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Epinephrine Increases Facility of Outflow and Cyclic AMP Content in the Human Eye in Vitro

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The physiologic mechanism that underlies the epinephrine-induced increase in facility of outflow (C) in glaucomatous human eyes and normal primate eyes is not completely understood. In this study, a recently developed in vitro human eye perfusion model was used to simultaneously monitor facility and cyclic adenosine monophosphate (AMP) changes in response to epinephrine (EPI). In this system, EPI (2.5×10^{-5} mol/l) resulted in a maximal 44% increase in C, with an ED_{50} occurring at approximately 8×10^{-6} mol/l. The C-increasing effect of 10^{-5} mol/l EPI was unaffected by 10^{-6} mol/l phentolamine. However, it was completely blocked in the presence of 10^{-6} mol/l timolol or 2×10^{-7} mol/l ICI118,551, suggesting the involvement of β-2 adrenergic receptors. In biochemical studies, 10^{-5} mol/l EPI induced a 12- to 14-fold increase in cyclic AMP in the perfusate of treated eyes. This increase was blocked by ICI118,551. In isolated intact human trabecular tissue, a 10 min incubation with 10^{-5} EPI stimulated cyclic AMP by a factor of 2.7 over control levels. After 90 min, cyclic AMP levels were increased 4.2 fold over control levels. Collectively, these results show that the intraocular pressure lowering effect of EPI in the human eye is mediated, at least in part, by an increase in facility of outflow. Furthermore, the facility increase appears to be mediated by β-2 adrenergic receptors and is correlated in time with increased cyclic AMP production. Invest Ophthalmol Vis Sci 33:2672–2678, 1992

Although epinephrine has been used for a number of years to treat primary open angle glaucoma, the mechanism of action of its intraocular pressure (IOP)-lowering effects remains debatable.[1] Most experimental evidence points to the role of the outflow pathway tissues in mediating the IOP decrease in the human and subhuman primate eye. Radioligand binding studies of cultured human trabecular cells isolated from the outflow pathway demonstrate the presence of β-adrenergic receptors, and β-adrenergic stimulation of these cultured cells leads to an accumulation of cyclic adenosine monophosphate (AMP) that is blocked by timolol.[2–4] Autoradiographic studies of human outflow tissue in situ show the presence of β-adrenergic receptors in the outflow pathway tissues, with a predominance of the β-2 subtype.[5] These in vitro studies are consistent with findings from clinical studies that demonstrate epinephrine-induced increases in total outflow facility (as measured indirectly by tonography) are blocked by coadministration of timolol.[6,7] In the monkey eye, epinephrine and cyclic AMP analogues increase outflow facility in the intact eye[8–16] and in the ciliary muscle-disinserted eye.[13] These observations support a role for β-adrenergic receptors and suggest a link between the effects of epinephrine and cyclic AMP on facility. However, some studies, which show that epinephrine's ocular hypotensive effect can be blocked by indomethacin, have raised the question of whether epinephrine's action may be mediated by prostaglandins, including some derivatives (eg, $PGF_{2\alpha}$) not known to stimulate cyclic AMP production.[17] In addition, other data have questioned the role of β-adrenergic receptors in epinephrine's ocular actions because of the unexpectedly potent effect on IOP of β-adrenergic R-enantiomers, which, on the basis of stereochemical interactions, should have a greatly reduced receptor effect.[18,19]

In the past, a methodology has not been available for correlating time-related changes in outflow facility induced by epinephrine with biochemical alterations in second messenger production. In the present study, a recently developed outflow pathway perfusion model[20] was used to simultaneously study epinephrine-induced alterations in facility and cyclic AMP metabolism.

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Table 1. Epinephrine dose/facility response

Epi ($\times 10^{-6}$ *mol/l*)	*n*	C_o	C_d	C_d/C_o
0	12	0.17 ± .02	0.16 ± .03	0.91 ± .04
1	5	0.29 ± .05	0.32 ± .07	1.09 ± .04
5	7	0.18 ± .03	0.23 ± .03	1.09 ± .04
10	13	0.27 ± .03	0.33 ± .03	1.24 ± .08*
25	4	0.35 ± .20	0.45 ± .24	1.44 ± .19†
50	7	0.25 ± .06	0.36 ± .09	1.43 ± .04‡

Anterior ocular segments of human eyes were perfused at a constant pressure, as described in Materials and Methods. After a baseline facility was established, (C_o), epinephrine bitartrate (epi), in the indicated concentrations, was added to the perfusion solution, and a post-drug facility (C_d) was determined. For C_d/C_o, the ratio of each eye's pre- and post-drug facility was calculated first, and the mean of the individual ratios was determined. Thus, C_d/C_o in the column above is a mean of ratios and not a ratio of the means. As a result, C_d/C_o is not the simple ratio of the preceding two columns. Data are mean ± SEM of facility determinations in the number (n) of eyes. The ratios of post-drug to pre-drug facilities (C_d/C_o) are significantly different from 1.0 by the two-tailed Student's t-test: *$P < 0.02$, †$P < 0.10$; ‡$P < 0.001$.

