Effects of H-7 on the Iris and Ciliary Muscle in Monkeys

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Objectives: To determine the effects of H-7 on (1) iris and ciliary muscles (CMs) in living monkeys; (2) isolated monkey CM strips; (3) actomyosin contractility in cultured Swiss 3T3 cells.

Methods: (1) Pupillary diameter (calipers) and accommodation (refractometer) in living monkeys were measured after topical, intracameral, or intravitreal administration of H-7 followed by systemic pilocarpine hydrochloride. (2) Pilocarpine-induced contraction of isolated monkey CM strips following administration of H-7 was measured in a perfusion chamber. (3) Actomyosin contractility in Swiss 3T3 cells cultured on thin silicone rubber film was determined by measuring cell-induced film wrinkles before and after administration of H-7.

Results: Topical H-7 prevented anesthesia-induced miosis but did not affect resting refraction. Intracameral or intravitreal H-7 dilated the pupil and inhibited miotic but not accommodative responses to pilocarpine. H-7 inhibited pilocarpine-induced contraction of isolated monkey CM strips and reduced Swiss 3T3 cell contraction.

Conclusions: H-7 inhibits actin-based contractility in non-muscle cells and in monkey iris sphincter and CM. Under our in vivo experimental conditions, the effect on the iris predominates over that on the CM.

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SMOOTH MUSCLE contraction is associated with Ca^{++}-dependent activation of myosin light chain kinase (MLCK) and consequent phosphorylation of myosin; MLCK activation is a primary factor increasing myosin light chain phosphorylation,[1] leading from receptor activation to cellular contraction.[2-4] H-7 [1-(5-isoquinolinyl-sulfonyl)-2-methylpiperazine], a nonselective protein kinase inhibitor, reduces actomyosin-driven contractility in chick lens cells and fibroblasts, thus leading to deterioration of the actin microfilament system,[2] and it inhibits endothelin 1–induced contraction of rabbit aortic [5] and airway smooth muscles.[6] Since H-7 disrupts microfilament bundles with accompanying decreases in the incorporation of phosphorus 32 into phosphoproteins,[7] and pretreatment with H-7 inhibits adenosine triphosphate–induced contraction of actin filaments,[2] the H-7-induced reduction of actomyosin contractility is likely consequent to the decrease of phosphoproteins. However, the effects of the target kinase(s) for H-7 on the actin cytoskeleton, actomyosin contractility, and smooth muscle cell relaxation remain unclear.

H-7 increases aqueous humor outflow facility and reduces intraocular pressure in living monkeys, probably by affecting the trabecular meshwork cytoskeleton.[8] Given potential therapeutic implications for glaucoma, and the effects of H-7 on cellular contractile systems, it seemed important to determine whether H-7 might interfere with contraction of the iris sphincter and ciliary muscles (CMs), which are responsible for miosis and accommodation respectively. We therefore determined the effects of H-7 on pupillary diameter and accommodation in the living monkey eye, and on contraction of isolated monkey CM strips in vitro. To gain insights at the cell biological level, we also studied the effects of H-7 on the contractility of cultured Swiss 3T3 cells.

RESULTS

PUPILLARY MEASUREMENTS AND REFRACTION IN LIVING MONKEYS

Protocol 1

The slight time-dependent miosis that occurred under anesthesia with ketamine and pentobarbital was inhibited by 150-mmol/L (1.1 mg) topical H-7 ($P<.05$, **Figure 2**), but resting refraction was not affected (**Figure 3**).

MATERIALS AND METHODS

ANIMALS AND ANESTHESIA

Normal cynomolgus monkeys (*Macaca fascicularis*) weighing 2.1 to 4.3 kg were studied in accordance with University of Wisconsin-Madison, National Institutes of Health, and Association for Research in Vision and Ophthalmology guidelines. Anesthesia was induced by intramuscular ketamine (10 mg/kg), and maintained by intramuscular pentobarbital sodium (35 mg/kg).

DRUGS AND CHEMICALS

H-7, Medium 199 (containing glutamine), penicillin G-streptomycin solution, fetal bovine serum, and pilocarpine hydrochloride were obtained from Sigma Chemical Co, St Louis, Mo; dimethyl sulfoxide, from Research Industries Corporation, Salt Lake City, Utah; 2.5% phenylephrine hydrochloride (Mydfrin), from Alcon Laboratories, Inc, Fort Worth, Tex; and cyanoacrylate adhesive from Tri-Point Medical L.P., Raleigh, NC. H-7 solution for transcorneal anterior chamber (AC) injection (3 mmol/L) was freshly prepared in Bárány mock aqueous humor.[9] For intravitreal injection, H-7 was formulated as 0.27 mg in 10 µL of 50% dimethyl sulfoxide (75 mmol/L) or 0.55 mg in 10 µL of 100% dimethyl sulfoxide (150 mmol/L). For topical application, H-7 was formulated as 1.1 mg in 20 µL of 25% dimethyl sulfoxide (150 mmol/L). Pilocarpine was dissolved in 0.1% citric acid buffer just before the experiments, so that intramuscular infusion of 3 mL delivered from 3 to 6 mg of pilocarpine hydrochloride (1.5 mg/kg of body weight).

