (n = 6)				
BL	0.28 ± 0.06	0.29 ± 0.09	1.13 ± 0.13	
0-30 min	0.93 ± 0.30	0.39 ± 0.13	2.69 ± 0.51‡	2.34 ± 0.30‖
30-60 min	2.00 ± 0.61	0.50 ± 0.20	6.01 ± 2.10	4.83 ± 1.30†
60-90 min	2.58 ± 0.80	0.66 ± 0.30	7.89 ± 3.15	6.14 ± 2.02
BL comparisons (n = 22)				
BL$_1$	0.37 ± 0.03	0.41 ± 0.04	0.97 ± 0.06	
BL$_2$	0.42 ± 0.06	0.44 ± 0.04	1.07 ± 0.17	
BL$_2$/BL$_1$	1.24 ± 0.19	1.19 ± 0.10	1.12 ± 0.15	

Data are means ± SEM for n animals, each contributing one LAT-A-treated and one vehicle-treated eye.

BL, baseline; Veh, vehicle-treated eye; LAT-A, LAT-A-treated eye; BL$_1$, baseline before LAT-A or vehicle administration; BL$_2$, baseline at the following perfusion; BL$_L$, mean baseline for LAT-A-treated eye; BL$_V$, mean baseline for vehicle-treated eye; 0-30 min, 30-60 min, 60-90 min, mean facility measurements over 30 minutes beginning immediately after, 30 minutes after, or 60 minutes after LAT-A or vehicle administration, respectively.

† $P < 0.05$, ‡ $P < 0.025$, ‖ $P < 0.01$, ¶ $P < 0.005$, # $P < 0.001$ for ratios different from 1.0 by the two-tailed paired t-test.

22, corrected proportionally for changes in the contralateral control eyes) than the baseline immediately before receiving LAT-A (Table 1).

DISCUSSION

One must ask whether the DMSO in the vehicle could have affected the findings in either the in vivo or in vitro experiments. Based on the procedures and assumptions indicated in the Methods section, the highest intracameral DMSO concentrations achieved in the live monkey AC bolus infusion, AC exchange, and topical eyedrop protocols were 2%, 0.25%, and approximately 0.05%, respectively. Such intracameral DMSO concentrations do not affect outflow facility in cynomolgus monkey eyes. Only when the intracameral DMSO concentration exceeds 4% is there a significant reduction in outflow facility.[25] Similarly, the DMSO concentrations in the BAEC experiments were 0.04%, 0.2%, 0.4%, and 1% for the 0.2, 1, 2, and 5 μM LAT-A concentrations, respectively. Although these DMSO concentrations would not be expected to affect either our in vitro or in vivo results, we cannot unequivocally exclude such a possibility, because DMSO could theoretically enhance ocular or cellular penetration of itself or LAT-A.[26] However, vehicle control eyes in all protocols allowed the identification of LAT-A effects from potential DMSO effects.

We saw no corneal effects attributable to LAT-A on biomicroscopy 3 and 7 days after topical drug administration/perfu-

sion. In noninvasive experiments studying other aspects of anterior segment physiology not described here, biomicroscopy showed that within 3 hours monkey eyes treated with topical LAT-A (42 μg) or LAT-B (3.95 μg) developed corneal thickening and endothelial cell shape changes. The effects were transient, lasting at most 24 hours.[27,28] Nonetheless, agents that enhance facility by perturbing the ubiquitous actin microfilament system may require special cautions and delivery strategies if they are to be used for clinical glaucoma therapy.

