Latrunculin-A Causes Mydriasis and Cycloplegia in the Cynomolgus Monkey

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Purpose. To determine the effect of latrunculin (LAT)-A, which binds to G-actin and disassembles actin filaments, on the pupil, accommodation, and isolated ciliary muscle (CM) contraction in monkeys.

Methods. Pupil diameter (vernier calipers) and refraction (coincidence refractometry) were measured every 15 minutes from 0.75 to 3.5 hours after topical LAT-A 42 μg (~10 μM in the anterior chamber [AC]). Refraction was measured every 5 minutes from 0.5 to 1.5 hours after intracameral injection of 10 μl of 50 μM LAT-A (~5 μM in AC), with intramuscular infusion of 1.5 mg/kg pilocarpine HCl (PILO) during the first 15 minutes of measurements. Pupil diameter was measured at 1 and 2 hours, and refraction was measured every 5 minutes from 1 to 2 hours, after intravitreal injection of 20 μl of 1.25 mM LAT-A (~10 μM in vitreous), with intramuscular infusion of 1.5 mg/kg PILO during the first 15 minutes of measurements (all after topical 2.5% phenylephrine), and contractile response of isolated CM strips, obtained <1 hour postmortem and mounted in a perfusion apparatus, to 10 μM PILO ± LAT-A was measured at various concentrations.

Results. Topical LAT-A of 42 μg dilated the pupil without affecting refraction. Intracameral LAT-A of 5 μM inhibited miotic and accommodative responses to intramuscular PILO. Intravitreal LAT-A of 10 μM had no effect on accommodative or miotic responses to intramuscular PILO. LAT-A dose-dependently relaxed the PILO-contracted CM by up to 50% at 3 μM in both the longitudinal and circular vectors.

Conclusions. In monkeys, LAT-A causes mydriasis and cycloplegia, perhaps related to its known ability to disrupt the actin microfilament network and consequently to affect cell contractility and adhesion. Effects of LAT-A on the iris and CM may have significant physiological and clinical implications. (*Invest Ophthalmol Vis Sci.* 1999;40:631-638)

Latrunculin-A (LAT-A), a macrolide derived from the Red Sea sponge *Latrunculia magnifica*, is a specific actin-disrupting agent that disassembles actin filaments by sequestering monomeric actin[1-3] while leaving the organization of other cytoskeletal systems unchanged.[2-4] Cytochalasins B and D (CB, CD) are compounds that also destroy the actin-based cytoskeleton but by a complex mechanism different from that of LAT-A.[5-8] CB and CD alter the shape of human trabecular meshwork (TM) cells in culture[9,10] and increase trabecular outflow facility in the living monkey eye[11-13] due to separation of cells in the juxtacanalicular region of the meshwork and the lining of the inner wall Schlemm's canal, with subsequent distension of the meshwork, ruptures in the inner wall, and washout of extracellular material.[14,15] LAT-A lowers intraocular pressure by increasing outflow facility up to several-fold in living monkeys.[16,17] The parasympathetically dominated iris sphincter and ciliary (CM) smooth muscles play important roles in controlling pupillary diameter, and accommodation and outflow facility, respectively.[18] Given LAT-A's subcellular mechanism of action and glaucoma therapeutic potential, we sought to characterize LAT-A's functional effects on the primate iris and CM.

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Methods

Animals and Anesthesia

For in vivo pupil/refraction experiments, normal adult cynomolgus (*Macaca fascicularis*) monkeys were anesthetized with intramuscular (i.m.) ketamine (10 mg/kg) followed by pentobarbital sodium (35 mg/kg, i.m.). For in vitro CM contraction experiments, globes were enucleated from rhesus (*Macaca mulatta*) or cynomolgus monkeys under deep pentobarbital intravenous (i.v.) anesthesia (25 mg/kg) just before or within 5 minutes of euthanatization (for nonocular protocols or untreatable illness) by pentobarbital overdose. All experiments were conducted in accordance with NIH and University of Wisconsin Animal Care and Use guidelines, and with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research.

