Prostanoid EP_4 Receptor Stimulation Produces Ocular Hypotension by a Mechanism That Does Not Appear to Involve Uveoscleral Outflow

David F. Woodward,[1] *Siv F. E. Nilsson,*[2] *Carol B. Toris,*[3] *Alexander B. Kharlamb,*[1] *Amelia L. Nieves,*[1] *and Achim H.-P. Krauss*[1,4]

Purpose. As part of a systematic elucidation of the pharmacology of prostaglandin's (PG) effects on intraocular pressure in the monkey, the prototypical selective prostanoid EP_4 receptor agonist (3,7-dithia PGE_1) was examined. It was found to be highly efficacious in nonhuman primates, and its mechanism of ocular hypotensive activity was investigated.

Methods. Intraocular pressure (IOP) was measured by pneumatonometry in conscious monkeys restrained in custom-designed chairs. All other animal experiments were performed in animals sedated with ketamine or anesthetized with ketamine/diazepam and given drug or vehicle for various lengths of time. Aqueous flow was determined by fluorophotometry. Total outflow facility was measured by the two-level, constant-pressure method and by 2-minute tonography in both normotensive and hypertensive monkey eyes. Uveoscleral outflow was measured by perfusing the anterior chamber with FITC-labeled dextran for 30 minutes at a fixed IOP of approximately 15 mm Hg. Isometric responses to drugs were measured in longitudinal and radial preparations of monkey and human isolated ciliary smooth muscle specimens.

Results. The selective EP_4 receptor agonist 3,7-dithia PGE_1 and an isopropyl ester prodrug thereof reduced IOP in monkeys. A single dose of 3,7-dithia PGE_1 isopropyl ester, at a 0.01% or 0.1% dose, decreased IOP in the glaucomatous monkey in the range of 40% to 50%. Studies on total outflow facility by the two-level, constant-pressure perfusion method and tonography indicated that EP_4 receptor stimulation facilitated aqueous humor outflow facility. No effect on aqueous flow was apparent. In contrast to all PGs and prostamides studied to date, 3,7-dithia PGE_1 exerted no effect on uveoscleral outflow measured directly. Moreover, it did not relax longitudinal or radial preparations of isolated human or monkey ciliary muscles.

Conclusions. The EP_4 receptor agonist 3,7-dithia PGE_1 is a highly efficacious IOP-lowering drug in monkeys. It has no effect on uveoscleral outflow but does increase total outflow facility, which accounts for a substantial proportion of the ocular hypotensive activity. (*Invest Ophthalmol Vis Sci.* 2009; 50:3320–3328) DOI:10.1167/iovs.08-3031

Natural prostaglandins are potent and efficacious ocular hypotensive agents. They are now known to exert their effects by interacting with at least nine distinct target receptors, but detailed understanding of their role in aqueous humor dynamics is an ongoing endeavor. Over the past few years, selective agonists and antagonists for each of the prostanoid receptors have emerged. These represent invaluable tool drugs for completing a detailed investigation of the ocular pharmacology of PGs on a functional basis. To date, selective agonists for DP, EP_2, and FP receptors all have been shown to reduce intraocular pressure (IOP) in nonhuman primates.[1–7] Of interest, the underlying mechanisms involved in ocular hypotension produced by DP, EP_2, and FP receptor stimulation in nonhuman primates appear to exclusively involve increased uveoscleral outflow.[6–11] This finding may be regarded as unexpected, as these receptors are not only discrete entities but do not couple with the same signal-transduction mechanisms. As part of a systematic attempt to completely characterize the pharmacology of PG-induced ocular hypotension, we investigated the effects of the first selective EP_4 receptor agonist to be described, 3,7-dithia PGE_1.[12]

It is not surprising that selective EP_4 agonists only recently have been described, because the EP_4 receptor was the last prostaglandin E_2 (PGE_2)–sensitive receptor to be proposed as part of the original pharmacologic classification for prostanoid receptors.[13] The mouse and human EP_4 receptors were cloned contemporaneously[14,15] but were misnamed EP_2 until the authentic EP_2 receptor sequence was reported.[16] Structurally, the EP_4 receptor is characterized by a long cytoplasmic terminus that is claimed to be involved in agonist-induced receptor desensitization and internalization.[17–20] The EP_4 receptor is widely distributed.[13–15,21–23] EP_4 receptors have been reported to be widely represented in human ocular tissues, according to extensive immunolocalization studies[24] and were present in every tissue and cell, with the following exceptions: iris epithelium, ciliary process blood vessels, retinal blood vessels, and Müller cells.[24] Studies involving reverse transcription–polymerase chain reaction (RT-PCR)[24–27] and function provide corroborative evidence for the immunolocalization results. The expression of EP_4 receptors in trabecular meshwork cells, endothelial cells lining Schlemm's canal, collector channels, aqueous veins, ciliary epithelium, ciliary muscle, and ciliary body stromal cells[24,26,27] is particularly relevant to aqueous humor dynamics and drugs that reduce IOP.

Given the abundant message for prostanoid EP_4 receptors in the trabecular meshwork,[24] this tissue could be regarded as a likely target for EP_4 receptor–mediated ocular hypotensive activity. Increased trabecular outflow facility is an attractive mechanism of action for an antiglaucoma medication, since

From the [1]Department of Biological Sciences, Allergan, Inc., Irvine, California; the [2]Department of Medical and Health Sciences, Division of Drug Research Faculty of Health Sciences, University of Linköping, Linköping, Sweden; and the [3]University of Nebraska Medical Center, Omaha, Nebraska.

[4]Present affiliation: Ophthiris, GlaxoSmithKline, King of Prussia, Pennsylvania.

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Corresponding author: David F. Woodward, Department of Biological Sciences (RD2–2C), Allergan, Inc., 2525 Dupont Drive, Irvine, CA 92612-1599; woodward_david@allergan.com.

such an agent would treat the disease closer to its origin.[28–31] Other potential mechanisms also are possible, given the wide ocular distribution of EP_4 receptors.[24] With this background information, it was considered worthwhile to study the ocular hypotensive activity and related mechanisms by using the first selective EP_4 receptor agonist 3,7-dithia PGE_1.[12]

METHODS

The experiments adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the animal care and use committees of each institution.