Materials and Methods

Dissection and Perfusion of Eyes

Postmortem eyes from 36 donors with a mean age of 73 ± 10.9 yr (mean ± SD; range 42–87 yr) with no prior history of ocular disease or surgery were used in these studies. The eyes were obtained from the National Disease Research Interchange, Philadelphia PA. Eyes were enucleated within 3 hr of the donors' deaths and stored refrigerated in a humid saline environment until they were dissected. Within 12 hr of donors' deaths, eyes were dissected at the eye bank, placed in Dexol (Chiron Ophthalmics, Irvine CA), and shipped chilled to Boston via overnight mail. Prior to perfusion, the eyes were rinsed in sterile Dulbecco's modified Eagle's medium (DMEM) containing 50 U/ml penicillin, 50 μg/ml streptomycin, and 5 μg/ml amphotericin B (DMEM + PSA), mounted on a specialized perfusion chamber, and placed in an incubator at 37°C with 5% CO_2/95% O_2.

Perfusion was carried out at a constant pressure of 15 mm Hg using DMEM + PSA. Determinations of facility of outflow began when steady state conditions were reached—usually 20–30 min after the onset of perfusion or after a drug exchange. Outflow facility was calculated as the ratio of the rate of flow of perfusion solution into the eye (μl/min) to the perfusion pressure (mmHg). Baseline facility measurements consisted of the mean of four consecutive 15 min periods. For the data in Tables 1 and 2 and in Figure 1, post drug facilities consisted of the mean of the four consecutive 15 min periods commencing 90 min after drug exchange. A detailed description of the method of tissue dissection, the perfusion apparatus, and the perfusion method can be found in previous publications.[20,21] As noted previously, this dissection removes iris, lens, and ciliary process, leaving intact the trabecular meshwork and distal outflow pathways.

In the present experiments, we determined that, during facility experiments, cyclic AMP could be easily measured in the perfusate that exited the organ-cultured eye and pooled between the outside of the anterior sclera and the clamping ring of the apparatus. With a micropipette, all fluid (30–70 μl) was collected in microfuge tubes during each 15 or 30 min period. Immediately after collection, tubes were acidified with 1/10 volume of 0.75 mol/l sodium acetate, capped, heated at 90°C for 4 min, and frozen at −90°C for later radioimmunoassay.

Drugs and Effects on Outflow Facility

DL-epinephrine bitartrate, timolol maleate, DMEM, and PSA were obtained from Sigma Chemical Co., Inc. (St. Louis, MO). ICI118,559 (Erythro-DL-1-(7-methylindan-4-yloxy)-3-isopropylamindoutan-2-01) was a gift from Imperial Chemicals Industries Inc. (Wilmslow, Cheshire, England). Phentolamine hydrochloride was a gift of Ciba-Geigy (Suffern, NY). Drug stock solutions and their working dilutions were prepared in DMEM + PSA immediately prior to perfusion experiments.

For studies with epinephrine alone, a dose-response relationship for outflow facility was established. After a steady state baseline facility was determined, the reservoir contents, tubing, and perfusion chamber con-

Table 2. Outflow facility and cyclic AMP after 10^{-5} mol/l epinephrine and 2×10^{-7} mol/l ICI118,551

Drug	*Exp no.*	C_o	C_d	C_d/C_o	$cAMP_o$	$cAMP_d$	$cAMP_d/cAMP_o$
Epi	105 (OS)	0.29	0.36	1.24	22.24	278.86	12.0
	108 (OD)	0.31	0.47	1.52	40.78	584.58	14.3
Epi + ICI	98	0.23	0.38	1.65	167.60	4550.60	27.2
	105 (OD)	0.22	0.18	0.82	38.61	52.48	1.4
	108 (OS)	0.49	0.48	0.98	12.43	15.28	1.2

Outflow facility was calculated in perfused human ocular anterior segments, and perfusate cyclic adenosine monophosphate (AMP) was measured, as described in Materials and Methods. After a baseline facility was determined (C_o), the chamber contents were exchanged with 10^{-5} mol/l epinephrine alone (Epi) or in combination with 2×10^{-7} mol/l ICI118,551 (epi + ICI), and post-drug facilities (C_d) were determined. Outflow facility data C_o and C_d are expressed as μl/min/mmHg. Baseline cyclic AMP ($cAMP_o$) and post-drug ($cAMP_d$) measurements are expressed as fmol per sample.