PUPILLARY DIAMETER AND ACCOMMODATION MEASUREMENT IN LIVING MONKEYS

Accommodation (difference between baseline and post-drug refraction) was determined with a Hartinger (Zeiss-Jena, Jena, Germany) coincidence refractometer. Pupillary diameter was measured with vernier calipers under normal room light (350 lux).

Protocol 1

Baseline refraction and pupillary diameter were measured (average of 2-3 readings). Four 5-µL drops of 150-mmol/L H-7 or 25% dimethyl sulfoxide was administered to the central cornea of opposite eyes of supine monkeys at 30-second intervals, with blinking prevented between and for 1 minute after the last drops. Beginning 45 minutes later, refraction and pupillary diameter were measured every 15 minutes for 75 minutes (ie, until 2 hours after H-7 treatment). The H-7 dosage was chosen to give a 300-µmol/L H-7 concentration in the approximately 100-µL monkey AC,[10] assuming 1% intracameral penetration and no drug loss from the AC.[11-13] A concentration of 300-µmol/L H-7 reduces actomyosin contractility and cytoskeletal cell junctional effects in cultured chick lens cells and fibroblasts,[2] and it maximally increases outflow facility in living monkeys.[8]

Protocol 2

Baseline refraction and/or pupillary diameter were measured, followed by topical application of 2.5% phenylephrine (which stimulates the iris dilator muscle without influencing the iris sphincter and CM,[14,15] thereby facilitating measurement of miosis and accommodation[16]). Refraction and/or pupillary diameter were measured again about 25 minutes later, after which 10 µL of 3-mmol/L H-7 was administered intracamerally (300 µmol/L in the approximately 100-µL AC), or 10 or 20 µL of 75- or 150-mmol/L (respectively) H-7 was administered intravitreally (300 µmol/L to 1.2 mmol/L in the approximately 2.5-mL vitreous[17]) to one eye and vehicle to the other. Refraction and pupillary diameter were determined 25 minutes after intracameral H-7 or 55 minutes after intravitreal H-7. Five minutes later, about 3 mL of pilocarpine hydrochloride solution was infused intramuscularly into the thigh (1.5 mg/kg) for from 10 to 15 minutes. Refraction was determined every 5 minutes after pilocarpine infusion until stable, and then final pupillary diameter was measured (**Figure 1**).

Anterior chamber injections were made under an operating microscope, using a 30-gauge needle connected via polyethylene tubing to a micrometer syringe (Gilmont Instruments, Barnant Co, Barrington, Ill). The needle was threaded through the corneal stroma for approximately 6 mm, then directed into the AC so that the wound was self-sealing. For intravitreal injections, the needle was inserted 4 mm through the temporal pars plana 4 mm posterior to the limbus.

CLINICAL EXAMINATION

Slitlamp examination and indirect ophthalmoscopy were conducted by an ophthalmologist (B.T. or P.L.K.). Corneal epithelial integrity, the presence or absence of AC flare or cells, and lens clarity were noted 2 hours after topical or intracameral administration of H-7 and of corresponding vehicle in the contralateral eye. Vitreous transparency, retinal color and structure, and the state of the anterior segment were noted 1 week and 1 month or longer after intravitreal H-7 injection.

CONTRACTION OF ISOLATED CM

CM Preparation

Globes were enucleated from 6- to 18-year-old rhesus monkeys (*Macaca mulatta*) that were under deep anesthesia with intravenous pentobarbital sodium (25 mg/kg) just before or within 5 minutes of death by pentobarbital overdose. The CM was dissected, strips mounted in a perfusion chamber, and contractile responses to drugs in the circular (coronal) and longitudinal (sagittal) vectors measured simultaneously.[18-20]

H-7 Experiments

After equilibration, 10-µmol/L pilocarpine hydrochloride (just maximal for contraction) was perfused through the chamber for 15 minutes. H-7 was then added to the Krebs-pilocarpine mixture at successive concentrations (1 to 300 µmol/L), each perfusing for 15 minutes. After the final dose, the strip was perfused with plain Krebs solution for 90 minutes (to reestablish baseline tension), and then rechallenged with pilocarpine (10 µmol/L) for 15 minutes. The CM strip was then perfused with plain Krebs solution for another 60 minutes, and then again challenged with pilocarpine for 15 minutes. Responses to H-7 were expressed as absolute Δ force from resting tension and as percent average pilocarpine response for each CM strip, the latter

normalizing the data for interstrip variations due to muscle dimensions, mass, and mounting.

CULTURED CELLS

To test the effect of H-7 on the mechanical forces exerted by substrate-attached cells, Swiss 3T3 cells were plated on a silicone rubber film.[21]

Silicone Rubber Substrates

Dimethyl polysiloxane (silicone rubber, 12 000 centistokes) was aliquotted into Petri dishes, allowed to spread for several hours, and then coated with gold-palladium using an evaporator sputter coater (Edwards High Vacuum International, Wilmington, Mass)[22] at settings of 3 kV and 15 mA for 3 minutes. The UV light from the sputter coater polymerized the top layer of the silicone rubber to form a thin film floating on the unpolymerized rubber. The gold-palladium reduced the film's hydrophobicity and facilitated cell adhesion.