Intraocular Pressure

IOP studies were performed in conscious female cynomolgus monkeys (*Macaca fascicularis*) weighing 2 to 4 kg. All treatments were masked for the experimenters. Cynomolgus monkeys trained to accept pneumatonometry were restrained in chairs custom designed for the procedure. Ocular normotensive monkeys and monkeys rendered unilaterally ocular hypertensive by circumferential argon laser treatment of the trabecular meshwork were used. IOP was measured by means of an applanation pneumatonometer. One minute before pneumatonometry, 25 μL of proparacaine was applied to minimize ocular discomfort during the measurement. Determination of the effects of drugs on IOP involved administration of 25 μL of drug solution (3,7-dithia PGE_1 and its isopropyl ester, 0.01%) to one eye (lasered eye in the case of glaucomatous monkeys) and an equal volume of vehicle (sterile saline with 1% polysorbate 80, 5 mM Tris-HCl) to the contralateral, normotensive eye as a control. IOP was measured at 1 hour before drug administration, just before drug administration, and at 2, 4, and 6 hours thereafter.

Aqueous Humor Dynamics

Cynomolgus monkeys of both sexes, approximately 2 to 8 years of age and weighing between 2.2 and 4 kg, were used. For aqueous flow studies, ocular normotensive monkeys were treated bilaterally with a single dose of study drug on one day and vehicle on a separate day. For uveoscleral outflow studies, ocular normotensive monkeys were treated once daily for 5 days with 25 μL of drug solution administered to one eye and 25 μL of vehicle administered to the contralateral eye. For total outflow facility studies, ocular normotensive monkeys were given a single morning dose of study drug in one eye and vehicle in the contralateral eye. For tonography, both eyes were treated once a day for 4 days. Between 4 and 6 hours after drug administration, the animals were anesthetized and measurements were made as described in the next section.

Total Outflow Facility: Two-Level, Constant-Pressure Method. Monkeys were injected intramuscularly with a mixture of ketamine (100 mg/mL) 0.15 mL/kg body weight and diazepam (5 mg/mL) 0.3 mL/kg body weight, to provide a deep level of anesthesia and an absence of eye movements. Supplemental doses of ketamine (0.075 mL/kg body weight) were given at 30-minute intervals during the course of the experiment, and additional doses of diazepam (0.15 mL/kg body weight) were given at 60-minute intervals. Vital signs (i.e., heart rate and body temperature) were continuously monitored.

After a stable and deep level of anesthesia had been achieved, IOP was determined with a calibrated pressure transducer system. Total outflow facility was then determined by the two-level, constant-pressure method described by Bárány.[32] Briefly, the anterior chamber was connected through a 25-gauge stainless-steel needle to a fluid reservoir containing a modified mock aqueous humor solution.[33] In this system, the actual IOP is dependent on the hydrostatic pressure of the solution in the reservoir and can be adjusted as necessary. The pressure in the eye was changed at 10-minute intervals between two different pressure levels approximately 2.5 (p_1) and 10 mm Hg (p_2) above the real IOP level, which, in this context, describes the IOP that would be present in the eye if the pressure were not artificially maintained by means of a reservoir. Keeping the artificial pressure above real IOP ensures the flow of fluid from the reservoir. For each 10-minute measurement period at p_1 or p_2, the flow rate of fluid from the reservoir was determined as F_1 or F_2, respectively. Flow rates were calculated as $F = \Delta W/t$ with ΔW representing the weight difference of the reservoir between the beginning and end of the measurement period and t representing the duration of the period. Total outflow facility C could then be calculated with the equation

$$C = (F_2 - F_1)/(p_2 - p_1)$$

Total outflow facility in each eye was averaged over five measurement periods. The outflow facility studies were performed twice in the same animals approximately 4 months apart, at baseline, and after treatment.

Tonography. Eight female cynomolgus monkeys with laser-induced glaucoma in one eye were used in this crossover experiment. Baseline IOPs and administration of drug or vehicle were performed in conscious trained animals. Doses of 25 μL of either drug or vehicle were administered once daily at 9:00 AM for 4 days. Four hours after the last dose, the animals were sedated with ketamine and a 2-minute tonography measurement was performed in each eye with the tonography mode of a pneumatonometer (Reichert, Depew, NY). Data were stored on a computer (PowerLab software; ADInstruments, Colorado Springs, CO). Calculations were performed in commercial software (Excel; Microsoft, Redmond, WA). One week later, the animals were given the alternate solution, and the tonography was repeated.

Uveoscleral Outflow. To determine the effect on uveoscleral outflow, we used the anterior chamber perfusion method originally developed by Bill[34] and subsequently modified by Toris et al.,[35] using a fluorescein-tagged tracer. This method employs the principle of determining the amount of tracer in ocular tissues after perfusing the anterior chamber with a solution containing a high-molecular-weight tracer. Each eye was cannulated with three 25-gauge needles, two of which were connected to 5-mL gas-tight syringes mounted in a reciprocal syringe pump (Harvard Apparatus, Holliston, MA), the third was connected to an open fluid reservoir with an in-line pressure transducer, to record IOP. A reciprocal pump (Harvard Apparatus) allowed perfusion of the anterior chamber at a predetermined flow rate without affecting IOP. Both eyes were perfused for 30 minutes with a mock aqueous humor solution[33] containing 0.7% (1×10^{-4} M) FITC-dextran 70,000 as a tracer. To provide for a quick exchange of the anterior chamber content with the tracer, the eyes were perfused for the first 5 minutes at a rate of 0.2 mL/min and then during the remaining 25 minutes at a rate of 0.05 mL/min. The perfusates from those two perfusion periods were collected separately as primary and secondary perfusates, respectively. The IOP in both eyes was clamped during the anterior chamber perfusion at approximately 15 mm Hg, via the open reservoir. At the conclusion of the 30-minute perfusion period the animal was euthanatized with an overdose of pentobarbital sodium (2 mL; Eutha-6 CII; Wester Medical, Santa Ana, CA). Both eyes were then perfused for approximately 10 minutes with mock aqueous humor solution, without tracer, to wash the tracer out of the anterior chamber. The eyes were then enucleated and immediately dissected into anterior sclera, posterior sclera (posterior to ora serrata), extraocular tissues, ciliary body, choroid, and retina, and vitreous humor and other fluids including wash fluid were collected. The cornea, lens, and iris were excluded from the measurements because these tissues are not thought to contribute to uveoscleral outflow.

