Indomethacin and Epinephrine Effects on Outflow Facility and Cyclic Adenosine Monophosphate Formation in Monkeys

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Purpose. To investigate the effect of indomethacin inhibition of prostanoid production on the epinephrine-stimulated increase in outflow facility and cyclic adenosine monophosphate (cAMP) production in the anterior segment of the monkey eye.

Methods. Topical indomethacin was given 1 hour before the intracameral administration of epinephrine to living cynomolgus monkeys. Outflow facility was measured for 45 to 60 minutes, beginning 3 hours after epinephrine administration, by two-level constant pressure perfusion of the anterior chamber. Cyclic adenosine monophosphate formation was measured in cell membranes isolated from rhesus monkey ciliary muscle, ciliary processes, trabecular meshwork, and iris in the presence of forskolin, indomethacin, epinephrine, or indomethacin and epinephrine combined.

Results. Three hours after the intracameral administration of 5.5 μg epinephrine, facility increased by ~40%, a putatively maximal response, at which time the intracameral epinephrine concentration was ~15 μM. Pretreatment with topical indomethacin produced a dose-dependent inhibition of epinephrine’s facility-increasing effect; the maximum inhibition of 50% to 70% occurred at an indomethacin dose of 50 to 125 μg. Doubling the indomethacin dose (250 μg) produced no further inhibition, whereas a fivefold larger epinephrine dose (27.5 μg) did not overcome the inhibition. Forskolin and epinephrine both stimulated cAMP production in vitro, whereas [indomethacin] 10^-4 M partially inhibited both basal and epinephrine-stimulated cAMP production in all four tissues.

Conclusions. Approximately half of the epinephrine-induced facility increase is inhibited by indomethacin, but it is unclear whether the indomethacin-inhibitable fraction is mediated by epinephrine-stimulated prostanooid production or release.

Topical epinephrine (EPI), or its dipivylated prodrug, is used commonly to lower intraocular pressure (IOP) in the management of glaucoma.6-9 However, controversy remains as to the exact mechanism of action and the cell type(s) modulating the IOP-lowering effect.6-8 Epinephrine affects three major parameters of aqueous humor dynamics: aqueous production,6-11 trabecular outflow,10,12-15 and uveoscleral outflow.15 Adrenergic α and β receptors have been identified and characterized in the tissues of the anterior segment of the eye by ligand binding16,17 and, more recently, by in situ hybridization and autoradiography.18-20 Because ligand-receptor binding for both β and α-adrenergic receptors is known in many systems to affect adenylate cyclase activity, the production or inhibition of production of the second-messenger cyclic adenosine monophosphate (cAMP) has been implicated in the regulation of outflow facility, aqueous humor production, and ciliary muscle contraction or relaxation and, therefore, may serve as a mediator

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of changes in intraocular pressure. Additionally, forskolin (FSK) and its analogues, which are direct stimulators of adenylylate cyclase, as well as of cAMP itself, lower IOP in a number of species. In the rabbit, a relationship between IOP lowering and stimulation of adenylylate cyclase by adrenergic agonists and FSK analogues was observed. Various studies have suggested that the increase in trabecular outflow facility in response to EPI may be caused by a β-adrenergic receptor–cAMP-mediated mechanism located in the meshwork and/or canal endothelium.

The IOP-lowering effects of several drugs that stimulate cAMP formation, including EPI and FSK, are inhibited by the systemic administration of indomethacin (INDO) or the topical administration of flurbiprofen, both of which block cyclooxygenase and thus inhibit prostaglandin (PG) synthesis from arachidonic acid. In addition to inhibiting cyclooxygenase, these anti-inflammatory agents can have nonspecific effects, including inhibition of phosphodies-terase, cAMP-dependent protein kinase, and calcium flux across membranes. However, because certain PGs themselves can reduce IOP, because topical application of EPI stimulates PG synthesis in the eye, and because systemic or topical INDO can partially block the epinephrine-induced IOP decrease, some investigators have concluded that the ocular hypertensive effect of EPI is mediated, at least in part, by the local synthesis of PGs from endogenous precursors.

In the current study, we sought to determine whether topical INDO would affect EPI’s ability to increase outflow facility, measured by two-level constant pressure perfusion in the subhuman primate in vivo. Additionally, the in vitro effects of INDO on basal and EPI-induced cAMP formation in primate ciliary muscle, ciliary processes, trabecular meshwork, and iris were determined.