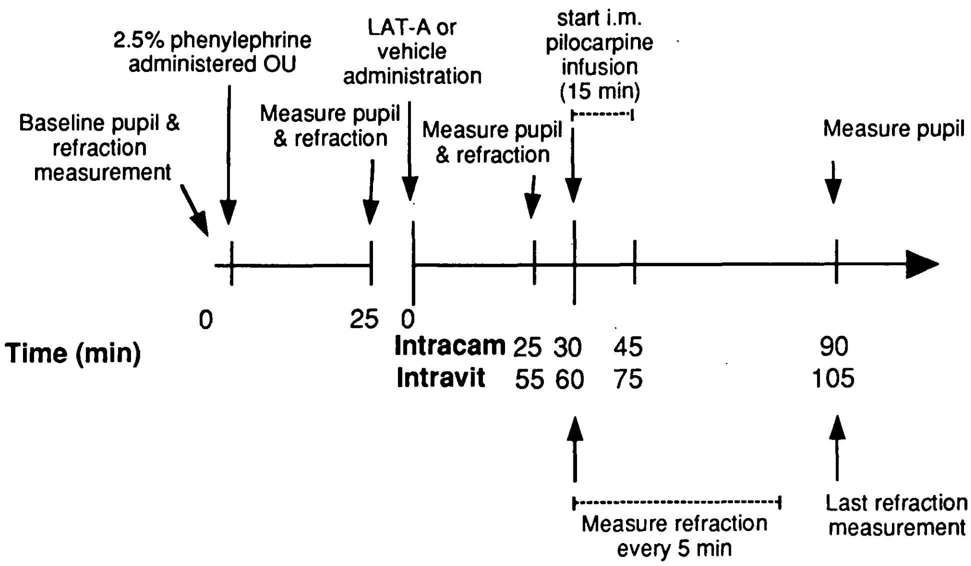

FIGURE 1. Time line for protocols determining the effects of intracameral (Intracam) and intravitreal (Intravit) LAT-A on pupillary and accommodative responses to intramuscular pilocarpine. OU, in each eye.

Drugs and Chemicals

LAT-A was obtained from Yoel Kashman, Department of Organic Chemistry, Tel-Aviv University, Tel-Aviv, Israel. Medium-199 (containing glutamine), penicillin G-streptomycin solution, fetal bovine serum, pilocarpine HCl (PILO), and dimethylsulfoxide (DMSO) were obtained from Sigma Chemical (St. Louis, MO); 2.5% phenylephrine HCl (PHE, Mydfrin) from Alcon (Fort Worth, TX); and cyanoacrylate adhesive was supplied by Tri-Point Medical (Raleigh, NC). LAT-A 5 mM solution for topical applications was formulated as 11.25 μl of 20 mM LAT-A stock solution and 33.75 μl of Bárány's mock aqueous humor solution.[19] LAT-A 50 μM solution for intracameral transcorneal injection was formulated as 0.75 μl of 20 mM LAT-A stock solution and 22.5 μl of Bárány's. LAT-A 1.25 mM solution for intravitreal injection was formulated as 3.75 μl of 20 mM LAT-A stock solution and 56.25 μl of Bárány's. PILO was dissolved in 0.1% citric acid buffer just before the experiments so that systemic intramuscular infusion of 3 ml delivered ~3 mg to 6 mg PILO (1.5 mg/kg body weight).

Effect of LAT-A on Pupil Diameter and Accommodation in Living Monkeys

Topical Eye Drops. Baseline refraction (an average of 2 to 3 readings) was measured with a Hartinger coincidence refractometer (Jenoptik, Jena, Germany). Accommodation was recorded as the difference between baseline and postdrug refraction. Baseline pupillary diameter was measured with vernier calipers under normal room light (350 lux). Four 5-μl drops of 25% DMSO ± 5 mM LAT-A (total LAT-A = 42 μg) were administered to the central cornea of opposite eyes in supine monkeys at 30-second intervals, with blinking prevented between and for 5 minutes after the last drops with lid speculums. Beginning 45 minutes later, refraction and pupillary diameter were measured every 15 minutes for 3.5 hours after LAT-A administration. The dosage was chosen to give a 10-μM LAT-A concentration, a near maximal dose for increasing outflow facility,[4] in the ~100-μl monkey anterior chamber (AC),[20] assuming 1% penetration and no drug loss from the AC.[21-23]

Intracameral Injection. Baseline refraction and pupillary diameter were measured bilaterally. The pupils were then dilated by topical application of 2 or 3 drops of PHE (an α_1-adrenergic agonist, which stimulates the sympathetically dominated iris dilator muscle without influencing the parasympathetically dominated iris sphincter and CM[18,24]) to facilitate subsequent refractometry in the presence of PILO.[24] Refraction and pupillary diameter were measured again 25 minutes later, after which 10 μl of 0.25% DMSO ± 50 μM LAT-A was administered intracamerally to opposite eyes (5 μM LAT-A and 0.025% DMSO in the AC). Intracameral injections were made under a Zeiss operating microscope using a 30-gauge needle connected via polyethylene tubing to a Gilmont (Barrington, IL) micrometer syringe. The needle was threaded through the corneal stroma for 6 mm and then directed into the AC so that the wound was self-sealing and prevented leakage of aqueous humor after the needle was withdrawn. Refraction and pupillary diameter were measured 25 minutes after LAT-A administration. Five minutes later, 3 ml of PILO solution was infused into the quadriceps muscle of the thigh, delivering 1.5 mg/kg body weight over 15 minutes. Refraction was determined every 5 minutes beginning at the start of PILO infusion until stable. The final pupillary diameter was then measured (see Fig. 1).