Each tissue was homogenized in Dulbecco's phosphate-buffered saline (D-PBS) and centrifuged. The FITC-dextran concentration in the supernatant was determined with a spectrometer (LS-50B; Perkin-Elmer, Wellesley, MA) with excitation and emission wavelengths of 493 and 515 nm, respectively, and 5.0 nm excitation and emission slits. Total uveoscleral outflow (F_u) was calculated with the equation

$$F_u = \sum(\text{tissue-FITC}) \times (\text{perfusate-FITC})^{-1} \times \text{time}^{-1}$$

TABLE 1. In Vitro Pharmacology of 3,7-Dithia PGE_1 at Human Recombinant Prostanoid Receptors

hDP	hEP_1	hEP_2	hEP_3	hEP_4	hFP	hIP	hTP
>10,000	304	380	36	0.26	>10,000	>10,000	561

$[Ca^{2+}]_i$ as measured by FLIPR. Data are EC_{50} (nM); $n = 6$.

where Σ(tissue-FITC) represents the total amount of FITC-dextran present in all ocular tissues, perfusate-FITC represents the mean FITC-dextran concentration in the anterior chamber during the perfusion (secondary perfusate), and time represents the duration of the perfusion.

Aqueous Flow. Female cynomolgus monkeys between the ages of 3 and 8 years were included in the study. Sedation was produced by ketamine hydrochloride, 1 to 5 mg/kg, administered by intramuscular or intravenous injection to achieve pharmacologic restraint. Animals were seated for tonometry and pachymetry measurements and prone for fluorophotometry measurements. Corneal thickness and anterior chamber depth were measured by ultrasound pachymetry (Sonomed, Inc., Lake Success, NY) and corneal diameter was measured with a ruler. Cornea and anterior chamber volumes were calculated from these values.[36] Next, fluorescein (10% in 2% agar gel) was applied to the cornea using iontophoresis for various lengths of time. One 25-μL drop of vehicle was applied to each eye at approximately 9 AM. The fluorescence of the cornea and anterior chamber were measured with a scanning ocular fluorophotometer (Fluorotron; OcuMetrics, Palo Alto, CA). Scans were taken in duplicate at 60-minute intervals for four sets. These data were used to determine aqueous flow (F_a).[36] F_a measurements were repeated 3 days later, 4 hours after a topical 25-μL drop of drug.

Statistical Analyses

Comparisons were made by Student's two-tailed *t*-test for paired observations. Differences were assumed to be statistically significant at $P < 0.05$.

Isolated Ciliary Muscle

Ciliary smooth muscle specimens were surgically excised from enucleated human eyes (obtained from the National Disease Research Interchange, Philadelphia, PA), in accordance with the guidelines of the Declaration of Helsinki for research involving human tissue, and monkey eyes and were set up as either longitudinal or radial (circular) preparations in 15-mL organ baths containing Krebs buffer, 10^{-6} M indomethacin, and gassed with 95% O_2/5% CO_2. Longitudinal ciliary muscle preparations were tied securely at each end by thread and then mounted in organ baths under a final tension of 200 mg. Circular ciliary muscle preparations were very short (~2 mm) and were therefore mounted with custom-made microclamps and placed under 80 mg tension. Responses were recorded isometrically on a physiograph (Grass; Astro-Med, West Warwick, RI). After a 30-minute equilibration period, each ciliary muscle preparation was precontracted to approximately 50% maximum by carbachol to enable any relaxant responses to readily occur. 3,7-dithia PGE_1, butaprost, or PGE_2 were then applied cumulatively in 10-fold increments up to 10^{-5} M. Finally, atropine 10^{-7} M was applied at the end of the experiment and responses to 3,7-dithia PGE_1, butaprost, and PGE_2 were calculated as percentage relaxant response to atropine.

Human Recombinant Receptor Studies

The use of chimeric G protein cDNAs allowed responses to G_s- and G_i-coupled prostanoid receptors to be measured as a Ca^{2+} signal, as previously described.[37] Prostanoid DP, EP_2, and EP_4 receptor cDNAs were cotransfected with chimeric G_{qs} cDNA containing a hemagglutinin (HA) epitope. The prostanoid EP_3 receptor was cotransfected into HEK-293 EBNA cells, using $pCEP_4$ as a vector, with chimeric G_{qi}-HA. G_{qs} and G_{qi} chimeric cDNAs (Molecular Devices, Sunnyvale, CA) were cloned into a $pCEP_4$ vector and also selected by using a hygromycin B selection marker. Transfection into HEK-293 EBNA cells was then performed by a commercial method (FuGENE 6; Roche, Indianapolis, IN). Because G_{qs} and G_{qi} contained an HA epitope, protein expression was detected by Western blot analysis with anti-mouse HA monoclonal antibody and horseradish peroxidase (HRP)–conjugated secondary antibody. For the human recombinant EP_1, FP, IP, and TP receptors, stable transfectants were obtained as previously described.[37] Briefly, $pCEP_4$ was used as the expression vector, and transfection into HEK-293-EBNA cells was performed (FuGENE 6; Roche). Stable transfectants were again selected according to hygromycin resistance.

Ca^{2+} signaling studies were performed with a FLIPR (fluorometric imaging plate reader). The cells were seeded at a density of 5×10^4 cells/well in poly-D-lysine–coated, black walled, clear-bottomed 96-well plates (Biocoat; BD Biosciences, Franklin Lakes, NJ) and allowed to attach overnight in an incubator at 37°C. The cells were then washed twice with HBSS-HEPES buffer (Hanks' balanced salt solution without bicarbonate and phenol red, 20 mM HEPES; pH 7.4) using a plate washer (Denley Cellwash; Labsystems, Franklin, MA). After 45 to 60 minutes of dye-loading in the dark using the Ca^{2+}-sensitive dye Fluo-4AM, at a final concentration of 2×10^{-6} M, the plates were washed four times with HBSS-HEPES buffer to remove excess dye and leaving 100 μL of buffer in each well. The plates were then placed in the FLIPR instrument and allowed to equilibrate at 37°C. Compound solutions were added in a 50-μL volume to each well to give the desired final concentration. The cells were excited with an argon laser