In BAECs, LAT-A caused destruction of the actin bundles, most noticeably in the cell-cell junctions, and separation of the cells in a dose- and time-dependent manner. These effects were reversible on drug removal, also in a dose- and time-dependent manner. Actin filaments are present in almost all cells, including the cells of the juxtacanalicular endothelial meshwork and the inner wall Schlemm's canal.[29-31] Actin filaments in these cells are often associated with junctional complexes.[31] LAT-A may "loosen" these junctions, as in BAECs, causing the separation of cells from each other and from the extracellular matrix, and alteration of overall meshwork architecture. Such changes could facilitate flow across the meshwork in several ways, for instance by opening paracellular pathways through the juxtacanalicular meshwork and the inner wall endothelium[32] (cell-cell separation) or by facilitating separation of the meshwork layers and overall distension of the meshwork by the pressure-flow gradient induced by AC perfusion (cell-extracellular matrix separation). These para-
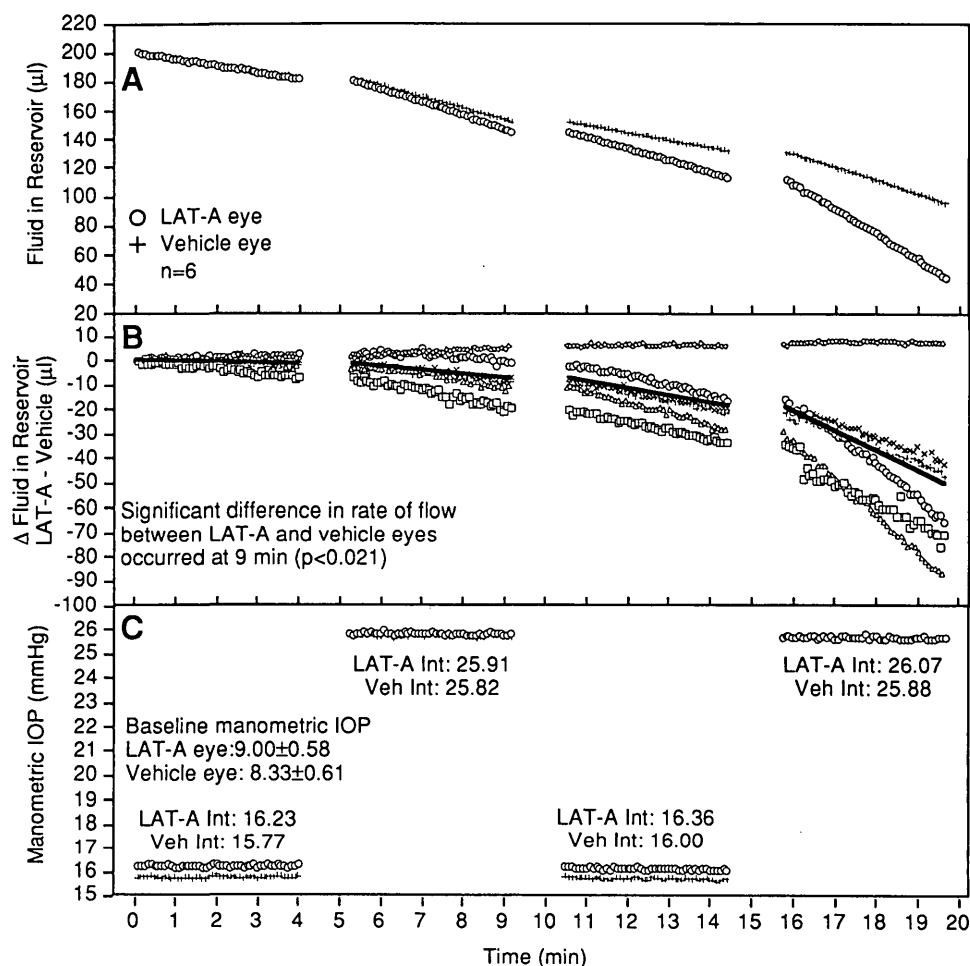
FIGURE 5.   Mean flow from external reservoir into eye (A), individual monkey flow differences (LAT-A − vehicle; B), and corresponding mean IOP (C) versus time after AC exchange with 5 μM LAT-A or vehicle in contralateral eyes of 6 monkeys. *Dark line* in (B) is mean of individual slopes obtained from individual monkey flow differences regressed on time and adjusted for repeated measures. Veh, vehicle; Int, intercept. See the Methods and Results sections for a detailed explanation.

digms are analogous to comparable findings with cytochalasins B and D.[7-11] Several other classes of compounds (e.g., H-7,[33] ethacrynic acid,[34-36] epinephrine,[37,38] and staurosporine[39]) may increase facility at least in part by affecting the microfilament system in outflow pathway tissues. LAT-A might also affect other outflow-relevant cells in the chamber angle (e.g.,

collector channels, episcleral veins), with uncertain physiological consequences.