Visualization and Analysis of Cell Contractility

Swiss 3T3 cells were grown for 48 hours on thin silicone rubber film in Dulbecco modified Eagle medium plus 10% newborn calf serum. Two days after plating, cell-induced wrinkles appeared on the film. The cells were then treated with 300-µmol/L H-7. Time-lapse video cinematography was performed using National Institutes of Health Image software, taking 1 frame every 30 seconds. Intensity vs x-coordinate profiles were taken for the cross section of each of 5 wrinkles at each time point. The amplitudes of the profiles were measured and normalized so that the amplitude at the zero time points of each wrinkle were equal to 1.0.

Protocol 2

Pupillary Diameter. Twenty-five minutes after phenylephrine administration, both pupils dilated equally (to 7.17 ± 0.28 mm vs 7.20 ± 0.26 mm [mean ± SEM], *P* = not significant (*P*<.6), **Figure 4**, B, **Figure 5**, A). Twenty-five minutes after AC injection of 10 µL of 3-mmol/L H-7 ([H-7]AC = 300-µmol/L), the pupils in the H-7–treated eyes dilated further relative to the contralateral controls (to 7.88 ± 0.19 mm vs 6.78 ± 0.46 mm [mean ± SEM], *P*<.05, Figure 4, C, Figure 5, A). When pilocarpine was infused intramuscularly into the thigh, the control pupils constricted but the H-7–treated pupils did not (to 4.80 ± 0.20 mm in controls vs 7.25 ± 0.13 mm [mean ± SEM] in H-7–treated eyes, *P*<.001, Figure 4, D, Figure 5, A). H-7 also dilated the pupil and inhibited the miotic response to pilocarpine dependent on the dose after intravitreal injection of 10 or 20 µL of 75- or 150-mmol/L H-7, respectively (300-, 600- or 1200-µmol/L H-7 in the vitreous, Figure 5, B-D).

Accommodation. No significant differences between pilocarpine-induced accommodation in H-7–treated vs control eyes were observed at 300-µmol/L intracameral H-7 or at 300- or 600-µmol/L intravitreal H-7 (Figure 5, E-H). At 1.2-mmol/L intravitreal H-7, a physiologically minimal but statistically significant difference was observed only at 40 and 45 minutes after intramuscular administration of pilocarpine, with the H-7–treated eyes accommodating around 1 diopter (D) less than the controls (Figure 5, H).

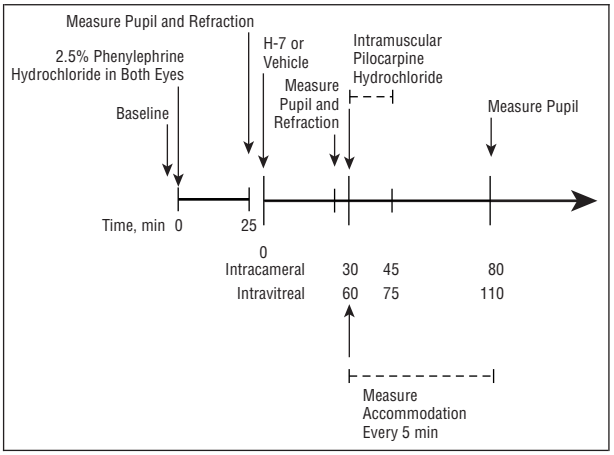

Figure 1. *Time line for protocols determining the effects of intracameral and intravitreal H-7 [1-(5-isoquinolinyl-sulfonyl)-2-methylpiperazine] on pupillary and accommodative responses to pilocarpine in monkeys anesthetized with ketamine and pentobarbital sodium.*

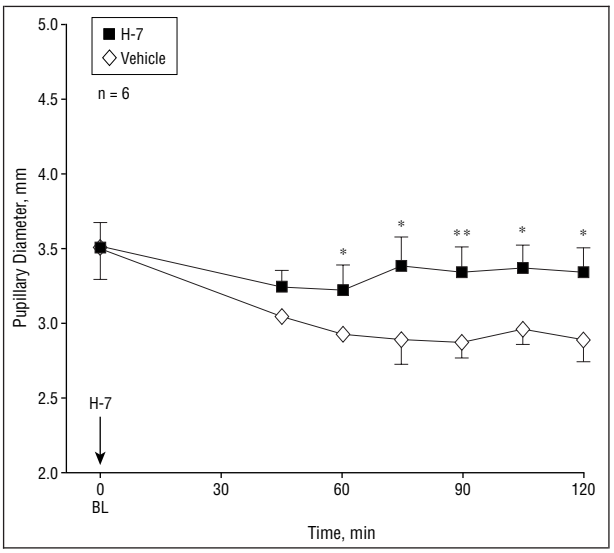

Figure 2. *Inhibition of anesthesia-induced miosis by topical H-7 [1-(5-isoquinolinyl-sulfonyl)-2-methylpiperazine] (150 mmol/L = 1.1 mg). BL indicates pre–H-7 baseline. Data are given as the mean ± SEM for the number of (n) monkeys anesthetized with ketamine and pentobarbital sodium. Significant difference between eyes: *, P<.05; **, P<.01.*

CLINICAL EXAMINATION

Mild punctate corneal epithelial defects and slight epithelial cloudiness were seen in H-7–treated eyes compared with controls 2 hours after 150-mmol/L (1.1-mg) topical H-7 or vehicle. No AC, lens, or retinal abnormalities were seen at any time following topical, intracameral, or intravitreal administration of H-7 or vehicle.