Intravitreal Injection. Baseline refraction and pupillary diameter were measured and topical PHE applied to both eyes as outlined above. Refraction and pupillary diameter were measured again 25 minutes later, after which 20 μl of 1.25 mM LAT-A or 6.25% DMSO was administered intravitreally (10 μM LAT-A and 0.05% DMSO in the 2.5 ml vitreous[25]) to opposite eyes by inserting the 30-gauge needle through the temporal pars plana 4 mm from the limbus to a depth of 4 mm. Refraction and pupillary diameter were measured 55 minutes after injection. Five minutes later PILO was administered intramuscularly as above, and refraction was determined every 5 minutes beginning at the start of PILO infusion until stable. The final pupillary diameter was then measured (see Fig. 1).

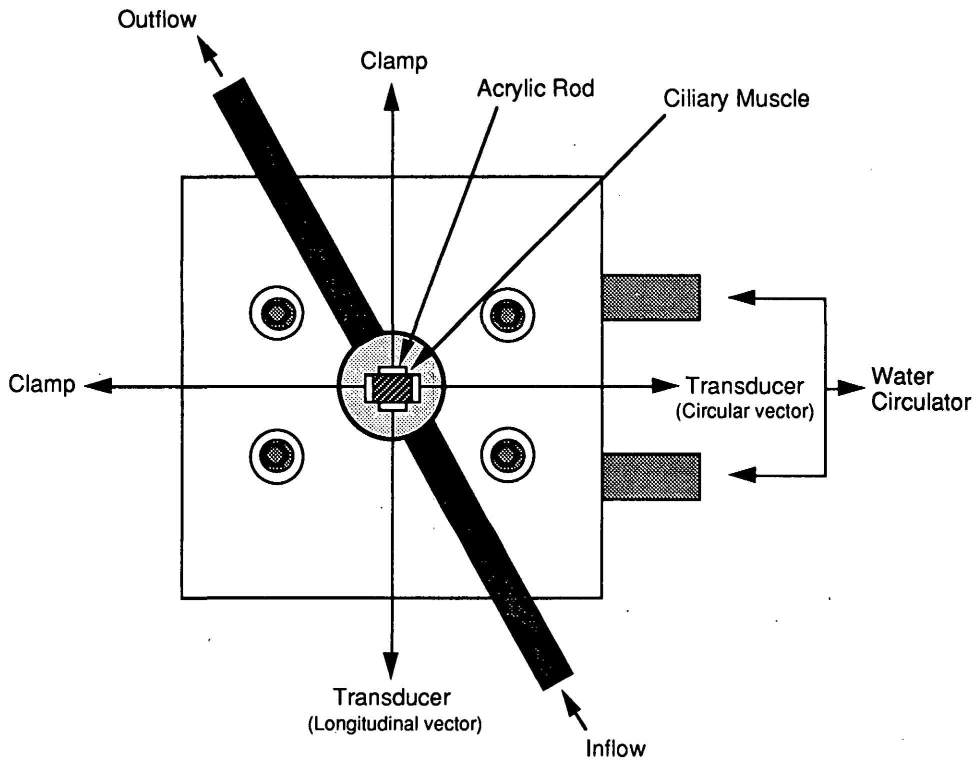

FIGURE 2. Top view of the in vitro CM chamber apparatus, modified from Poyer JF, Kaufman PL, Flügel C. Age does not affect contractile responses of the isolated rhesus monkey ciliary muscle to muscarinic agonists. *Curr Eye Res.* 1993;12:413-422, with permission.

LAT-A Inhibition of PILO-Induced Contraction of Isolated CM Strips

Ciliary Muscle Preparation. The muscle chamber and the protocol for tissue collection, CM dissection, and chamber mounting have been described in detail elsewhere.[26-28] Briefly, globes were enucleated under deep pentobarbital anesthesia just before or within 5 minutes of euthanatization, and placed in cell culture medium at 4°C. The CM was dissected under a surgical microscope, yielding a section of muscle approximately 4-cm circular × 4-mm meridional and including the entire anterior-posterior extent of the CM from the scleral spur to the ora serrata. A 5-mm circular × 4-mm meridional strip was then cut from the section, secured to four acrylic attachment rods with cyanoacrylate adhesive, and mounted in the apparatus (Fig. 2). The remainder of the original section was stored in culture medium at 4°C for future use.

The muscle chamber was maintained at 34°C and perfused continuously with warmed oxygenated Krebs' solution (ionic composition [in mM]: Na^+ 143.3, K^+ 5.9, Ca^{2+} 2.6, Mg^{2+} 1.2, Cl^- 128.3, $H_2PO_4^{2-}$ 2.2, HCO_3^- 24.9, SO_4^{2-} 1.2, glucose 11.1, pH 7.4) or Krebs' solution containing drug. The muscle attachment rods were secured via paraffin to two force transducers (one monitoring contractile responses in the circular and the other in the longitudinal vector of the muscle) mounted on micropositioners for precise control of muscle resting tension. Output from both channels was recorded on a two-channel flatbed recorder.