The induction and dose-time relationship of perturbations of actin filaments and cell adhesions in cultured BAECs were remarkably predictive for the in vivo facility effect of LAT-A. In the living monkey eye, AC exchange with LAT-A produced a

TABLE 2.   Intracameral 5 μM LAT-A Exchange and Ciliary Muscle Disinsertion ($n = 9$)

	Facility (μl/min per mm Hg)		
	Irid + CM Dis	Irid	Irid + CM Dis/ Irid
BL	0.13 ± 0.01	0.38 ± 0.09	0.45 ± 0.08
PRx	0.65 ± 0.23	1.51 ± 0.30	0.43 ± 0.15
PRx/ BL	4.53 ± 1.29†	4.28 ± 0.35#	1.06 ± 0.27

Data are means ± SEM for n animals (3 cynomolgi, 6 rhesus) with bilateral total iridectomy and unilateral ciliary muscle disinsertion receiving 5 μM LAT-A bilaterally. BL, baseline; PRx, postdrug administration; Irid, total iridectomy; CM Dis, ciliary muscle disinsertion.

† $P < 0.05$, # $P < 0.001$ for ratios different from 1.0 by the two-tailed paired t-test.
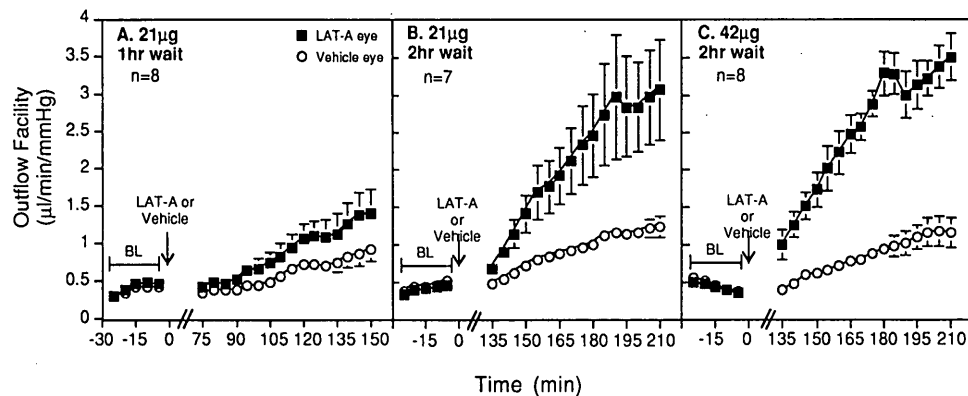
FIGURE 6.   Outflow facilities versus time for LAT-A topical protocols. Each data point is the mean ± SEM of the facility readings at that time for *n* monkeys. Drug administration begins at time 0. BL, baseline.

dose-dependent two- to threefold increase in outflow facility, not dependent on ciliary muscle interaction with the TM. Topical administration of LAT-A also produced a dose-dependent two- to threefold increase in outflow facility. However, the initial facility values after a 1-, 2-, 6-, or even 24-hour wait were not as substantially elevated; only with continued perfusion did facility rise. The absence of a large initial increase is not likely related to decay of AC drug concentration below threshold, because the 0.5 μM LAT-A exchange was effective after restarting the infusion, and the AC concentration after the 21-μg topical dose (~5 μM in AC, assuming 1% corneal pene-

tration and minimal early drug loss from the AC) would not likely have decreased below 0.5 μM within a 2-hour waiting time. In the AC exchange experiments, in which the interval between drug administration and initial postdrug facility measurement was much shorter, the initial postdrug facility values were not elevated at any dose. However, analysis of the flow rates from the external reservoir into the eye indicated the onset of drug effect within 9 minutes after 5 μM LAT-A exchange and within 18 minutes 24 hours after topical 42-μg LAT-A treatment. It is not clear whether LAT-A increases AHF, but LAT-A surely does not reduce AHF[40]; for the 5 μM ex-