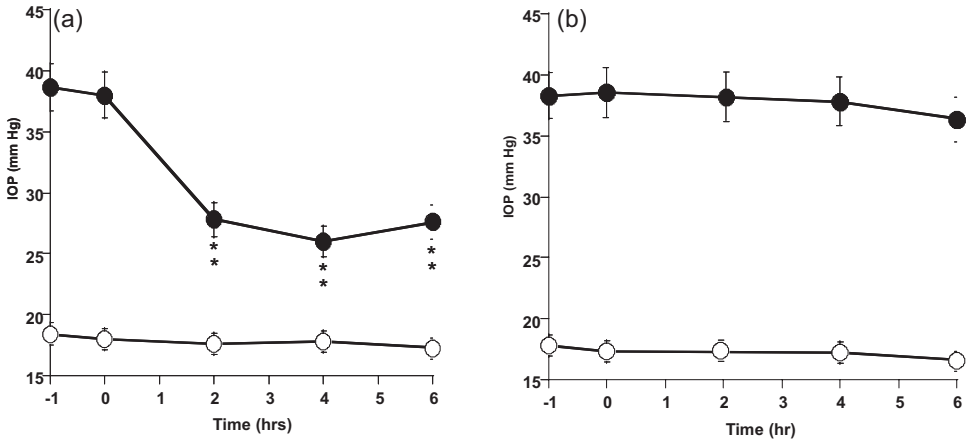

FIGURE 1. The effects of (**a**) 3,7-dithia PGE_1 (0.1%) and (**b**) saline on the IOP of laser-induced, ocular hypertensive monkeys. IOP is shown in (●) ocular hypertensive and (○) the untreated, normal control eyes. Mean ± SEM; $n = 6$. $^{**}P < 0.01$, decrease relative to baseline.

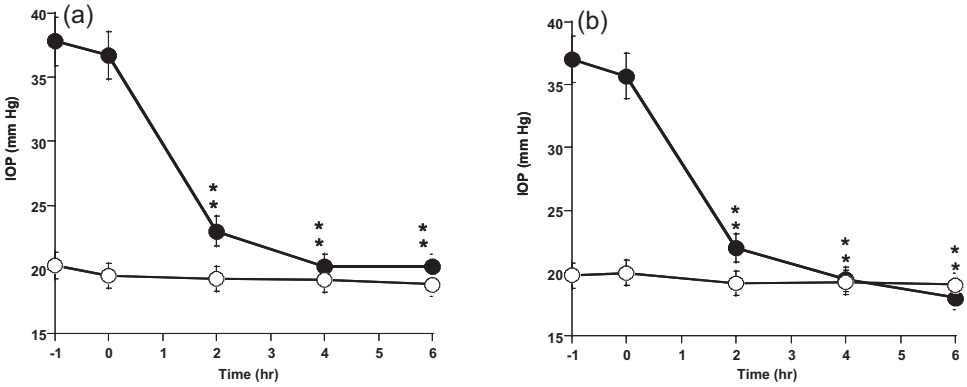

FIGURE 2. The effects of 3,7-dithia PGE_1-isopropyl ester on the IOP of laser-induced, ocular hypertensive monkeys. The effects of (**a**) 0.01% and (**b**) 0.1% doses on IOP is shown in (●) drug-treated, ocular hypertensive and (○) untreated, normal, control eyes. Mean ± SEM; $n = 6$. $^{**}P < 0.01$, decrease relative to baseline.

at 488 nm, and emission was measured through a 510- to 570-nm band width emission filter (FLIPR; Molecular Devices). The peak increase in fluorescence intensity was recorded for each well. On each plate, four wells each served as negative (HBSS-HEPES buffer) and positive controls (standard agonists: DP, BW 245C; EP_1-EP_4, PGE_2; FP, $PGF_{2\alpha}$; IP, carbaprostacyclin; and TP, U-46,619). The peak fluorescence change in each well containing drug was expressed relative to the controls. To obtain concentration–response curves, we tested compounds in duplicate in each plate over the desired concentration range. Each compound was tested on at least three separate plates, using cells from different passages to give $n = 3$.

Materials

3,7-dithia PGE_1 and its isopropyl ester were synthesized by and purchased from Target Molecules (Southhampton, UK), according to published methods.[12] PGE_2 and butaprost-free acid were purchased from Cayman Chemicals (Kalamazoo, MI). Krebs buffer composition was NaCl 118 × 10^{-3} M, KCl 4.7 × 10^{-3} M, KH_2PO_4 1.2 × 10^{-3} M, $CaCl_2.2H_2O$ 1.9 × 10^{-3} M, $MgSO_4$ 1.18 × 10^{-3} M, $NaHCO_3$ 25 × 10^{-3} M, and glucose 11.7 × 10^{-3} M (pH adjusted to 7.4).

RESULTS

The Ca^{2+} signaling studies, using stable human prostanoid receptor stable transfectants, confirmed the original radioligand binding studies that reported 3,7-dithia PGE_1 as a selective EP_4 agonist.[12] It was >100-fold selective for EP_4 than for EP_3 and the relative selectivity at other receptors was 1000-fold or more (Table 1).

The effects of 3,7-dithia PGE_1 on the IOP of laser-induced, ocular hypertensive monkeys are depicted graphically in Figure 1. A 0.1% dose of 3,7-dithia PGE_1 produced a decrease in IOP of more than 10 mm Hg (Fig. 1a). Vehicle produced no significant IOP effect (Fig. 1b). The ocular hypotensive efficacy in monkeys was improved by esterification of the carboxylic acid of 3,7-dithia PGE_1. Thus, the isopropyl ester derivative, at both 0.01% and 0.1% doses, caused more than a 15-mm Hg (40%–50%) decrease in IOP (Fig. 2). The IOP of the ocular hypertensive eye was essentially reduced to that of the nonlasered, vehicle-treated control eye. No effect on pupil diameter was noted.

The effects of 3,7-dithia PGE_1 isopropyl ester on the IOP of normotensive monkey eyes is shown in Figure 3. Both a 0.01% (Fig. 3a) and 0.1% (Fig. 3b) dose produced a decrease in IOP of 2 to 3 mm Hg.

The effects of 0.01% 3,7-dithia PGE_1 isopropyl ester on total outflow facility (μl/min/mm Hg) are summarized in Table 2. A 35% increase in pressure-dependent outflow was achieved at 4 to 6 hours after treatment. $P < 0.05$ was obtained when the ratio of treated over control eye outflow facility (1.34 ± 0.15) was compared against the difference between eyes before treatment (ratio, 1.04 ± 0.05).

Tonography studies revealed that outflow facility was significantly less in ocular hypertensive (OHT) eyes than in ocular normotensive (ONT) eyes treated with vehicle ($P = 0.003$), a result to be expected in a laser-induced glaucoma model. When drug was administered, outflow facility was not different between the two eyes, which suggests that outflow facility in the lasered eyes recovered to some extent. The EP_4 agonist significantly ($P = 0.04$) increased outflow facility in OHT but not ONT eyes (Table 3).