METHODS

Animals and Anesthesia

All in vivo experiments were conducted in young adult, female cynomolgus monkeys (Macaca fascicularis) weighing 2 to 3.5 kg each. All eyes underwent slit lamp examination by a trained ophthalmologist and were free of biomicroscopically detectable cells and flare before the study. Perfusions and intracameral injections or aspirations were performed after intramuscular injections of ketamine (10 mg/kg) followed by pentobarbital Na (35 mg/kg), supplemented, if needed, by intramuscular pentobarbital Na (10 mg/kg) after 2 or 3 hours. After the experiment, each animal was given intravenous fluids and topical and systemic antibiotics and was placed in a heated cage until fully recovered.

Outflow Facility

Each eye was cannulated with a branched needle: One branch was attached to a reservoir containing mock aqueous humor, and the other was attached to a pressure transducer. For intracameral drug injections, a t-piece was inserted in the inflow tubing close to the eye. A 10 μl volume of the infusate was then injected through this t-piece, and 5 minutes were allowed for the drug to wash into the anterior chamber at the elevated pressure. The anterior chamber contents were next mixed by cold air convection, after which the inflow system was shut down and the eyes were left undisturbed for 3 to 4 hours before outflow facility measurements began. Total outflow facility was determined by two-level constant pressure perfusion (2.5 and 11.9 mm Hg above spontaneous IOP) of the anterior chamber with mock aqueous humor for 45 to 60 minutes, correcting for the internal resistance of the perfusion apparatus as appropriate for a one-needle technique. Ten facility values were obtained; three to nine consecutive values were averaged, depending on where the values were most stable, to give the final facility value. Approximately two thirds of the experiments used at least five values, but, for all experiments, the same interval was averaged for treated and control eyes of a given animal.

Drugs. 1-Epinephrine bitartrate (EPIBT, Sigma Chemical, St. Louis, MO) was prepared as a 0.1% solution in mock aqueous humor. NaOH was added so that the pH would be between 7.1 and 7.4 after dilution of the 10 μl injectate into the ~100 μl anterior chamber. The 10 μg dose of the bitartrate salt was equivalent to 5.5 μg of free base EPI (3 × 10⁻¹⁴ M initial dose in the anterior chamber).

Indomethacin (Sigma) was prepared as a 0.25% solution in 1.9 mM Na₂CO₃. The pH was adjusted to ~7.5 with 1 N HCl. Indomethacin was administered topically to the cornea of ketamine-anesthetized monkeys in the supine position, with 30 to 60 seconds between drops; 1, 2, 5, or 10 μl drops were administered, giving a dose of 25, 50, 125, or 250 μg, respectively.

Specific Protocols. Epinephrine Alone: A single 10 μg dose of EPIBT was administered intracameral to one eye of the experimental animals. The opposite eye received an equal volume of mock aqueous humor without EPIBT. The animals were then left undisturbed for 3 hours before measurement of outflow facility.

Indomethacin Alone: A single 125 μg topical dose of INDO was applied to one eye of each animal, with the opposite eye receiving an equal volume of vehicle without INDO. One group of animals was left undisturbed for 30 minutes before measurement of outflow facility (to rule out the possibility that INDO might have an early onset effect on facility); another group
was left undisturbed for 4 hours before measurement of outflow facility.

**Indomethacin + Epinephrine:** Topical INDO (25, 50, 125, or 250 µg) was applied to one eye, and vehicle without INDO was applied to the opposite eye of an animal 1 hour before the intracameral administration of EPI, (10 or 50 µg) bilaterally. The animals were then left undisturbed for 3 more hours before outflow facility was measured.

**Intracameral Concentration of Epinephrine Three Hours After Intracameral Infusion**

Three hours later, the eyes were cannulated with an unbranched 27-gauge needle into one eye of each of four cynomolgus monkeys as described. As a control, the opposite eyes were injected with 10 µl of Bárany’s solution containing the same volume of 0.2 N acetic acid. Three hours later, the eyes were cannulated with an unbranched 27-gauge needle attached to a syringe, and ~80 µl of anterior chamber aqueous was aspirated. Triplicate 5 µl aliquots of the original injection solution and duplicate 20 or 40 µl aliquots of the anterior chamber samples were counted in 10 ml Beta Max (ICN Biomedicals, Irvine, CA) in a liquid scintillation counter. Control eye background radioactivity (~30 cpm) was subtracted before calculation of anterior chamber EPI concentrations.