TABLE 3.   Topical LAT-A and Facilities by 30-Minute Intervals

	Facility (μl/min per mm Hg)			
LAT-A Dose	LAT-A	Veh	LAT-A/Veh	(LAT-A/BL$_L$)/(Veh/BL$_V$)
21 μg 1-h wait (n = 8)				
BL	0.41 ± 0.06	0.37 ± 0.06	1.16 ± 0.16	
0-30 min	0.54 ± 0.09	0.39 ± 0.06	1.53 ± 0.28*	1.34 ± 0.17*
30-60 min	0.92 ± 0.18	0.62 ± 0.09	1.62 ± 0.36	1.40 ± 0.21*
60-90 min	1.24 ± 0.28	0.80 ± 0.14	1.65 ± 0.33*	1.45 ± 0.19*
21 μg 2-h wait (n = 7)				
BL	0.43 ± 0.06	0.46 ± 0.06	0.93 ± 0.09	
0-30 min	1.39 ± 0.25	0.71 ± 0.07	2.07 ± 0.43†	2.31 ± 0.62*
30-60 min	2.32 ± 0.52	1.23 ± 0.28	2.37 ± 0.68*	2.69 ± 0.93
60-90 min	2.93 ± 0.66	1.18 ± 0.11	2.48 ± 0.50‡	2.74 ± 0.70†
42 μg 2-h wait (n = 8)				
BL	0.43 ± 0.04	0.46 ± 0.08	1.16 ± 0.26	
0-30 min	1.75 ± 0.22	0.61 ± 0.09	3.52 ± 0.77§	3.19 ± 0.51¶
30-60 min	2.89 ± 0.21	0.87 ± 0.11	3.96 ± 0.72‖	3.70 ± 0.42#
60-90 min	3.24 ± 0.24	1.12 ± 0.18	3.83 ± 1.04†	3.33 ± 0.33#
42 μg 6-h wait (n = 5)				
0-30 min	0.98 ± 0.11	0.47 ± 0.05	2.10 ± 0.22‖	
30-60 min	1.53 ± 0.16	0.65 ± 0.05	2.40 ± 0.32§	
60-90 min	1.88 ± 0.24	0.78 ± 0.06	2.49 ± 0.37§	
42 μg 24-h wait (n = 4)				
0-30 min	0.49 ± 0.08	0.38 ± 0.07	1.31 ± 0.10†	
30-60 min	0.63 ± 0.09	0.49 ± 0.09	1.32 ± 0.14	
60-90 min	0.81 ± 0.11	0.60 ± 0.10	1.37 ± 0.07§	

Data are mean ± SEM for *n* animals, each contributing one LAT-A-treated and one vehicle-treated eye. BL, baseline; Veh, vehicle-treated eye; LAT-A, LAT-A-treated eye; BL$_L$, mean baseline for LAT-A-treated eye; BL$_V$, mean baseline for vehicle-treated eye; 0-30 min, 30-60 min, 60-90 min, mean facility measurements over 30 minutes beginning immediately after, 30 minutes after, or 60 minutes after LAT-A or vehicle administration, respectively.

* P < 0.1, †P < 0.05, ‡P < 0.025, §P < 0.02, ‖P < 0.01, ¶P < 0.005, #P < 0.001 for ratios different from 1.0 by the two-tailed paired *t*-test.
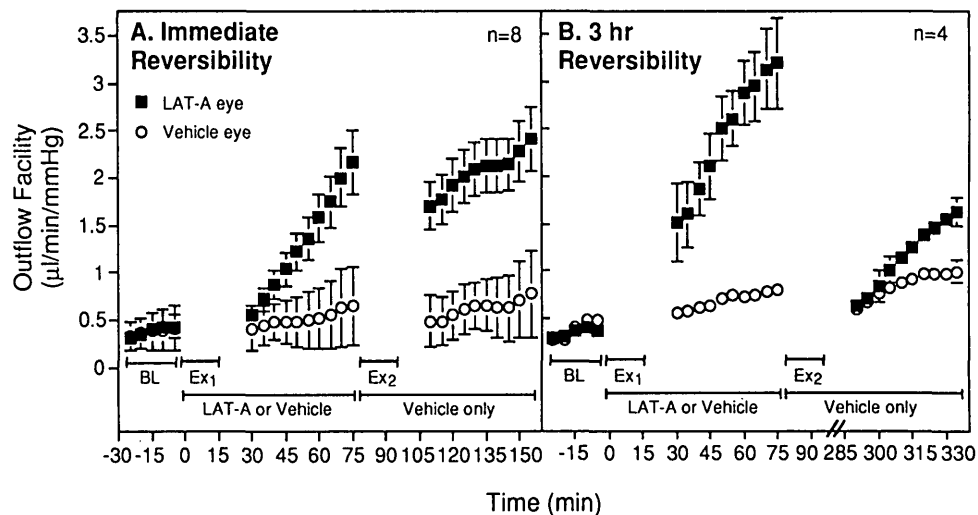
FIGURE 7. Outflow facilities versus time for LAT-A exchange followed by vehicle exchange protocols. Each data point is the mean ± SEM of the facility readings at that time for n monkeys. BL, baseline; Ex_1, unilateral 5 μM LAT-A AC exchange; Ex_2, bilateral vehicle exchange.