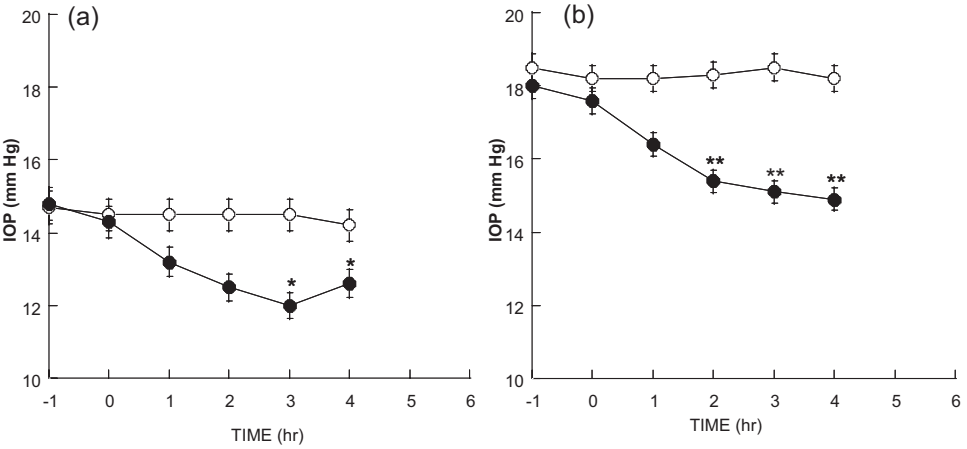

FIGURE 3. The effects of 0.01% (**a**) and 0.1% (**b**) 3,7-dithia PGE_1 isopropyl ester on the IOP of ocular normotensive monkeys. IOP is shown in (●) drug-treated and (○) vehicle-treated eyes. Mean ± SEM; $n = 6$. $^{*}P < 0.05$, $^{**}P < 0.01$, decrease relative to baseline.

TABLE 2. The Effect of 3,7-Dithia PGE_1 Isopropyl Ester (0.01%) on Total Outflow Facility

	Baseline	Treatment
Treated eye	0.71 ± 0.12	0.55 ± 0.09
Control eye	0.69 ± 0.11	0.43 ± 0.07
Ratio of treated to control	1.04 ± 0.05	1.34 ± 0.15*

Outflow facility was measured by two-level, constant-pressure perfusion in ocular normotensive cynomolgus monkeys. Data are the mean total outflow facility (μl/min/mmHg) ± SEM; $n = 6$.

* $P < 0.05$.

The EP_4 agonist produced no significant difference in uveoscleral outflow compared with that in vehicle-treated control eyes (Table 4) and did not alter aqueous flow in normotensive eyes compared with that at baseline (Table 5).

The effects of graded doses of 3,7-dithia PGE_1, butaprost, and PGE_2 relative to a single dose of atropine on longitudinal and radial preparations of the human ciliary muscle are depicted in Figure 4. 3,7-dithia PGE_1 produced virtually no relaxation of the precontracted longitudinal (Fig. 4a) or radial (Fig. 4b) human ciliary muscle, even when a 10^{-5}-M concentration was reached, which is far in excess of that necessary to fully activate EP_4 receptors. Butaprost-free acid and PGE_2 were more active (Figs. 4c–f) and the magnitude of the relaxant responses to PGE_2 was comparable to those previously reported for human ciliary muscle for relaxation measured in milligrams.[38] In marked contrast, a single 10^{-7} concentration of atropine reversed carbachol-induced contraction of both longitudinal and radial ciliary muscle preparations (see Fig. 6). Given the modest relaxant effects of PGE_2 compared with that of atropine, it was considered preferable to measure relaxant responses as a percentage of atropine response. Similar results were obtained in the isolated monkey ciliary muscle (Fig. 5), although fewer tissues were examined, as monkey eyes rarely became available for study. The EP_4 agonist 3,7-dithia PGE_1 produced no meaningful relaxation of longitudinal (Fig 5a) or radial (Fig. 5b) monkey ciliary muscle preparations. Butaprost-free acid had little effect on longitudinal monkey ciliary smooth muscle (Fig. 5c) but appeared to produce a greater effect in radial preparations (Fig. 5d). PGE_2 also produced a greater effect in radial ciliary muscle preparations (Figs. 5e, 5f). Atropine produced a pronounced relaxation of ciliary muscle specimens precontracted with carbachol, thereby again indicating that the ciliary muscle preparations were viable.

Actual traces of monkey longitudinal (Fig. 6a), human longitudinal (Fig. 6b), monkey radial (Fig. 6c), and human radial (Fig. 6d) experiments are provided in Figure 6. It can be seen that 3,7-dithia PGE_1 produced no meaningful relaxant effect, whereas atropine substantially reversed carbachol-induced contraction in all cases.

TABLE 3. The Effects of 3,7-Dithia PGE_1 Isopropyl Ester (0.01%) on Outflow Facility Measured by Tonography in Cynomolgus Monkeys with Laser-Induced Hypertension in One Eye (OHT) and Normotension in the Contralateral Eye (ONT)

	ONT	OHT	*P**
ONT	0.31 ± 0.12	0.21 ± 0.08	0.34
OHT	0.04 ± 0.01	0.13 ± 0.04	0.04
P†	0.003	0.29	

Data are the mean tonographic outflow facility (μL/min/mm Hg) ± SEM; $n = 8$.

* Vehicle treatment versus drug treatment.

† ONT versus OHT.

TABLE 4. The Effect of 3,7-Dithia PGE_1 Isopropyl Ester (0.01%) on Uveoscleral Outflow

Eye	Outflow
Treated eye	0.52 ± 0.16
Control eye	0.45 ± 0.12

Outflow was measured by an intracameral tracer method in ocular normotensive cynomolgus monkeys. Data are the mean uveoscleral outflow (μL/min) ± SEM; $n = 6$.

DISCUSSION

The selective prostanoid EP_4 receptor agonist, 3,7-dithia PGE_1 and its isopropyl ester were found to possess clear ocular hypotensive activity in normal and ocular hypertensive monkeys. In laser-induced ocular hypertensive monkeys, a single 0.01% or 0.1% dose of 3,7-dithia PGE_1 isopropyl ester reduced IOP to the level of that measured in the untreated, ocular normotensive control eyes. Simply stated, 3,7-dithia PGE_1 isopropyl ester normalized elevated IOP. The IOP results obtained with 3,7-dithia PGE_1 and its isopropyl ester were pursued further by studying the underlying aqueous humor dynamics.