**Intracameral Penetration of Topical Epinephrine and Indomethacin**

Trace amounts of ²H INDO (specific activity, 64.14 Ci/mmol; DuPont, Wilmington, DE) in 0.2 N acetic acid–ethanol (9:1) was evaporated partially under nitrogen. A trace amount was added to cold EPI—1 mg/ml in Bárany’s perfusand—and the pH was adjusted to ~6.5 to ~7. Immediately, 10 µl was injected, through a t-piece attached to a 27-gauge branched needle, into one eye of each of four cynomolgus monkeys as described. As a control, the opposite eyes were injected with 10 µl of Bárany’s solution containing the same volume of 0.2 N acetic acid.

Cyclic Adenosine Monophosphate Formation

Anterior segment quadrants from approximately 40 rhesus monkeys (*Macaca mulatta*) of both sexes, 1 to 13 years of age, killed by pentobarbital overdose for use in other protocols or because of untreated illness or debilitation, were dissected into quadrants within 1 hour of death and stored at −80°C for 1 week to approximately 2 to 3 years. Only one animal was killed at a time, and this usually occurred at 1- to 2-week intervals. Because eye tissue from only one animal was insufficient for a single experiment, pooled frozen tissue was required. The levels of adenylyl cyclase activity—and the responses to forskolin, EPI, and other drugs tested but not included in this study—were comparable to values reported in both fresh and frozen ocular and brain tissues from other species (W. Heideman, unpublished data, 1984). Freezing and thawing commonly is performed in these types of assays; therefore, we were confident about our results, especially because our primary interest was the relative activity, rather than the absolute values, in the tissues and under the pharmacologic milieux studied.

Cyclic and under the pharmacologic milieux studied.

The quadrants were thawed slightly, and the iris, ciliary processes, ciliary muscle, and trabecular meshwork were dissected under an operating microscope and placed separately in ice-cold buffer solution (20 mM Tris, pH 7.4, 5 mM ethylenediaminetetraacetic acid, 300 mM sucrose, 5 mM MgCl₂, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). Histologic examination of dissected tissues confirmed the tissue separations but revealed that the trabecular meshwork specimens also contained some cells from the anterior ciliary muscle, and some ciliary process specimens may have contained some peripheral retina. Pooled tissue separations from three to seven monkeys were then homogenized using a Dounce ground glass homogenizer. To isolate the membrane fraction, the homogenate was centrifuged at 4°C for 5 minutes at 1000g, the supernatant was centrifuged for 20 minutes at 14,000g. The pellet was resuspended in homogenization buffer to a final protein concentration of 1 to 10 mg/ml and
stored at −70°C until needed. Protein was assayed by the method of Lowry.

Adenylate cyclase was assayed by a modification of the method of Salomon. Briefly, each assay tube contained ice-cold (4°C) buffer consisting of 160 mM Tris, pH 7.4, 4 mM MgCl₂, 2 mM ethylenediaminetetraacetic acid, 200 μM 3-isobutyl-1-methylxanthine, and 30 μM sucrose; an adenosine triphosphate (ATP) regenerating system (10 mM phosphocreatine, 100 μg creatine phosphokinas); 32P-labeled substrate (400 μM ATP, 10⁶ cpm α-[32P] ATP/assay); 3H cAMP (2 mM, 10⁶ cpm/assay) to monitor product recovery; the drug solutions under investigation (EPI BT, INDO, EPI BT+INDO or FSK [Calbiochem, La Jolla, CA]); and membrane aliquots. EPI BT doses ranged from 10⁻⁹ to 10⁻⁵ M; INDO doses were 10⁻⁷ to 10⁻³ M, with EPI BT concentrations of 10⁻⁴ and 10⁻³ M; the FSK dose was 10⁻⁴ M. Assays were run in duplicate or triplicate for each membrane pool. All components of the assay were combined at 4°C without preincubation with any one component because corneal penetration was not an issue in vitro and all drugs had direct and immediate access to the target tissues. (The assay volume is small, the cells are disrupted, and the process is controlled strictly by aqueous diffusion across very small spaces; the receptors need only to pluck the ligand from the well-mixed solution. As proof, these assays are often linear from the beginning, indicating that the substrate ATP, which has diffusion rates similar to those of the drugs, has arrived swiftly at the cyclase unimpeded.) The reaction was begun by incubating the mixture at 30°C for 20 minutes and was terminated by the addition of 10% sodium dodecyl sulfate. The assay mixture was then transferred onto Dowex and Alumina columns to isolate the cAMP. Samples were counted in Ultima Gold (Packard Instrument, Downers Grove, IL) cocktail in a liquid scintillation counter.

Each experiment used pooled membranes from three to seven animals, with virtually no overlap between groups. Expressed as pmol per min per mg protein in each tissue, cAMP formation is shown as mean ± SEM for n experiments. Most experiments also were replicated using the same pooled tissue preparation on separate days, with similar results. Because of the relative quantities of available tissue, more experiments were performed using ciliary muscle and processes than trabecular meshwork or iris.