change data the LAT-A-treated eyes had minimally higher average spontaneous IOP, and minimally higher pressures at both the lower and higher pressure settings (but more so at the lower setting), so that the pressure differences between periods was minimally smaller in the LAT-A-treated eyes (none of these differences were significant). Thus, onset of LAT-A-induced outflow enhancement was no later than our estimate; it might have been slightly earlier (see the Methods section).

Collectively, these results suggest that interference with the actin filament network destabilizes cell junctions within the TM and promotes time-dependent resistance washout at physiological flow rates but that a higher than physiological flow rate is required to destabilize overall TM geometry sufficiently to substantially reduce flow resistance, at least within the drug dose and exposure duration limits of our experiments. This hypothesis requires electron microscopic confirmation
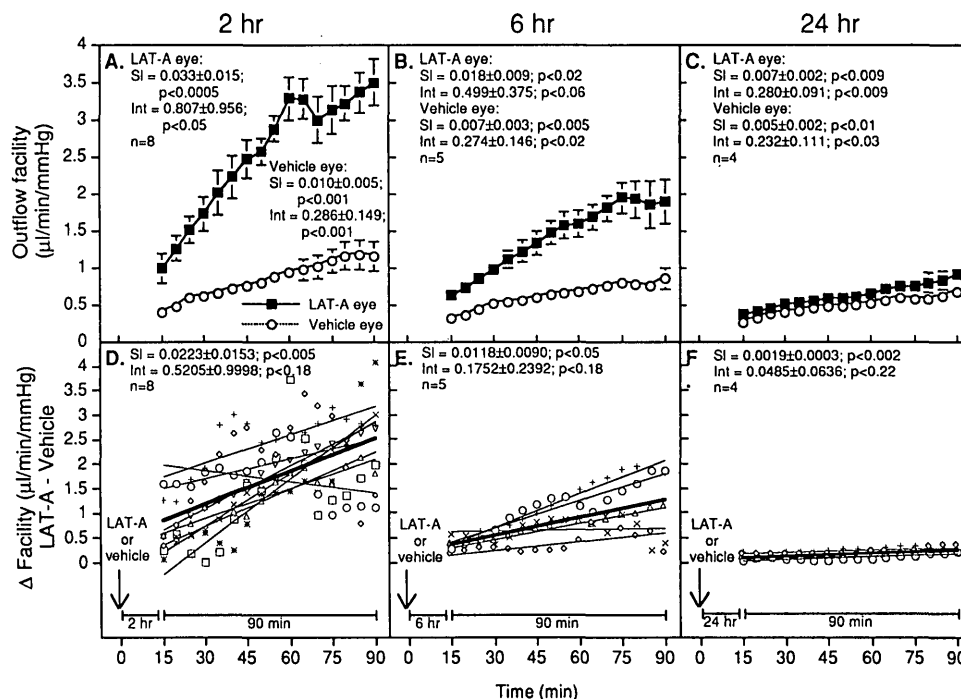