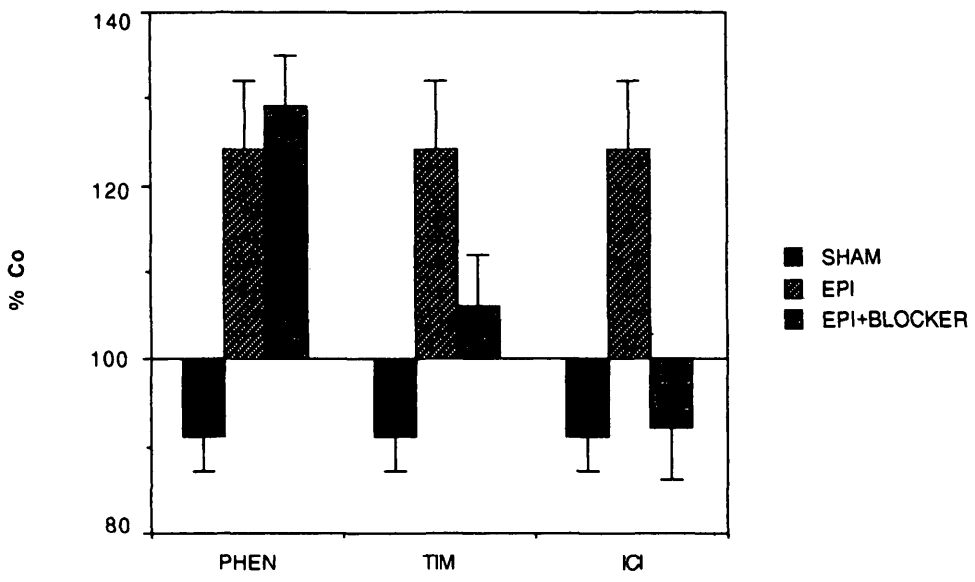

Fig. 1. Characterization of adrenergic receptors mediating the epinephrine-induced outflow facility response. Outflow facility was measured in human ocular anterior segments, as described in Materials and Methods. After a baseline facility was established (C_o), subsequent facility determinations were made after a sham manipulation (SHAM; n = 12), or exposure to 10^{-5} mol/l epinephrine alone (Epi; n = 13), or in combination with 10^{-6} mol/l phentolamine (PHEN; n = 5), 10^{-6} mol/l timolol (TIM; n = 7), or 2×10^{-7} mol/l ICI118, 551 (ICI; n = 6). Data are mean ± SEM.

tents were exchanged with perfusion solution containing 10^{-6}, 5×10^{-6}, 10^{-5}, 2.5×10^{-5}, or 5×10^{-5} epinephrine bitartrate. After volume equilibration, post epinephrine facility measurements were made for at least 3 hr. In early studies, the fellow eye was used as a medium-only control to ensure that the drug exchange procedure per se did not alter outflow facility. In subsequent studies, the fellow eye, if available, was used in antagonist studies (described later) or in unrelated perfusion studies. Drug effects were evaluated in each eye as the ratio between post drug facility (C_d) and predrug (C_o) facility. Statistical analysis consisted of a paired t-test using the individual ratios (C_d/C_o) (Table 1). This method of analysis normalizes for inter-eye differences in baseline outflow facility and has been used extensively in prior studies that use this technique.[10-13,20,21]

For receptor characterization studies, a baseline facility determination was made, after which the reservoir, tubing, and chamber contents were exchanged with culture medium containing a specific receptor antagonist. The eye was perfused with antagonist for 90–120 min, after which a second exchange occurred with medium containing antagonist + 10^{-5} mol/l epinephrine. Perfusion with epinephrine plus antagonist was carried out for at least 3 hr and outflow facility was calculated every 15 min.