The effects of 3,7-dithia PGE_1 isopropyl ester on IOP in normal and glaucomatous monkeys remained very evident at the termination of the studies. This ocular hypotensive activity profile could be viewed as consistent with long-term effects on aqueous humor dynamics—perhaps the ultrastructural changes that provide the morphologic basis of increased uveoscleral outflow.[6] Contrary to expectations and unlike the prostanoid DP,[7] the FP[8,10,11] and EP_2[6,8] agonists, and a prostamide,[8] the selective EP_4 agonist 3,7-dithia PGE_1 exhibited no significant effect on uveoscleral outflow.

In addition to direct measurement of uveoscleral outflow, EP_4 receptor–mediated regulation of IOP and uveoscleral outflow was investigated indirectly by studying its effects on ciliary muscle tone. It has often been suggested that relaxation or inhibition of ciliary muscle contraction may result in increased uveoscleral outflow, thus reducing IOP.[39] Studies in nonhuman primates have directly demonstrated that agents that relax ciliary smooth muscle may result in an increase in uveoscleral outflow.[39,40] To date PGs, atropine,[39–44] and inhibitors of Rho-associated coiled coil-forming protein kinase (ROCK) have been proposed to increase uveoscleral outflow with the potential for lowering IOP. We therefore elected to study the effects of 3,7-dithia PGE_1, PGE_2, butaprost, and atropine on the tone of carbachol precontracted human and monkey ciliary muscle. The longitudinal preparation has been suggested to more closely model the outflow route via the ciliary muscle,[43,44] but we also examined specimens prepared in the radial orientation. Although there are ultrastructural, histo-

TABLE 5. The Effect of 0.01% 3,7-Dithia PGE_1 Isopropyl Ester on Aqueous Humor Outflow

Test Group	Flow Rate
Vehicle	2.50 ± 0.44
Drug	2.39 ± 0.26

Outflow was measured by fluorophotometry in ocular normotensive monkey eyes. No significant drug effects were obtained, according to Students' two-tailed, paired *t*-test ($P = 0.9$). Data are the mean aqueous humor flow (μL/min) ± SEM. $n = 5–8$.

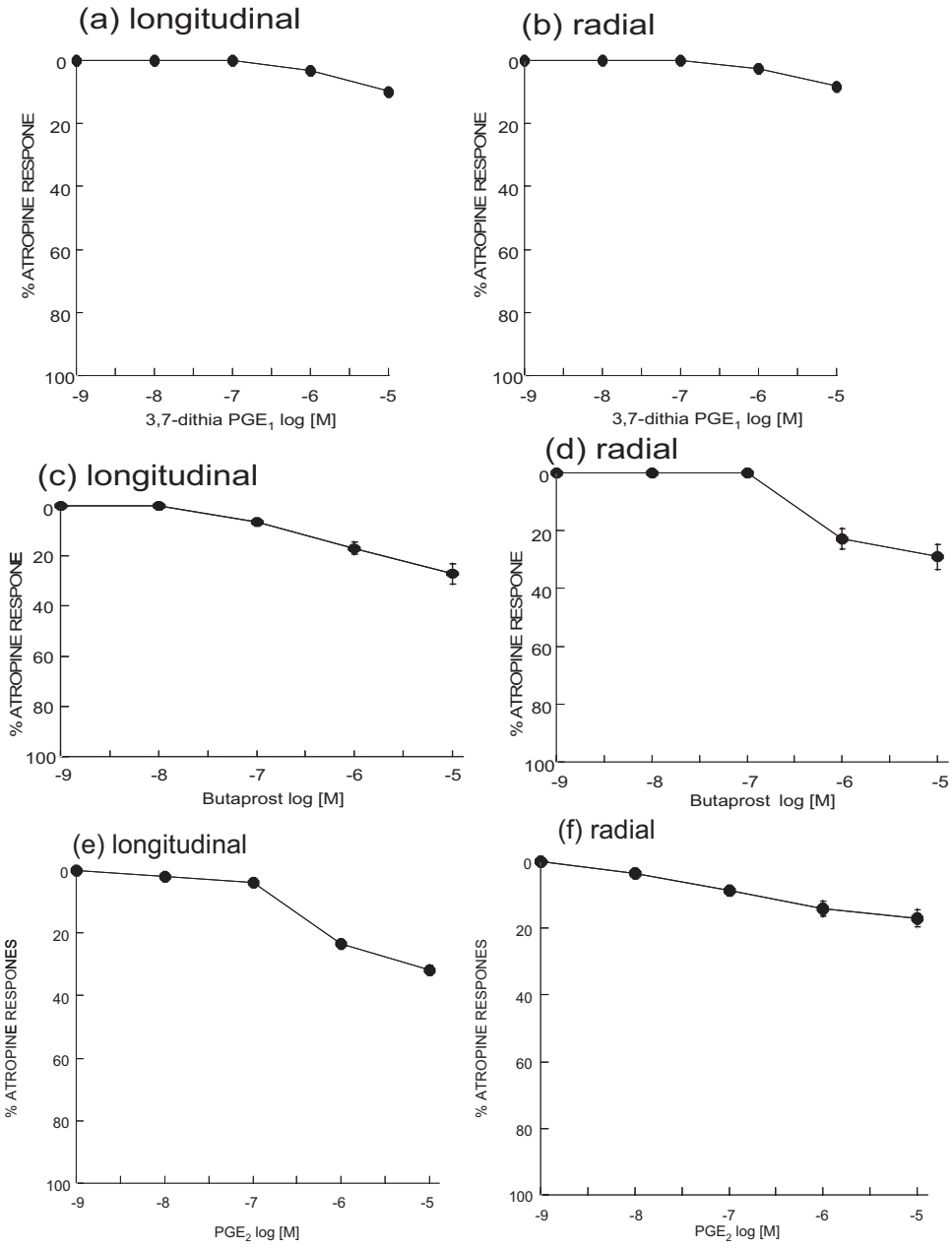

FIGURE 4. Comparison of the effects of graded doses of 3,7-dithia PGE_1, butaprost, PGE_2, and 10^{-7} M atropine on precontracted longitudinal and radial human ciliary smooth muscle preparations. 3,7-dithia PGE_1, (**a**) longitudinal, (**b**) radial; butaprost, (**c**) longitudinal, (**d**) radial; and PGE_2, (**e**) longitudinal, (**f**) radial. Mean ± SEM, $n = 6$.

chemical, and functional distinctions between longitudinal and circular ciliary muscle preparations,[43–45] responses to carbachol, PGE_2, butaprost, and atropine were similar. Atropine caused a pronounced relaxation of both circular and longitudinal ciliary smooth muscle, which is consistent with its reported effects on uveoscleral outflow.[39,40] In contrast, 3,7-dithia PGE_1 produced very little ciliary smooth muscle relaxation, less than that reported for $PGF_{2\alpha}$ relaxation of rhesus monkey ciliary muscle.[44] This virtual lack of relaxant effect apparent for 3,7-dithia PGE_1 further substantiates the finding that it does not alter uveoscleral outflow.