A muscle strip was mounted in the chamber beginning 1 hour after enucleation. Muscle strips were also used on the day after enucleation, having been kept in the cell culture medium at 4°C overnight. In this system, PILO induces reproducible dose-dependent contractions in both vectors, with essentially superimposable dose-response curves. The responses are similar for the use of fresh, same day-stored, and overnight-stored strips.[26]

LAT-A/PILO Experiments. After equilibration of each CM strip, 10 μM PILO (a just-maximal concentration for contraction) was added to the Krebs' reservoir and perfused through the chamber for 15 minutes. LAT-A was then added to the Krebs'-PILO mixture at successive concentration (100 nM to 3 μM), each concentration of LAT-A perfusing for 15 minutes. After the final dose of LAT-A, the strip was perfused with plain Krebs' solution for 60 minutes (to reestablish baseline tension) and then rechallenged with PILO (10 μM) for 15 minutes. The strip was then perfused with plain Krebs' solution for another 30 minutes.

Data Analysis

Live monkey data are presented as mean ± SEM for n eyes or animals. Differences between eyes or treatments were compared to 0.0 (Figs. 3, 4) by the two-tailed paired t-test; a value significantly $\neq$ 0.0 indicates a significant difference between eyes or treatments. In vitro CM responses were expressed as mean ± SEM absolute change in force from resting tension and as the mean ± SEM proportion of the PILO response remaining after exposure to LAT-A, for n CM strips (Table 1). The latter comparison normalizes the data to compensate for variations in the final dimensions and mass of the strips, which may affect contractility. This proportionate response was compared to 1.0 by the two-tailed paired t-test; a value significantly less than 1.0 indicates attenuation of the response to PILO by LAT-A.

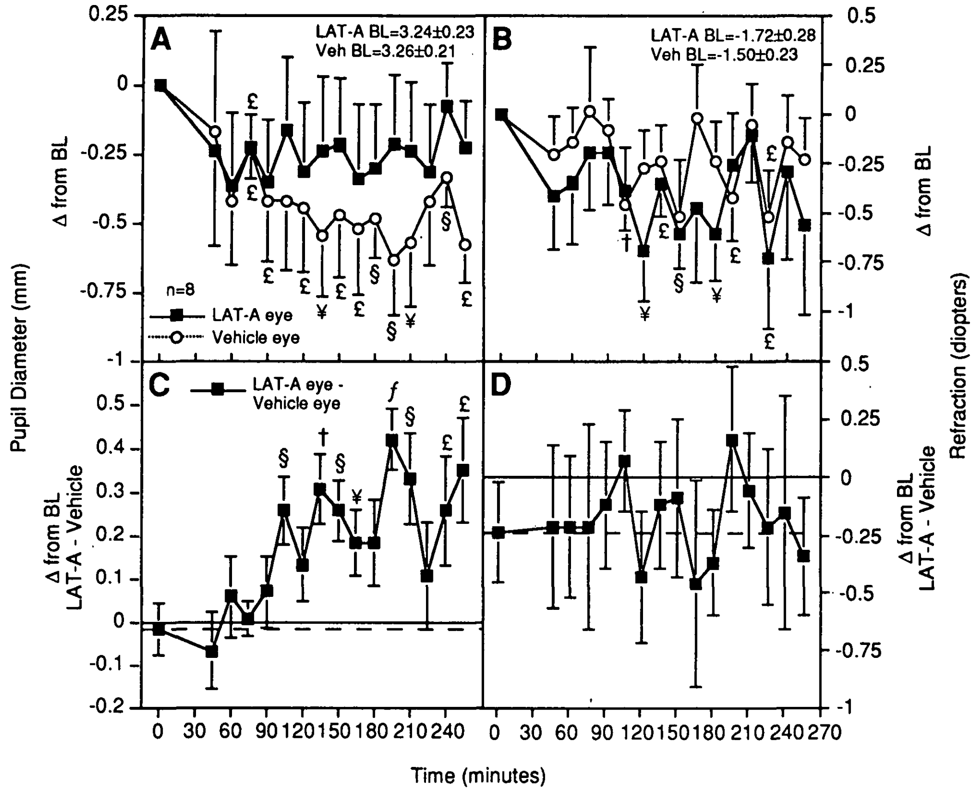

FIGURE 3. Effect of topical 42 μg LAT-A on pupil diameter (A, C) and refraction (B, D). Drug given at time 0, immediately after baseline (BL, time 0) measurements. In (C) and (D), *solid line* represents no change from BL; *dashed line* is the initial difference between eyes at time 0 (BL). Data are mean ± SEM for 8 monkeys, each contributing one LAT-A-treated and one vehicle-treated eye. Changes and differences in pupil diameters ≠ 0.0 by the two-tailed paired *t*-test for differences: £$P < 0.1$, ¥$P < 0.05$, §$P < 0.02$, †$P < 0.01$, $fP < 0.001$.