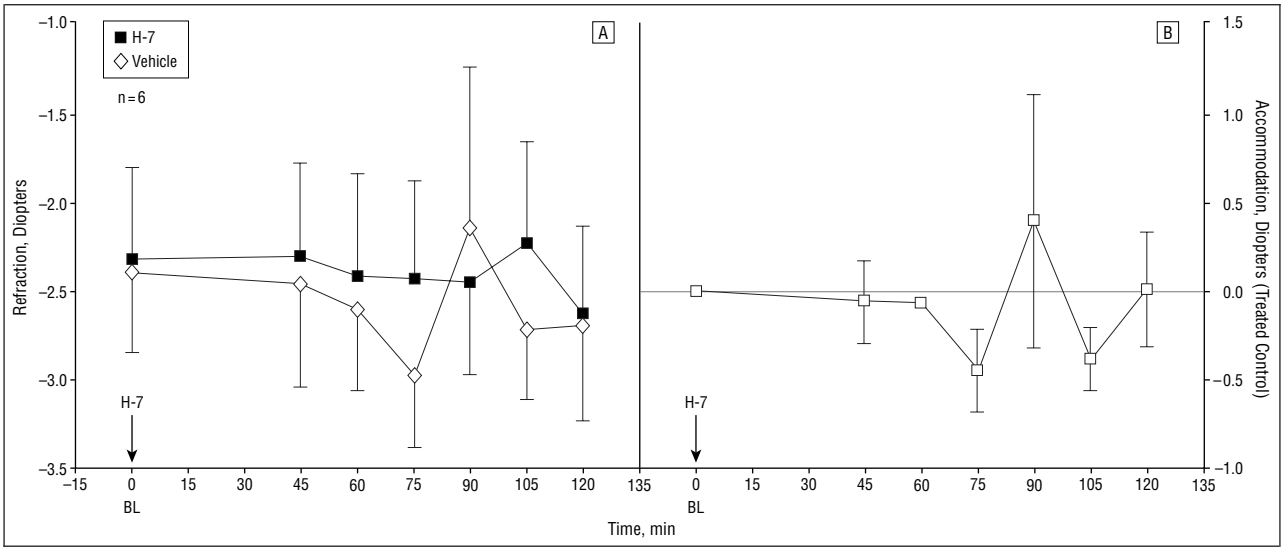

Figure 3. *Accommodative responses to topical H-7 [1-(5-isoquinolinyl-sulfonyl)-2-methylpiperazine] (150 mmol/L = 1.1 mg). A, Refraction; B, accommodation. BL indicates pre–H-7 baseline; dashed line, equality. Data are given as mean ± SEM for the number of (n) monkeys. No difference between eyes.*