Although the selective EP_4 agonist 3,7-dithia PGE_1 exhibited no significant effect on uveoscleral outflow, it did produce a significant 35% increase in total outflow facility, as measured by the two-level, constant-pressure method. This increase in outflow facility also was found in ocular hypertensive eyes according to tonographic studies, but not in normotensive eyes. This difference may reflect differences in the methodology and associated sensitivity. Taken together, they suggest that 3,7-dithia PGE_1 isopropyl ester lowers IOP by increasing trabecular outflow facility. Absence of effects on uveoscleral outflow and aqueous humor formation indirectly support this contention. These results set EP_4 agonist effects apart from those of prostanoid DP, EP_2, and FP agonists and prostamides, where increased uveoscleral outflow is the predominant mechanism of action. The EP_4 agonist increased total outflow facility, presumed largely to be trabecular outflow facility. A marked decrease in episcleral venous pressure could contribute to the ocular hypotensive effects of 3,7-dithia PGE_1. If episcleral venous dilatation were to occur, it would be a contributory factor in ocular surface hyperemia. However, no pronounced ocular surface hyperemic response to 3,7-dithia PGE_1 isopropyl ester was noted that was contemporaneous with the IOP readings in conscious monkeys.

Considering the effects on aqueous humor secretion, prostaglandins tend to increase aqueous humor inflow.[46] 3,7-dithia

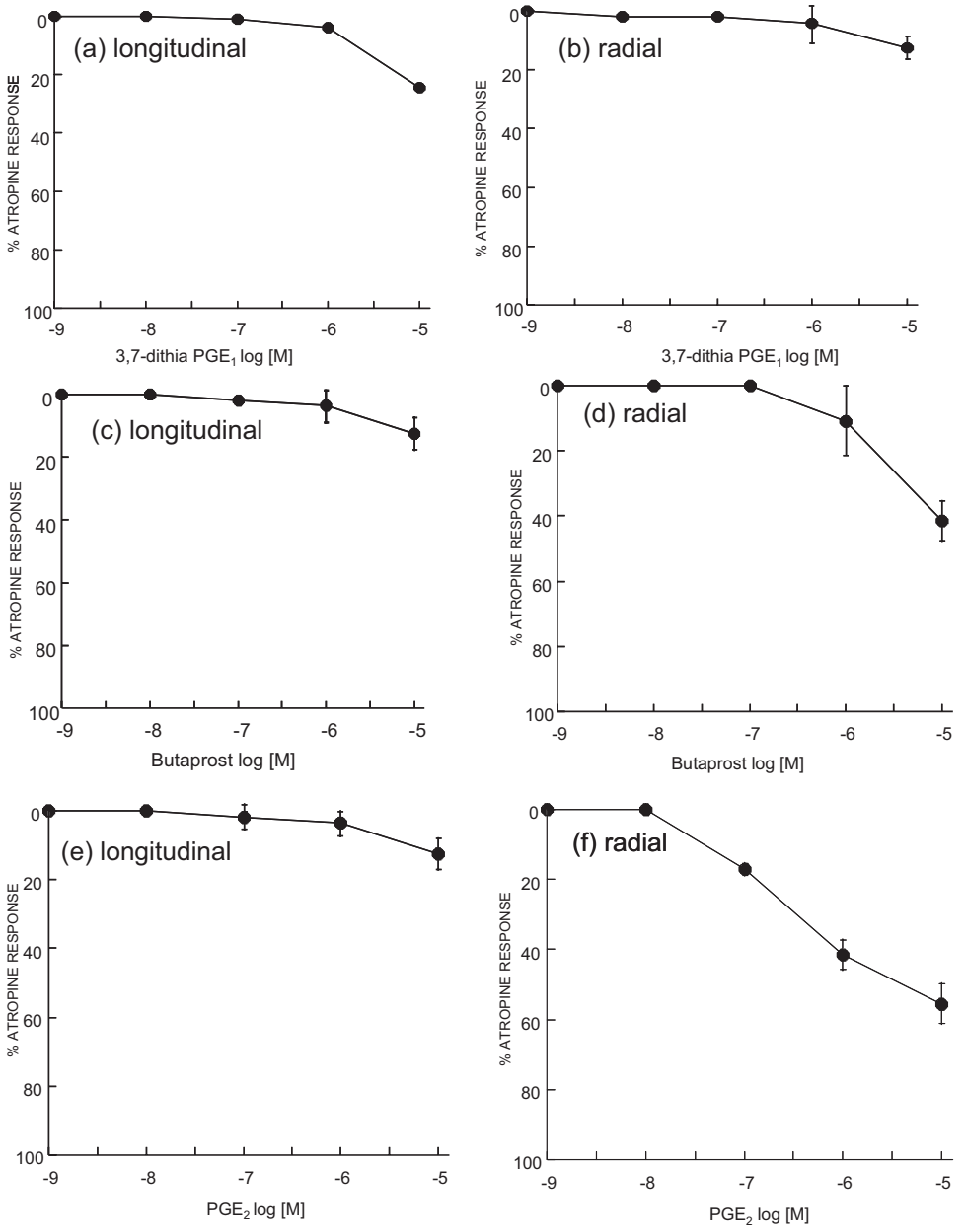

FIGURE 5. Comparison of the effects of graded doses of 3,7-dithia PGE_1, butaprost, PGE_2, and 10^{-7} M atropine on precontracted longitudinal and radial monkey ciliary smooth muscle preparations. Mean ± SEM; 3,7-dithia PGE_1 longitudinal (**a**) $n = 3$, radial (**b**) $n = 3$; butaprost longitudinal (**c**) $n = 3$, radial (**d**) $n = 2$; PGE_2 longitudinal (**e**) $n = 3$, radial (**f**) $n = 2$.