Statistical Analysis

All data are presented as mean ± SEM. Graphic examination of the outflow facility responses indicated that normal probability (i.e., parametric) models for testing differences between experimental and control eyes would be more appropriate after the data for the individual animals were converted to experimental:control ratios or were logarithmically transformed (log₁₀) transformed because the difference between the paired eyes and the different groups was dependent on the absolute level of the starting facility. (Normal distribution of the ratios and the logarithmically transformed data were verified as follows: For each pair of measurements in each dose group, the difference, ratio, and log ratio [i.e., difference of the logs] was computed. The data within each group were standardized by subtracting the group mean from each individual value and dividing that difference by the group standard deviation. After such standardization, normally distributed original measurements would still be normally distributed, but with mean = 0 and standard deviation = 1 for each group. The groups could then be combined and all the data compared to a normal [0, 1] distribution by a normal quantile-quantile [qq] plot. This exercise [not shown] revealed that both the ratios and the log ratios closely approximated a hypothetically “perfect” normal distribution, with the log ratios coming the closest; the difference plot was the farthest from normality). Two-sample t-tests were then used to examine whether the mean of the log₁₀ facility for the experimental and control eyes in each group was the same. The mean and 95% confidence intervals for the mean of the difference of the log₁₀ responses were transformed back to provide inferences on the ratio scale. Additionally, the mean ratio (without log₁₀ transformation) of experimental to contralateral control eyes in each group was compared to 1 using a two-tailed t-test because such data typically are expressed in this format.

To investigate the differences among tissue types with respect to cAMP, a one-way analysis of variance model was fit to the paired ratios of the FSK-stimulated cAMP to basal cAMP. Corresponding analysis of variance analyses were conducted after logarithmic transformation of the cAMP data; the results yielded conclusions identical to those of the ratio analyses of untransformed data and, therefore, are not presented. P < 0.05 was considered statistically significant; we have indicated values close to that for information purposes. All procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

RESULTS

Outflow Facility

Epinephrine or Indomethacin Alone. Three hours after a single 10 μg intracameral dose of EPI BT, total outflow facility was ~40% higher than in the contralateral vehicle-injected control eyes (Table 1). A single 125 μg topical dose of INDO had no effect on total outflow facility either 30 minutes or 4 hours after dosing.
were compared. The 25 values, was of borderline significance at the 250 T/C or log_m T-log_m C significantly different from 1.0 or 0.0, respectively, by the two-tailed t-test:

Three hours after intracameral injection of 10 µg of EPIBT, the concentration of EPI in the aqueous humor was 13.4 ± 4.3 µM and 4.4% ± 1.4% (n = 4) of the initial 3 × 10^-14 M intracameral concentration. These levels are at least 10^5 times greater than those found endogenously in human aqueous humor or plasma (aqueous EPI = 1.2 × 10^-13 M; plasma EPI = 2.7 × 10^-10 M). Calculation of the EPI remaining at 3 hours based on an aqueous humor flow rate of approximately 1.25 µl/minutes (this was the mid-point of the typical range of aqueous flow rates in this species) yields a similar value of 10.5% (e^{-1.25 µl/minute per 100 µl x 180 minutes} = e^{-2.25} = 10.5%).

### Intracameral Penetration of Topical Epinephrine and Indomethacin

After topical application of 500 µg of EPI or 125 µg of INDO (Fig. 1), the peak intracameral concentration of each drug, averaging ~45 µM and 15 µM, respectively (equivalent to 0.16% and 0.43% of the topically applied amounts, assuming a 100 µl anterior chamber volume), was achieved at approximately 1 hour. At 3 hours the EPI concentration could reasonably be estimated to be ~30 µM, whereas at 4 hours the INDO concentration was probably between 5 and 10 µM.