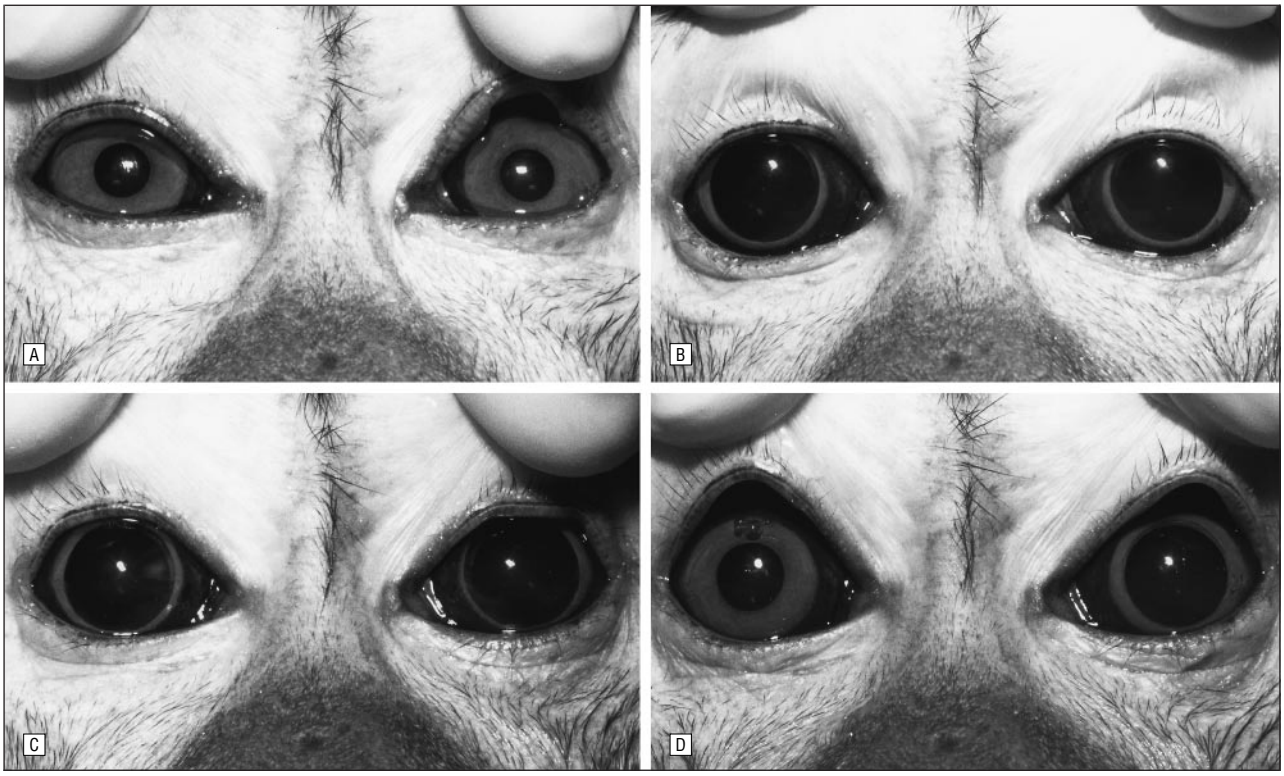

Figure 4. *Pupils, cynomolgus monkey, Figure 1 protocol. A, Baseline; B, 25 minutes after topical phenylephrine; C, 25 minutes after intracameral H-7 [1-(5-isoquinolinyl-sulfonyl)-2-methylpiperazine] (300 µmol/L) left eye, vehicle right eye; D, approximately 50 minutes after 1.5-mg/kg intramuscular pilocarpine hydrochloride.*

CONTRACTION OF ISOLATED CM

Resting CM tension was 100 to 200 mg (data not shown). Pilocarpine hydrochloride at a 10-µmol/L concentration induced reproducible, stable contractions averaging 22 and 37 mg above baseline in the circular and longitudinal vectors, respectively (**Figure 6**, A, **Figure 7**, A). H-7 dependent on the dose relaxed the pilocarpine-precontracted CM strips, the maximum effect occurring at 100 to 300 µmol of H-7 per liter and averaging 15 and 25 mg (69% and 75% of the pilocarpine response, respectively) in the respective vectors (Figure 6, A and B; Figure 7, A). H-7 was equipotent in the 2 vectors, with the dose-response curves for percent inhibition of pilocarpine-induced contraction virtually superimposable. After restoring the CM strips to baseline tension for 90 minutes by perfusion with drug-free Krebs solution, perfusion with 10-µmol/L pilocarpine hydrochloride for 15 min-