PGE_1 did not alter aqueous humor flow, measured fluorophotometrically, and it appears that decreased aqueous humor production is not a contributory factor to its effect on IOP.

We were interested in knowing whether ciliary muscle relaxation may represent a first phase of increased uveoscleral outflow, followed by a second phase that involves remodeling and the creation of new outflow channels. Within the scope of these present studies, we examined the effects of both 3,7-dithia PGE_1 and the EP_2 agonist butaprost on ciliary muscle tone. It is already known that EP_2 agonists lower IOP by increasing uveoscleral outflow exclusively.[6] Butaprost, like 3,7-dithia PGE_1, produces a rapid reduction in IOP that almost certainly precedes initiation of ciliary muscle remodeling to form new and widened outflow channels. The results of this present study suggest that ciliary muscle relaxation had minimal influence on IOP. 3,7-dithia PGE_1 caused no meaningful relaxation of ciliary smooth muscle. Butaprost did relax longitudinal and radial ciliary smooth muscle slightly more than 3,7-dithia PGE_1, but much less than atropine. These results may even be taken to suggest that butaprost causes a rapid-onset ocular hypotensive response that is not readily explained by current aqueous humor dynamics concepts.

In summary, the prototypical EP_4 receptor–selective agonist 3,7-dithia PGE_1 was found to be very efficacious in lowering IOP in cynomolgus monkeys. Unlike previously described receptor selective prostanoid analogues (FP, EP_2, DP) and the prostamide bimatoprost,[7–11] 3,7-dithia PGE_1 did not appear to exert a meaningful effect on uveoscleral outflow but increased trabecular outflow facility. Considering second-messenger pathways beyond G protein coupling and gene regulation, regulated by EP_2 and EP_4 receptors, it is not entirely unexpected that EP_4 agonist effects on aqueous humor dynamics exhibit a separate identity. For example, T-cell factor (Tcf) signaling also involves phosphatidyl inositol 3-kinase (PI3K) in the case of EP_4 receptors.[47] Further, EP_4 receptors, but not EP_2 receptors, can activate extracellular signal-regulated kinase (ERK)-1 and -2 via a PI3K pathway, to induce early growth response factor (ERG)-1.[48] The EP_4 receptor may therefore

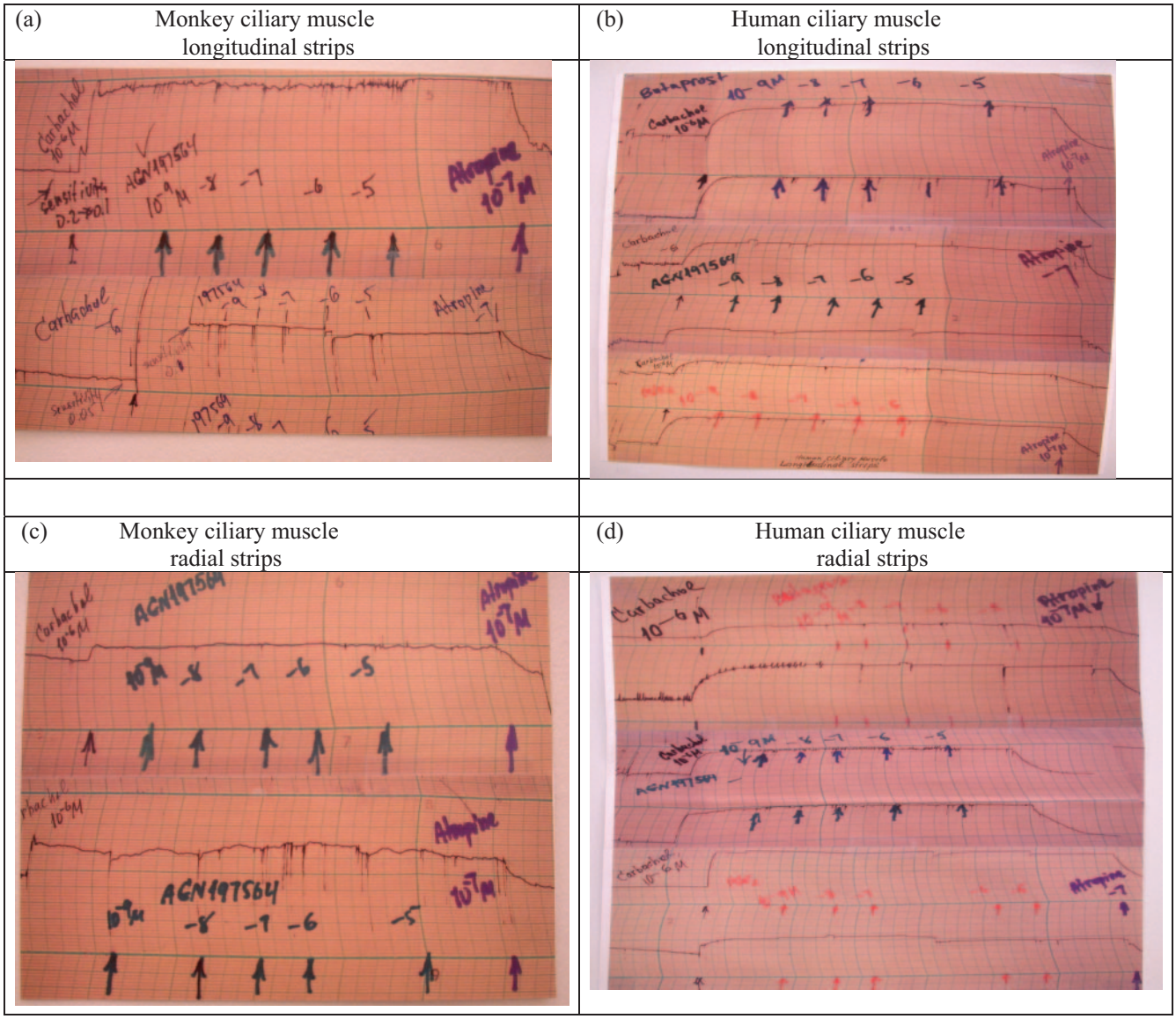

FIGURE 6. Example traces showing the sequential effect of carbachol, 3,7-dithia PGE_1 (AGN 197564), and atropine on ciliary smooth muscle preparations as follows (**a**) monkey longitudinal, (**b**) human longitudinal, (**c**) monkey radial, and (**d**) human radial.

provide a new focus for antiglaucoma drug design and perhaps provide a future new drug tool for the study of aqueous humor outflow mechanisms.

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