RESULTS

Pupil and Accommodation

Topical Eye Drops. The vehicle-treated eyes exhibited a slight but statistically significant time-dependent miosis of 0.5 mm, compared with baseline (Fig. 3A). The opposite eyes, which received topical LAT-A of 42 μg (10 μM in AC), exhibited only about half as much miosis (Figs. 3A, 3C). Resting refraction was slightly but statistically significantly myopic (1.5 D) and nearly identical in both eyes, with minimal variation over the 4.5-hour protocol and no differences between LAT-A- and vehicle-treated eyes at any time (Figs. 3B, 3D).

Intracameral Injection. Both pupils were dilated equally 25 minutes after topical PHE (to 6.68 ± 0.28 versus 6.56 ± 0.25 mm, P = NS, $n = 4$). By 25 minutes after intracameral injection of 10 μl of 50 mM LAT-A (5 μM in AC) or vehicle, both pupils dilated slightly further but the LAT-A-treated eyes more so (to 7.50 ± 0.35 versus 7.22 ± 0.42 mm, $P < 0.05$, $n = 6$; Figs. 4A, 4C). PILO infused intramuscularly constricted both pupils, but the LAT-A-treated eyes constricted significantly less than the controls (to 6.25 ± 0.38 versus 5.19 ± 0.65 mm, $P < 0.025$, $n = 6$, Figs. 4A, 4C).

Intracameral LAT-A significantly inhibited PILO-induced accommodation at 10 ($P < 0.01$), 15 ($P < 0.05$), and 20 ($P < 0.1$) minutes after the start of the PILO infusion. After 20 minutes the LAT-A-treated eye remained 3 D less accommodated than the contralateral controls (11 versus 14 Ds; Figs. 4B, 4D), but the difference was not significant.

Intravitreal Injection. LAT-A given intravitreally (10 μM) had no effect on pupil diameter, refraction, or their responses to intramuscular PILO at the dose used (data not shown).

Isolated C Strips

Resting CM tension ranged from 100 mg to 200 mg (not shown). Ten micromoles of PILO induced reproducible stable contractions averaging 68 mg and 72 mg above baseline in the longitudinal and circular vectors, respectively (Table 1, Figs. 5A, 5C; 6B). LAT-A dose-dependently relaxed the PILO-precontracted CM strips, with contraction at 3 μM LAT-A (the highest dose tested, but perhaps not maximal) averaging 34 mg and 37 mg beyond baseline tension (52% and 49% of the maximal PILO response) in the longitudinal and circular vectors, respectively (Table 1; Figs. 5B, 6B). After returning the CM strips to baseline tension for 60 minutes by perfusion with drug-free Krebs' solution, perfusion with 10 μM PILO induced contraction in both vectors of about one third the magnitude of that induced by the first exposure to PILO (Table 1; Figs. 5C, 6B).

DISCUSSION

Topical LAT-A prevented the time-dependent miosis that occurs in non-drug-treated monkey eyes under ketamine + pentobarbital anesthesia[29,30] and also dilated the pupil in monkeys anesthetized with ketamine alone (authors' unpublished observations). Intracameral LAT-A caused the pupil to dilate further