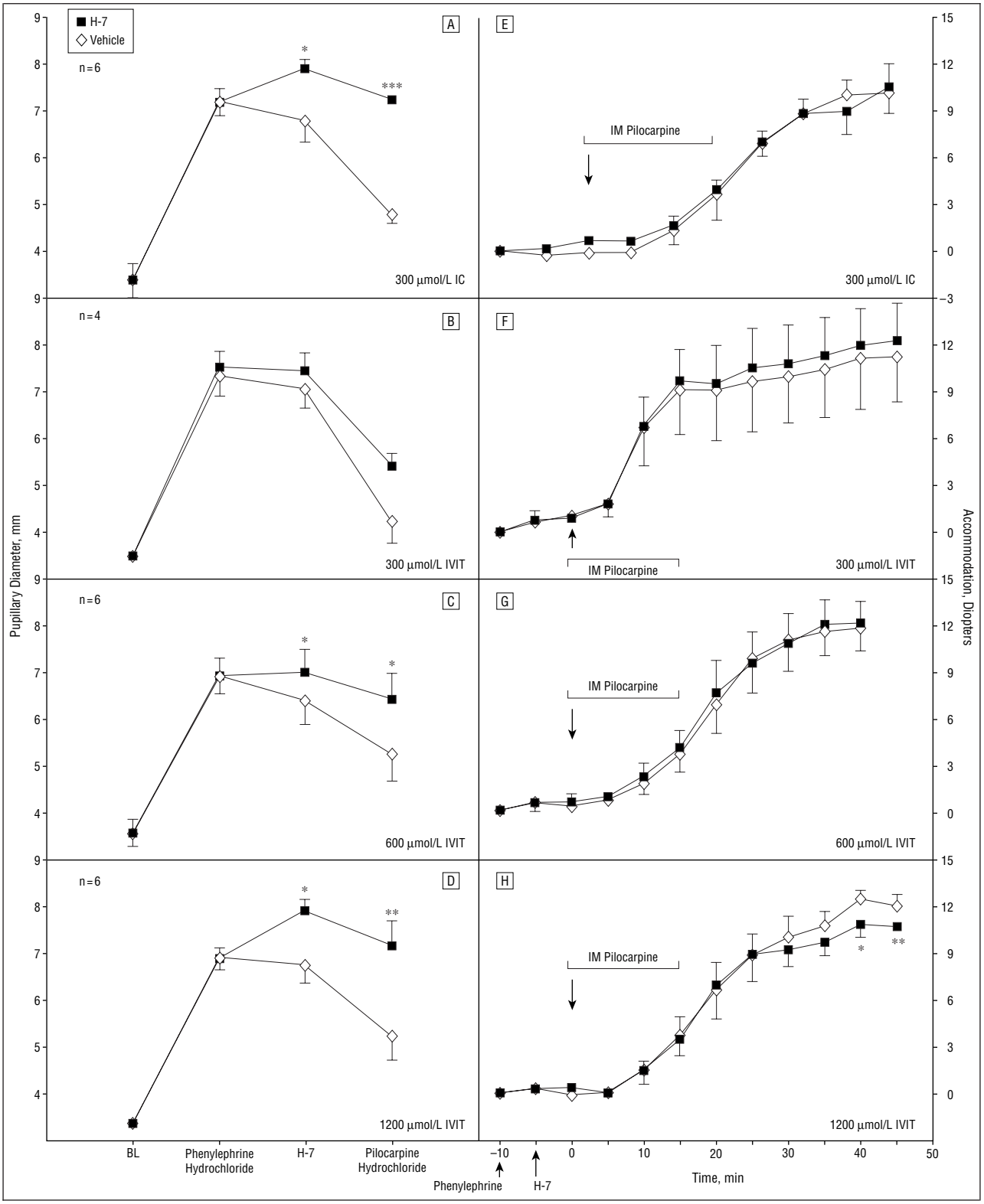

Figure 5. *Pupillary and accommodative responses to intracameral (IC) and intravitreal (IVIT) H-7 [1-(5-isoquinolinyl-sulfonyl)-2-methylpiperazine], topical phenylephrine hydrochloride), and intramuscular (IM) pilocarpine hydrochloride (1.5 mg/kg). Protocol per Figure 1. BL indicates baseline. Data are given as mean ± SEM for the number of (n) monkeys. Significant difference between eyes: *, P<.05; **, P<.01; and ***, P<.001.*

utes produced no contraction (Figure 7, B). After additional perfusion with drug-free Krebs solution for 60 minutes, perfusion with 10-µmol/L pilocarpine hydrochloride then yielded a weak, sluggish contraction in both vectors (Figure 7,C).

CULTURED CELLS

Microscopic examination of Swiss 3T3 cells cultured on a silicone rubber film revealed extensive wrinkles in the silicone substrate prior to H-7 treatment (**Figure 8**, A).

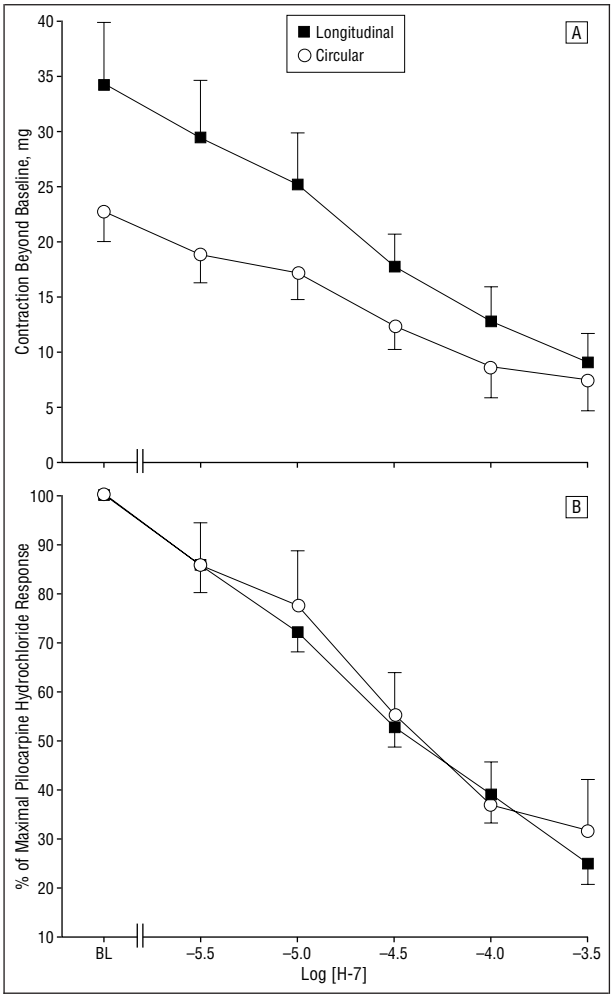

Figure 6. *Effect of H-7 [1-(5-isoquinolinyl-sulfonyl)-2-methylpiperazine] on contraction of ciliary muscle strips. A, 10-µmol/L pilocarpine hydrochloride for 15 minutes, followed by 10-µmol/L pilocarpine hydrochloride plus increasing H-7 concentrations for 15 minutes each. B, Percentage of maximum pilocarpine response at increasing H-7 concentrations plus 10-µmol/L pilocarpine hydrochloride. Data are given as mean ± SEM; the 5 ciliary muscle strips are from 4 rhesus monkeys.*