FIGURE 8. Outflow facility (A, B, C) and individual monkey facility differences (D, E, F) versus time for LAT-A- and vehicle-treated eyes measured for 90 minutes beginning 2 (A, D), 6 (B, E), or 24 (C, F) hours after drug administration. *Darker line* in (D, E, F) is mean of individual slopes obtained from individual monkey facility differences regressed on time (*thin lines*) and adjusted for repeated measures. Each data point (A, B, C) is mean ± SEM facility or facility difference (LAT-A − vehicle) at that time for n monkeys, each contributing one LAT-A-treated and one vehicle-treated eye. Sl, slope; Int, intercept; P, probability that slope or intercept = 0.0 by regression analysis.

with flow tracers in primate tissue (experiments are in progress) but is supported by the BAEC experiments in which the major effect of LAT-A at appropriate concentrations appears to be the separation of cell-cell adhesions, with only a limited effect on cell-extracellular matrix adhesions (see the Results section; also, Spector I, Volberg T, Geiger B, unpublished observations). Interestingly, H-7, which preferentially affects cell-extracellular matrix junctions over cell-cell adhesions, increased facility about as much as LAT-A without obvious acute or long-term adverse effects.[33] Clearly, more work, including ongoing ultrastructural studies of the outflow pathway in monkeys treated with H-7 or LAT-A, is needed to determine whether the facility-enhancing effects of the two drugs are attributable primarily to their capacity to perturb the microfilament system or to their different effects on cell-cell and cell-extracellular matrix adhesions and to define the most effective and safe target and approach to attacking junctional complexes in the TM to enhance facility as a possible antiglaucoma therapeutic strategy.

Longer exposure to lower concentrations of LAT-A caused more separation between BAECs than short exposure to higher concentrations, and LAT-A concentrations >1 μM caused cell detachment from the substrate, probably leading to cell death (data not shown). It is likely, albeit not yet tested, that lower concentrations for longer exposure times than the approximately 1 μM for approximately 1-hour effective range seen here with LAT-A in monkeys might also be effective, and perhaps "gentler." As with H-7,[33] bolus infusion of LAT-A was ineffective, probably because an adequate drug concentration was not maintained for sufficient time. The absence of substantial reversibility within 1 hour but nearly complete reversibility within 3 hours of LAT-A removal may derive from the high dose and short recovery time, as in cultured BAECs. A 3-hour recovery of junctional complexes and junction-dependent meshwork architecture and physiology seems reasonable, on the basis of the BAEC data. However, even after apparently complete reversal, perfusion with drug-free solution induced an accelerated facility increase well beyond that attributed to normal resistance washout in the control eyes. This also occurred for at least 24 hours after a single topical 42-μg dose of LAT-A, although the effect diminished with time. Whatever the basis for the persistent effect, essentially complete recovery occurred by 1 to 2.5 months. Reversibility studies using lower doses and intermediate recovery times would be of interest.

One might hypothesize that under the influence of LAT-A, the AJ between cells in the juxtacanalicular region, the FC between cells on the beams of the corneoscleral region and the extracellular matrix between the lamellae and the FC between cells and matrix in the juxtacanalicular region are all weakened, so that the overall meshwork architecture becomes less rigid or more tenuous. Perhaps fluid flow along a pressure gradient through such a "loosened" TM structure further loosens and separates the cell layers of the mesh, and separating cells of the juxtacanalicular region from each other and from their extracellular matrix, allowing easier fluid flow and resistance washout, manifested physiologically as increased outflow facility. This would be analogous to the mythical "house of cards easily disrupted by a puff of air" and is consistent with the facility increase and ultrastructural appearance of the meshwork in monkeys receiving EDTA and EGTA,[41] cytochalasin B,[42] and H-7 (Geiger B and Kaufman P, unpublished data). In this scenario, glaucoma patients with elevated IOPs may simply

need to apply digital pressure to the globe to enhance and prolong the IOP-lowering effect of a topical dose of such a drug.

We did not study LAT-A effects on uveoscleral outflow. Inhibition of ciliary muscle contractility (Peterson J et al., unpublished data) or adhesion between muscle cells or bundles and the intermuscular connective tissue matrix could conceivably affect this drainage pathway.

Acknowledgments

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