### TABLE 2. Total Outflow Facility After Unilateral Administration of Epinephrine or Indomethacin to Cynomolgus Monkeys

<table>
<thead>
<tr>
<th>Drug (dose)</th>
<th>n</th>
<th>EPI_T</th>
<th>INDO + EPI_T</th>
<th>95% CI for EPI_T/(INDO + EPI_T)</th>
<th>Ratio of Means</th>
</tr>
</thead>
<tbody>
<tr>
<td>INDO(25), EPI_T(10)</td>
<td>8</td>
<td>0.28 ± 0.06</td>
<td>0.27 ± 0.04</td>
<td>1.04 ± 0.08</td>
<td>1.02</td>
</tr>
<tr>
<td>INDO(50), EPI_T(10)</td>
<td>13</td>
<td>0.35 ± 0.05</td>
<td>0.31 ± 0.04</td>
<td>1.20 ± 0.13</td>
<td>1.13</td>
</tr>
<tr>
<td>INDO(125), EPI_T(10)</td>
<td>13</td>
<td>0.38 ± 0.09</td>
<td>0.30 ± 0.06</td>
<td>1.26 ± 0.10</td>
<td>1.21</td>
</tr>
<tr>
<td>INDO(250), EPI_T(10)</td>
<td>11</td>
<td>0.31 ± 0.05</td>
<td>0.29 ± 0.07</td>
<td>1.28 ± 0.13</td>
<td>1.22</td>
</tr>
<tr>
<td>INDO(125), EPI_T(50)</td>
<td>5</td>
<td>0.33 ± 0.05</td>
<td>0.27 ± 0.04</td>
<td>1.24 ± 0.09</td>
<td>1.23</td>
</tr>
</tbody>
</table>

Data are mean ± SEM (µl/minute per mm Hg) for n animals, each contributing one epinephrine bitartrate (EPI_T) -treated and one indomethacin (INDO)-treated eye. Facilities were determined 3 hours after intracameral EPI_T (4 hours after topical INDO). EPI_T(INDO + EPI_T) = mean EPI_T to INDO + EPI_T ratio for paired eyes. EPI_T(INDO + EPI_T) Estimated = ratio and corresponding 95% confidence interval (CI) obtained from log_2 transformation of the data as described in Statistical Analysis. EPI_T(INDO + EPI_T) or log_2 (EPI) - log_2 (INDO + EPI_T) significantly different from 1.0 or 0.0, respectively, by the two-tailed t-test: *P < 0.10, †P < 0.05, ‡P < 0.025.
Indomethacin and Epinephrine Effects

FIGURE 1. Intracameral indomethacin and epinephrine concentrations (mean μM) after topical application of 125 and 500 μg, respectively, of radiolabeled drug. Four animals were used for each drug, each contributing two eyes for two different time points. Background radioactivity (~30 cpm) was subtracted before concentrations were calculated.

based on the 1- to 3-hour data obtained and on reasonable extrapolation assumptions.

cAMP Formation

**Basal, Forskolin.** Basal adenylate cyclase activity was highest in the ciliary muscle, intermediate in the ciliary processes, and lowest in the iris and trabecular meshwork (Table 3). Forskolin, a direct activator of adenylate cyclase, significantly stimulated cAMP formation by ~5-fold in the iris and meshwork and by more than 10-fold in the ciliary processes and muscle. Compared to basal adenylate cyclase activity, forskolin-stimulated activity was significantly higher in both ciliary muscle and ciliary processes than in either iris or meshwork.

**Epinephrine Alone.** Epinephrine stimulated adenylate cyclase activity and cAMP formation in all tissues in a dose-dependent manner (Fig. 2). A representative experiment for cAMP formation, performed in duplicate and averaged, is shown in Fig. 2A; the results, averaged from multiple adenylate cyclase experiments, are shown in Fig. 2B. The maximal responses were consistently higher in the ciliary processes than in the other three tissues. All four tissues had Emax values in the 10−4 to 10−5 M range.

**Indomethacin + Epinephrine.** An INDO concentration of >100 μM was required for nearly complete inhibition of 10 μM EPI-stimulated cAMP production or for any noticeable inhibition of basal or 100 μM EPI-stimulated cAMP production in ciliary muscle (Fig. 3). In ciliary muscle (Fig. 3A), an INDO concentration of ~10 μM, which (based on extrapolation from the Fig. 1 data as described above) would be present intracameral after 1 to 4 hours after topical dosing with 125 μg of INDO, was ineffective at inhibiting basal cAMP production but reduced 10 μM EPI-stimulated cAMP production by approximately 30%. A similar trend was seen in the other three tissues, with data for trabecular meshwork shown in Figure 3B. The small available quantities of these tissues did not permit construction of a complete dose-response curve in any one experiment, and data for varying INDO concentrations at a given EPI concentration from different experiments could not be pooled because of the substantial baseline ([INDO] = 0) variability between experiments.