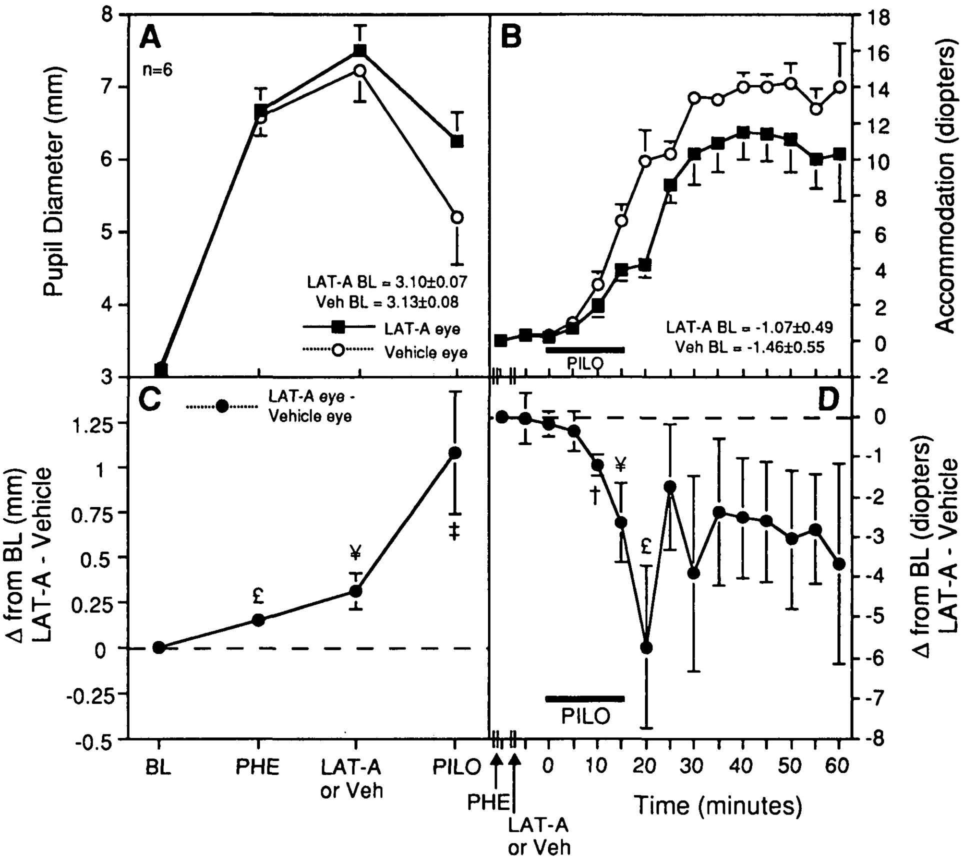

FIGURE 4. Inhibition of miotic and accommodative responses to intramuscular PILO by intracameral LAT-A. BL, baseline pupil diameter; PHE, pupil diameter or accommodation 25 minutes after topical 2.5% phenylephrine; LAT-A, Veh, pupil diameter or accommodation 25 minutes after transcorneal intracameral injection of 10 μl of 50 μM LAT-A (5 μM in AC) or 0.25% DMSO vehicle (Veh; 0.025% DMSO in AC); PILO, final pupil diameter (**A**, **C**) measured 60 minutes after starting intramuscular infusion of pilocarpine HCl (1.5 mg/kg; **B**, **D**). PILO infusion began 30 minutes after LAT-A administration and lasted for 15 minutes. Data are mean ± SEM pupil diameter or accommodation for 6 monkeys, each contributing one LAT-A-treated and one vehicle-treated eye. *Dashed line* (**C**, **D**) represents no change from BL. LAT-A, vehicle pupil diameter or accommodation ≠ 0.0 by the two-tailed paired *t*-test: £*P* <0.1, ¥*P* < 0.05, ‡*P* < 0.025, †*P* < 0.01.

after PHE and prevented miosis after systemic PILO infusion. Taken together this indicates that LAT-A interferes with the contraction of the iris sphincter smooth muscle. LAT-A was found to be very efficient in cultured cells in complexing with actin monomers and interfering with actin synthesis, leading to the destruction of the actin-based microfilament system.[1-3] Such effects would be expected to radically block cellular contractility, which is actomyosin driven. In principle, all the mechanical activities of actin in muscle and non-muscle cells alike depend on the presence of assembled actin filaments. This includes actomyosin contractility, which is most pronounced in smooth muscle. Filamentous actin also plays a major role in the formation and stabilization of cell-cell and cell-extracellular matrix adhesions. Thus, overall disruption of actin with such agents as LAT-A is expected to affect many cellular systems. However, in a recent study,[4,30] it was established that LAT-A has differential effects on different cellular systems. Cell-cell adhesions were shown to be considerably more sensitive to the drug than cell-extracellular matrix adhesions. A selective sensitivity of different muscular systems in the eye could, in principle, lead to differential effects not only on different adhesions but also on different muscle groups. Additionally, one cannot exclude the possibility that the effects of LAT-A on the iris are at least in part secondary to other consequences of the disruption of the microfilament network or even to cellular actions not primarily related to the microfilament system.

Theoretically, LAT-A should inhibit contraction of both the iris sphincter and dilator muscles. However, the sphincter predominates under normal physiological conditions,[18,24] perhaps accounting for LAT-A's pupillary dilating action. However, one cannot exclude the possibility of a specific effect on the sphincter muscle without affecting the dilator, attributable to a selective cellular sensitivity (see above) or even to differential penetration into the two cell types or other pharmacokinetic considerations.

In contrast to the pupil, topical LAT-A did not alter resting refraction, indicating the absence of an effect on the 1 D to 3 D of parasympathetically mediated tonic accommodation seen in ketamine- or pentobarbital-anesthetized monkeys[32,33] and in sleeping, anesthetized, or dark-surrounded humans.[32,34] In our monkeys, the refractions of eyes receiving topical LAT-A and their contralateral controls were similar and within this range, indicating that topical LAT-A did not substantially affect tonic

Table 1. Effect of LAT-A on Pilocarpine-Induced Contraction of Monkey CM Strips

	Contraction Force (mg)		(PILO + LAT-A)/PILO	
Drug	**Long**	**Circ**	**Long**	**Circ**
PILO 10 μM	68 ± 11	72 ± 12		
+				
LAT-A 100 nM	67 ± 11	72 ± 12	0.99 ± 0.01	1.00 ± 0.00
LAT-A 300 nM	57 ± 9	58 ± 9	0.87 ± 0.04‡	0.84 ± 0.05†
LAT-A 1 μM	43 ± 7	44 ± 9	0.64 ± 0.05§	0.61 ± 0.05§
LAT-A 3 μM	33 ± 6	37 ± 6	0.48 ± 0.06§	0.51 ± 0.04§
+				
PILO 10 μM*	25 ± 5	26 ± 5	0.37 ± 0.03§	0.39 ± 0.05§

Data are mean ± SEM mg contraction force beyond established baseline tension for 7 strips from 5 monkeys.