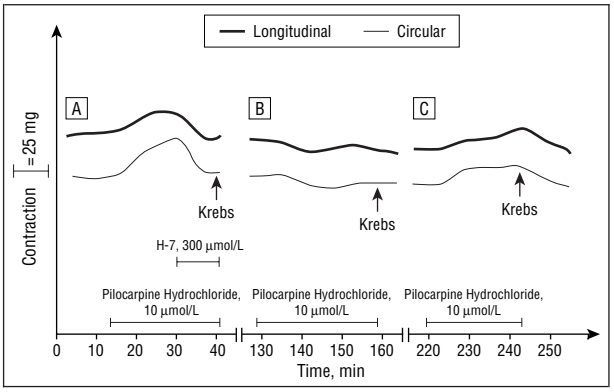

Figure 7. *Effect of H-7 [1-(5-isoquinolinyl-sulfonyl)-2-methylpiperazine] on contraction of rhesus monkey ciliary muscle strip. A, Ciliary muscle strip at baseline tone, then exposed to 10-µmol/L pilocarpine hydrochloride, then to 10-µmol/L pilocarpine hydrochloride plus 300-µmol/L H-7. Note the response to pilocarpine and its inhibition by H-7. B, Same ciliary muscle strip after a 90-minute washout; 10-µmol/L pilocarpine hydrochloride elicits no contraction. C, Same ciliary muscle strip after an additional 60-minute washout; 10-µmol/L pilocarpine hydrochloride elicits sluggish contraction in both vectors.*

After incubation with 300-µmol/L H-7 for up to 5.5 minutes, wrinkle amplitude was decreased by approximately 90% (Figure 8, B and C), indicating reduced cellular contractility.

COMMENT

H-7 increases outflow facility and reduces intraocular pressure in monkeys and therefore may have potential as an anti-glaucoma medication.[8] We determined the effects of H-7 on the contraction of intraocular smooth muscles in vivo and in vitro.

Topical H-7 prevented time-dependent miosis under anesthesia with ketamine and pentobarbital and further dilated the phenylephrine–treated pupil in living monkeys, indicating that H-7 interferes with contraction of the iris sphincter muscle. H-7 theoretically should inhibit contraction of both the iris sphincter and dilator muscles. Therefore, our data also suggest that the iris sphincter predominates in determining pupillary diameter under our experimental conditions. However, an in vitro dose-response comparison between the iris sphincter and dilator would be helpful to identify potentially different sensitivities to H-7.

Resting refraction in monkeys under pentobarbital anesthesia is –1 to –3 D,[23,24] The mild myopia is sensitive to atropine sulfate, and probably represents tonic accommodation.[24] Eyes receiving topical H-7 and their contralateral controls both fell within this range (–2.26 to –2.65 D in H-7–treated eyes vs –2.17 to –2.99 D in controls) and did not differ significantly, indicating that topical H-7 did not affect tonic CM contraction. Possible reasons may be (1) weakness of tonic contraction, making small changes difficult to detect; (2) inadequate drug concentration at the posteriorly situated CM after topical administration[25] (see paragraph below); or (3) that H-7 does not penetrate or is ineffective in CM cells.

Intracameral and intravitreal H-7 inhibited pupillary but not accommodative responses to intramuscular pilocarpine, excluding reason 1 and militating against reason 2 in the preceding paragraph. The experiments on isolated CM strips were designed to determine whether the pupillary-accommodative dissociation had a biological or a pharmacokinetic basis. Dependent on the dose, H-7 both prevented and reversed pilocarpine-induced contraction of isolated CM strips. Furthermore, the same dose of H-7 that prevented the pupillary response to pilocarpine in vivo prevented and reversed contraction of CM strips induced by a maximal pilocarpine dose in vitro, the effect lasting for several hours after removal of H-7 from the medium. Thus, H-7 inhibits contraction of both the iris sphincter and CM, but the effect on the latter is negligible under our in vivo experimental conditions. This has potential therapeutic implications for preserving pilocarpine's effect on outflow facility (which requires CM contraction)[26] while inhibiting its vision-reducing miotic effect, especially in elderly patients with incipient cataract.[27]

Pilocarpine administered topically or intracamerally in monkeys induced miosis at a 100-fold lower dose