DISCUSSION

Indomethacin itself has little or no effect on IOP after topical or systemic administration30,31 in humans, monkeys, or rabbits, and it had no effect on total outflow facility after topical administration in the current study. However, INDO pretreatment produced a dose-

![Graph](image)

**TABLE 3. Basal and Forskolin-Stimulated Adenylated Cyclase Activity in Rhesus Monkey Ocular Tissues**

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>FSK/Stimulated</th>
<th>FSK/Basal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>pmol/min/mg Protein</td>
<td>n</td>
</tr>
<tr>
<td>CM</td>
<td>4</td>
<td>3.74 ± 2.40</td>
<td>4</td>
</tr>
<tr>
<td>CP</td>
<td>4</td>
<td>5.81 ± 1.45</td>
<td>4</td>
</tr>
<tr>
<td>IR</td>
<td>4</td>
<td>3.44 ± 1.00</td>
<td>4</td>
</tr>
<tr>
<td>TM</td>
<td>4</td>
<td>3.34 ± 0.93</td>
<td>4</td>
</tr>
</tbody>
</table>

Data are pmol cAMP/minute per mg protein ± SEM (Basal, FSK), or mean ± SEM ratio between forskolin (FSK, 10−4 M)-stimulated and basal activity (FSK/Basal) in cell membranes isolated from rhesus monkey ciliary muscle (CM), ciliary processes (CP), iris (IR), and trabecular meshwork (TM), for n experiments. Each experiment, incorporating pooled tissue from three to seven different animals, was performed in duplicate or triplicate and the replicates averaged.

* FSK-stimulated versus BASAL activity for both CM and CP was significantly different by analysis of variance from corresponding activity in either the IR or TM (CM versus IR, P = 0.0009; CM versus TM, P = 0.0007; CP versus IR, P = 0.0027; CP versus TM, P = 0.0086).

Similar results were obtained by analysis of variance of the log10-transformed ratios (CM versus IR, P = 0.0007; CM versus TM, P = 0.0020; CP versus IR, P = 0.0029; CP versus TM, P = 0.0089).

FSK/basal ratio for each tissue was significantly different from 1.0 by the two-tailed t test: †P < 0.005, ‡P < 0.01, §P < 0.05.
dependent suppression of the EPI-stimulated facility by up to 50% to 70%. Thus, in our monkeys, EPI by itself increased facility by ~40% compared to contralateral untreated control eyes and by ~25% compared to contralateral INDO + EPI-treated eyes. Paired comparisons were required to elicit these differences because the interanimal variability in starting facility and EPI-stimulated facility was substantial compared to the magnitude of the drug effects, and this variability obscured the differences when group means were compared.

The validity of using a paired comparison of the ratios was substantiated by a paired analysis of the differences of logarithmically transformed data reaching essentially the same conclusions. The use of paired ratios and logarithmic transformations for analyzing epinephrine responses in this system has been discussed. Both types of analyses minimize the confounding effects of interanimal variability in baseline values and the fact that epinephrine responses in absolute terms increase with increasing starting facility but are a relatively constant percentage of starting facility over a wide range of starting facilities. Statistical purity demands that data be distributed normally to qualify for a parametric analysis such as a paired t-test, and both ratio and logarithmic transformation have such a normalizing effect. Linearity of normal quantile-quantile plots of the ratios and log ratios of the combined data after standardization (see Statistical Analysis in Methods section) indicate that both data sets are distributed normally and are more appropriate for parametric analysis than the arithmetic comparison of group means or their paired differences.

Baseline facilities were not determined in this study because of the assumption that, as in numerous previous studies, baseline values for paired eyes will be the same over a group of animals. Omitting the baseline saves time in an already long experiment, concomitant with the reduction of artifacts (see next paragraph) and anesthetic risks associated with long perfusions. Theoretically, the possibility exists that an early tissue response to INDO in vivo could have affected the subsequent response to EPI treatment. An alternative design would have been to administer INDO intracameraly and to follow it with EPI after a shorter waiting period than we used after the topical INDO administration. Although we did not do that, we did determine that 125 μg of topical INDO had no effect on outflow facility 30 to 60 minutes later. Additionally, INDO has no effect on IOP after topical or systemic administration in humans, and it seems unlikely that any alternative design for our experiments would have yielded different results. We have shown previously that the facility response of an eye 3 hours after intracameral administration of 10 μg EPI relative to its own baseline is essentially the same as in the simultaneous treated versus control comparison used in the current study.