Long, longitudinal vector; Circ, circular vector.

* After 60-minute washout of PILO and LAT-A.

† $P < 0.025$.

‡ $P < 0.02$.

§ $P < 0.001$ for ratios different from 1.0 by the two-tailed paired t-test.

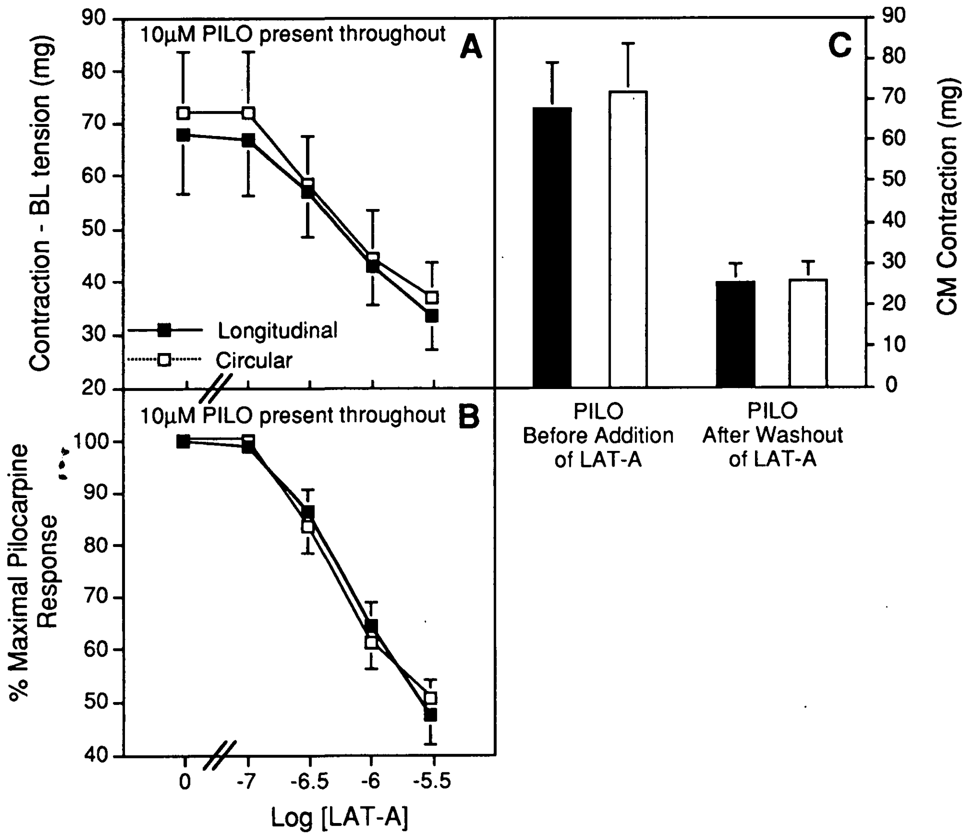

Figure 5. Effect of LAT-A on PILO-induced contraction of monkey CM strips. (**A**) Equilibrated CM strips exposed to PILO (10 μM) for 15 minutes, followed by PILO (10 μM) + LAT-A (at successive concentrations) for 15 minutes each. Data are contraction in milligrams beyond baseline tensions. (**B**) Percent inhibition of the maximal PILO response by LAT-A. (**C**) After the highest LAT-A dose (3 μM), strips were returned to baseline tension by perfusion with drug-free Krebs' solution for 60 minutes. Subsequent perfusion with 10 μM PILO yielded contraction ~⅓ that of first exposure in both vectors. Data are mean ± SEM for 7 CM strips from 5 monkeys.