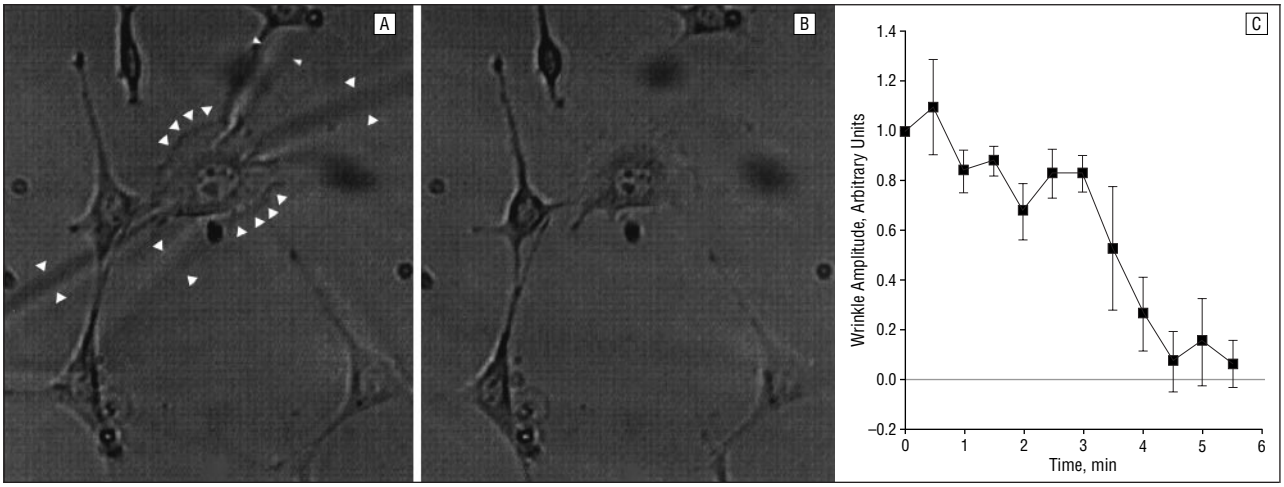

Figure 8. *Effect of H-7 [1-(5-isoquinolinyl-sulfonyl)-2-methylpiperazine] on cell contractility. Swiss 3T3 cells on silicone rubber film (A) prior to and (B) after 5.5-minute incubation with 300-µmol/L H-7. Note reduction in film wrinkling (arrowheads in A) after exposure to H-7. C, Wrinkle amplitude vs H-7 exposure time.*

than accommodation, but no difference in sensitivity occurred with systemically administered pilocarpine, suggesting that the pilocarpine concentration at the CM was 100 times less than at the iris following topical or intracameral dosing.[25] Such differing drug concentrations could account for the dissociation of pupillary-accommodative responses to pilocarpine following intracameral H-7 in our in vivo experiments. H-7 was administered intravitreally to afford possibly better access to the circular, putatively more accommodation-relevant, portion of the CM.[28,29] However, the circular CM is separated from the vitreous by the ciliary epithelium, the highly vascular ciliary process stroma, and the dense connective tissue ground plate between the stroma and the muscle. The first and third of these might constitute barriers and the second an efflux route, all preventing the drug from reaching the circular CM. Aqueous humor flow might carry some drug away from the ciliary body and into the AC. This may explain why even 1.2-mmol/L intravitreal H-7 only minimally inhibited the accommodative response to pilocarpine. Drug diffusion toward the target tissues was probably not a limiting factor, since the pupillary response was inhibited by intravitreal H-7. Further studies, such as an in vitro dose-response comparison between the iris sphincter and CM to identify potentially different sensitivities to H-7, are needed to unequivocally exclude a biological basis for the pupillary-accommodative dissociation, but a pharmacokinetic explanation seems plausible.

Smooth muscle contraction is an actin-myosin-based mechanical process. Activation of protein kinase C (PKC) and MLCK elicit muscle contraction by increasing myosin light chain phosphorylation. However, the inositol triphosphate -Ca^{++}-MLCK signaling system, rather than the diacylglycerol–PKC system plays the major role in smooth muscle contraction.[1] H-7 is a broad-spectrum, serine-threonine kinase inhibitor, inhibiting PKA, PKG, and PKC and MLCK.[30,31] Since (1) H-7 has a specific and rapid effect on the actin cytoskeleton of cultured Swiss 3T3 and PTK2 cells, but a potent PKC inhibitor (sangivamycin) and a specific PKA/PKG inhibitor (HA1004) do not[32]; and (2) H-7 and a selective MLCK inhibitor (KT5926) inhibit actomyosin-driven contractility in chick lens cells and fibroblasts that leads to deterioration of the actin microfilament system, but highly potent PKC inhibitors (Ro31-8220 and GF109203X) do not,[2] H-7 may inhibit actomyosin-driven contraction primarily by inhibiting MLCK or other enzymes involved in cellular contractility such as rho-kinase.[22,33] Furthermore, cultured Swiss 3T3 cells grown on a silicone rubber film substrate induce wrinkling in the film by their normal contractility, and this wrinkling was reduced dramatically following the addition of 300-µmol/L H-7. A similar effect on smooth muscle cell contractility could explain the inhibition by H-7 of pilocarpine-induced contraction of the iris sphincter and CM in our monkeys.

Further studies of this interesting compound, especially with regard to ocular safety, seem warranted.

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100 Years Ago in the ARCHIVES

A look at the past . . .

Conclusions.—(1) The normal conjunctiva always contains bacteria. Of these the staphylococcus epidermidis albus is found with such frequency that it must be regarded as a regular inhabitant of this situation. This coccus and probably other bacteria found in this locality are usually of only slight if any pathogenic power. It should be remembered, though, that bacteria, ordinarily non-pathogenic, may become harmful under certain favoring conditions, such as the bruising of the tissues by instruments or the irritation resulting from chemical substances.

(2) Neither the irrigation with sterilized water nor the instillation of sublimate solution (1:5000) produces sterility of the conjuctiva, and inasmuch as both measures are futile and possibly harmful they may just as well be abandoned. These methods of sterilizing the conjunctiva are the ones usually employed by ophthalmologists, and hence the choice of them for testing this question.

The most important essential of a germicide which is to be used upon the conjunctiva is that it be absolutely free of irritating properties, and furthermore, it should be demonstrable that this germicide will destroy the germs most commonly met within the normal conjunctiva. It goes without saying that an antiseptic which has these qualities would be indispensable in all operations on the eye.

(3) In operating upon the normal conjunctiva, as in cataract operations, the surgeon in the present state of our disinfecting armamentarium would do well to consider the subject of antisepsis and asepsis chiefly, if not solely, in connection with the hands, instruments, cocaine, and atropine.

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