Bill and Sperber provided evidence suggesting that the permeability of the blood–aqueous barrier increases after several hours of continuous perfusion after anterior chamber cannulation with three needles. Such increased blood–aqueous barrier permeability could result in increased pressure-sensitivity of aqueous humor formation (i.e., increased pseudofacility), which would, in turn, be reflected as increased total facility in our measurement technique. Furthermore, INDO might stabilize the blood–aqueous barrier, thereby reducing both pseudofacility and total facility and giving the spurious impression of an inhibition of epinephrine’s effect on true facility.
Indomethacin and Epinephrine Effects

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A. Ciliary Muscle

B. Trabecular Meshwork

FIGURE 3. Indomethacin effects on basal and epinephrine (EPI)-stimulated adenylate cyclase activity (mean ± SEM) using pooled tissue from three to seven different animals in (A) ciliary muscle from a single representative experiment performed in triplicate or (B) trabecular meshwork from single or multiple (n = 2 or 4, as indicated) experiments, each performed in duplicate or triplicate. The limited amount of trabecular meshwork tissue available from any given tissue pool permitted only one concentration of indomethacin to be tested in addition to baseline in any given experiment. Therefore, multiple individual experiments are shown in B for basal and 10^-5 M EPI. Similar results were obtained in ciliary processes and iris.

ever, pseudofacility is actually low in shorter perfusions (<0.02 μl/minute per mm Hg), comprising <5% of total facility. Our experiments were a hybrid in this regard because the eyes were cannulated for up to 4 hours, but only one needle was used and the infusion of mock aqueous was limited to no more than 1 hour (reservoirs were turned off during the 3-hour waiting period for this reason and to minimize resistance washout). Therefore, we think the observed drug effects on total facility are more likely to represent effects on trabecular facility than pseudofacility.

Uveoscleral outflow is relatively independent of IOP under normal conditions; hence, the facility of uveoscleral outflow measured by perfusion techniques is also <0.02 μl/minute per mm Hg, again <5% of total facility in this monkey species. Epinephrine can increase uveoscleral outflow, perhaps by stimulating PG synthesis in or near the ciliary muscle. PGF2α and various congeners markedly enhance uveoscleral outflow, and indirect evidence suggests that they also increase its IOP dependence (i.e., they increase uveoscleral facility). It is thus possible that part or all of INDO’s inhibition of the EPI-stimulated facility increase represented inhibition of EPI-stimulated uveoscleral facility rather than trabecular facility. The physiological data reported in our study, therefore, do not permit an unequivocal conclusion as to the target tissue or physiological process affected by INDO to inhibit partially the facility-increasing effect of epinephrine.

The in vivo drug penetration–concentration studies and the in vitro studies of EPI stimulation and INDO inhibition of cAMP production by anterior segment tissues were an attempt to help identify the target tissue(s) for the EPI and INDO effects on facility. For intracameral administration of EPI, 10 μg is a near-maximal, facility-effective dose in cynomolgus, whereas 500 μg of EPI topically gives approximately the same facility response in cynomolgus and in vervet (African green) monkeys (Cercopithecus aethiops) and is near maximal in humans. Three hours after the administration of 10 μg of EPI intracameral or 500 μg of EPI topically, a time when facility was consistently and uniformly elevated (current data), we measured (intracameral dosing) or estimated (by extrapolation from 1- and 2-hour data points; topical dosing) the anterior chamber aqueous humor concentration of EPI to be ~13 and 30 μM, respectively, well within the pharmacologically effective range. The available EPI concentration at the effective tissue site might be different by virtue of pigment binding, local drug metabolism, or other pharmacokinetic factors, and EPI’s effect on facility and on cAMP production may have a relatively long latency and duration. Nonetheless, the observed concentrations are consistent with reasonable assumptions about corneal permeation and aqueous humor turnover and with the known EPI dose-facility response relationships for topical and intracameral administration. Furthermore, these EPI concentrations were at least half, and per-
haps near, maximal for stimulation of cAMP production by monkey ciliary muscle, ciliary processes, trabecular meshwork, and iris in vitro.

A topical INDO dose of 125 μg was slightly supramaximal for inhibition of the EPI-stimulated facility increase and resulted in an intracamer INDO concentration estimated to be ~5 to 15 μM from 1 to 4 hours after dosing. This INDO concentration was ineffective at inhibiting basal cAMP production by monkey ciliary muscle, ciliary processes, trabecular meshwork or iris in vitro but reduced 10 μM EPI-stimulated cAMP production by approximately 30%. An INDO concentration >100 μM was required for complete inhibition of 10 μM EPI-stimulated cAMP production or for any noticeable inhibition of basal or 100 μM EPI-stimulated cAMP production. At such high concentrations, INDO may lose its specificity as a cyclooxygenase inhibitor. Although INDO decreases basal cAMP production in human fetal lung tissue (at 28 μM INDO) and canine tracheal epithelium (at 1 μM INDO), other effects, such as decreases in ATP-dependent calcium binding and electrically induced calcium and sodium uptake (at 0.1 to 0.5 mM INDO), as well as decreased short-circuit current and increased tissue resistance in canine tracheal epithelium (at 1 μM INDO), have been reported. However, binding of INDO by pigment in both the in vivo and in vitro experiments (pigment was present in all membrane samples for in vitro assays) may have increased the INDO concentrations required for maximal effect; the fact that the physiologically specific INDO concentration of 10 μM partially inhibited EPI’s effect on facility in vivo and cAMP production in vitro suggests that the INDO effects are indeed related to cyclooxygenase inhibition and that the EPI effects may be related to stimulation of prostanoid biosynthesis. Measurement of prostaglandin synthesis, and its stimulation by EPI and inhibition by INDO under our experimental conditions, would help resolve these issues.