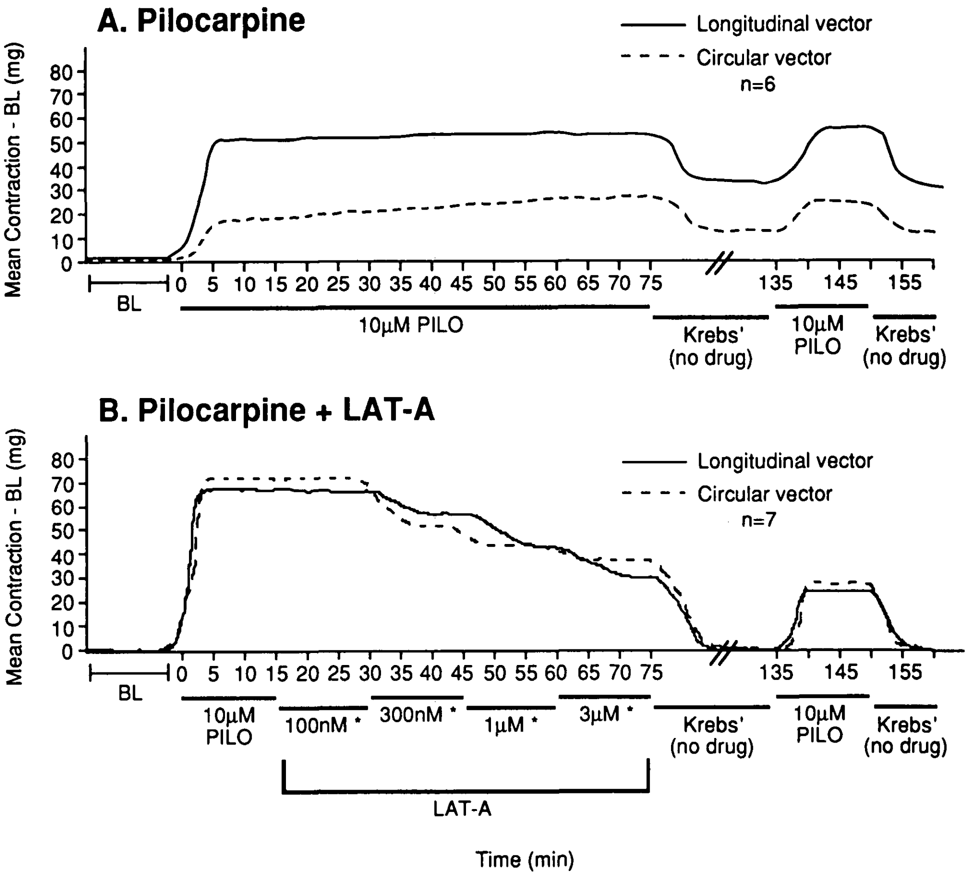

FIGURE 6. Mean contractile response to PILO (A, B) and LAT-A (B) beyond baseline tension in the longitudinal (*solid line*) and circular (*dashed line*) vectors of 6 CM strips from 1 cynomolgus and 2 rhesus monkeys (A) or 7 strips from 5 rhesus monkeys (B). BL, establishment of baseline tension (~60 minutes) while perfusing chamber with Krebs' solution; PILO, 10 μM pilocarpine added to Krebs' reservoir; *LAT-A added in successive concentrations to reservoir containing Krebs' and 10 μM PILO.

contraction of the CM. Partial inhibition of such minimal accommodation might have been hard to detect, or adequate drug concentration might not have reached the more posteriorly situated CM.[35] Although we did not measure refraction under conditions analagous to a conscious human focused at distance, it is hard to envision a pharmacological or physical mechanism by which ketamine or pentobarbital could have affected our findings. We chose LAT-A doses maximal for increasing outflow facility.[4] Higher topical LAT-A concentrations were not used, to avoid potential adverse corneal and other anterior segment side effects.[36,37] Intracameral LAT-A inhibited both the pupillary and accommodative responses to intramuscular PILO, indicating that LAT-A inhibits the contraction of both the iris sphincter and CM. LAT-A dose-dependently reversed and prevented PILO-induced contraction of isolated rhesus and cynomolgus CM strips, by 50% to 70% at 3 μm LAT-A, a dose lower than that used for the pupil and accommodation experiments. This dose may not have been maximal; limited supply of drug precluded testing higher doses. In contrast, the serine-threonine kinase inhibitor H-7, which also inhibits actomyosin contractility,[38,39] and in monkeys has effects similar to those of LAT-A on outflow facility and pupillary function in vivo, and on PILO-induced ciliary muscle contraction in vitro[4,40,41] does not inhibit the PILO-induced accommodation in monkeys at any rational intracameral or intravitreal dose.[40] The basis for the in vivo versus in vitro dissociation with H-7 but not LAT-A is unclear.

Smooth muscle contraction is dependent on the phosphorylation of the myosin light chain by myosin light chain kinase. Phosphorylation results in increased myosin ATPase activity, leading to increased rates of cross-bridge cycling and tension.[42] By disassembling actin filaments, LAT-A prevents cross-bridge formation between myosin and actin filaments, thus preventing contraction. In our experimental model, topical LAT-A caused mydriasis, and intracameral LAT-A inhibited the miotic response to PILO. These effects seem likely to be mediated by disassembly of actin filaments in the relevant tissues. However, our data do not address the cellular mechanism directly, and, as discussed above, other possibilities exist and different muscles may react differently to LAT-A.

In addition to its mydriatic and cycloplegic effects, LAT-A also increases outflow facility and decreases intraocular pressure[16,17] in monkeys. The latter two physiological effects may have potential clinical relevance for glaucoma therapy. LAT-A's mydriatic and cycloplegic effects are relatively weak compared with specific anticholinergic drugs, which may also be clinically relevant for glaucoma. For example, topical LAT-A given along with PILO may partially attenuate PILO's induction of clinically undesirable miosis, while still retaining enough PILO-induced CM contraction to increase outflow facility[43] and thereby complement LAT-A's direct facility increasing action on the trabecular meshwork.[4] Clearly more work is needed to evaluate LAT-A's potential antiglaucoma usefulness.

Acknowledgments

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