The basal and EPI-stimulated cAMP levels in our rhesus monkey tissues compare well to those reported in monkey and human ocular tissues by other investigators, and the in vitro concentrations of EPI required to produce maximal cAMP stimulation in each tissue are comparable to those required for a maximal physiologic effect in vivo. In the pentobarbital-anesthetized monkey, single 300- to 600-μg doses of topical EPI, which, based on extrapolation from our 1- and 2-hour data points, would result in intracamer concentrations on the order of 50 μM 3 hours after dosing, increase outflow facility by ~30% to 35% and (after multiple treatments) increase aqueous humor flow rate by ~40%. In our ciliary process membrane preparation, 50 μM EPI was ~70% maximal for cAMP stimulation, whereas 1 μM EPI had little effect. In the trabecular meshwork, 50 μM was near maximal, whereas 1 μM was ~50% maximal, comparable to other in vitro experiments using cultured trabecular meshwork cells.

In the human anterior segment perfusion model devoid of all uveal tissue, a maximal dose of 25 μM EPI produces a 44% increase in outflow facility. In vivo, initial intracamer doses of 5.5 μg of EPI (~500 μM) are necessary to increase outflow facility maximally in cynomolgus monkeys. The clinical topical dose (~500 μg) required to decrease IOP maximally in humans and to increase outflow facility in monkeys yielded intracamer concentrations of ~45, 35, and (by extrapolation) 30 μM in our animals 1, 2, and 3 hours after dosing. The order-of-magnitude higher initial intracamer dose required in the living monkey may be consequent to drug binding by uveal pigment or pharmacokinetic factors, among other possibilities.

Chronic EPI treatment resulted in elevated levels of prostaglandin E in the aqueous and vitreous humor of phakic and aphakic rabbits, and multiple doses of PGF2α, or its analogues can increase total outflow facility in monkeys and humans. Human trabecular cells in culture can synthesize both PGE2 and PGF2α, and PGE2 is readily converted to PGF2α. However, exogenously applied PGF2α does not increase trabecular facility in monkeys, and the increase in total facility probably represents increased pseudofacility or facility of uveoscleral outflow.

It is doubtful that the entire EPI effect on facility is inhibitible by INDO or is mediated by PG synthesis. Maximal topical doses of INDO (125 or 250 μg) apparently blocked only 50% to 70% of the facility increase induced by a putatively maximal intracamer dose of EPI (5.5 μg). The 125 μg topical INDO dose yielded an intracamer concentration of ~5 to 15 μM in our penetration studies. Other investigators have used comparable doses of INDO to inhibit the outflow facility increase, IOP decrease, and PGE release into the aqueous humor, induced by EPI in rabbit eyes. If INDO were acting directly on the EPI pathway, higher doses of EPI might overcome part of the inhibition. However, in our outflow facility studies, the effect of an intracamer dose of 27.5 μg of EPI (50 μg of
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EPI[β]t), which is well over the top of the dose-response curve,\textsuperscript{13} was inhibited by INDO to approximately the same extent as the 5.5 µg dose. Recent evidence suggests that EPI's acute facility-increasing effect is at least in part consequent to disruption of actin filaments within trabecular meshwork cells and the consequent changes in cell shape.\textsuperscript{8,66,78-92} Phalloidin, a fungal peptide that stabilizes actin filaments but by itself has no effect on facility, prevents as much as, but not more than, 50% of EPI's effect.\textsuperscript{20} Whether this is the same 50% inhibited by INDO is unknown, but it is of great interest in continuing attempts to unravel the still poorly understood mechanism(s) and site(s) of action responsible for EPI's facility increasing effect.

Key Words
aqueous humor outflow facility, cyclic adenosine monophosphate, epinephrine, indomethacin, monkey eye

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References

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