Based upon prior pharmacologic studies, the concentrations of antagonists used were chosen so as to be above their known K_i for the targeted receptor while maintaining selectivity for the receptor in question. Thus, phentolamine, a nonselective α-adrenergic antagonist, was used at 10^{-6} mol/l, a concentration at which phentolamine blocks binding of 10^{-5} mol/l epinephrine to α-adrenergic receptors,[22] but that does not significantly block binding of 10^{-5} mol/l epinephrine to β-adrenergic receptors.[23,24] Timolol was used at 10^{-6} mol/l, a concentration that causes a 99% inhibition of the effects of 10^{-5} mol/l epinephrine at β-adrenergic receptors.[24,25] ICI118,551, a β-2 adrenergic antagonist with a β-2/β-1 selectivity ratio of 500,[26-28] was used at 2×10^{-7} mol/l, a concentration that blocks the β-2 but not the β-1 adrenergic effects of 10^{-5} mol/l epinephrine.[28]

Stimulation of Cyclic AMP in Isolated Trabecular Meshwork

The trabecular meshwork was dissected from the anterior scleral shell as follows. After the scleral spur was removed, the trabecular meshwork was dissected along the anterior and posterior margins and was lifted away from the underlying sclera with the aid of a fine jeweler's forceps. Cyclic AMP accumulation then was measured in 1–4 mm strips cut from the meshwork of two eyes. Meshwork pieces were incubated at 37°C in a 5% CO_2 environment in one of four experimental conditions: culture medium alone for 10 min or 90 min or 10^{-5} mol/l epinephrine for 10 min or 90 min.

After incubation, meshwork pieces were homogenized in 300 μl of 0.075 mol/l sodium acetate, transferred to microfuge tubes, capped, heated to 90°C for 4 min, and centrifuged at 12,000 rpm for 5 min. Aliquots of the supernatant were frozen and later assayed by radioimmunoassay for cyclic AMP content. The pellet was solubilized in 1 N NaOH and assayed for protein by the Folin technique, using bovine serum albumin as the standard.

Radioimmunoassay for Cyclic AMP

Samples from the superfusion experiments or from the in vitro incubation of trabecular meshwork were thawed and duplicate (triplicate for meshwork) aliquots were assayed by radioimmunoassay kit (Biomedical Technologies, Stoughton, MA) using acetylation of endogenous cyclic AMP to increase sensitivity. Appropriate perfusion fluid or media blanks were used in the standard curve. In the perfusion experiments, basal cyclic AMP was calculated as a concentration (per microliter of original perfusate) and as a total per time period. Results from both methods showed similar hormone effects.

Results

Physiologic Studies

Perfusion with epinephrine resulted in a consistent dose-related increase in outflow facility of isolated anterior segments (Figs. 1, 2, 3; Table 1). The maximal response (44% increase over baseline facility) occurred at 2.5×10^{-5} mol/l (Table 1), and the dose resulting in a half-maximal increase in outflow facility (ED_{50}) was calculated to be 8.1×10^{-6} mol/l based upon a log dose-response curve generated using these data.

The adrenergic receptors involved in the facility response to epinephrine were characterized physiologically using various antagonists (Fig. 1). None of the antagonists changed the baseline facility when administered alone. The nonselective α-adrenergic blocker phentolamine had no effect on the facility increase mediated by 10^{-5} mol/l epinephrine, whereas the nonselective β-adrenergic antagonist timolol completely blocked epinephrine's effect. The highly selective β-2 adrenergic antagonist ICI118,551, at 2×10^{-7} mol/l, also blocked the facility effect of 10^{-5} mol/l epinephrine (Figs. 1, 2b).

Biochemical Studies

Analysis of the perfusate from epinephrine-exposed eyes showed that 10^{-5} mol/l epinephrine resulted in a 12- to 14-fold increase in cyclic AMP content, whereas the cyclic AMP increase in eyes simultaneously exposed to epinephrine in the presence ICI118,551 was negligible (Table 2, Fig. 2). The only exception to this was eye no. 98, which received epinephrine plus ICI118,551, and in which cyclic AMP levels were elevated. Interestingly, however, facility was also elevated in this eye (supporting the association between cAMP and facility increase; see Discussion).

After exposure to epinephrine alone, most eyes showed a large increase in cyclic AMP in the first 15 min post drug period, often peaking in the first or second period, then reaching a plateau or gradually decreasing over the next 20 min (Fig. 2a). The facility increase in response to epinephrine almost always lagged the cyclic AMP increase and typically did not occur until 90 min after exposure to epinephrine.

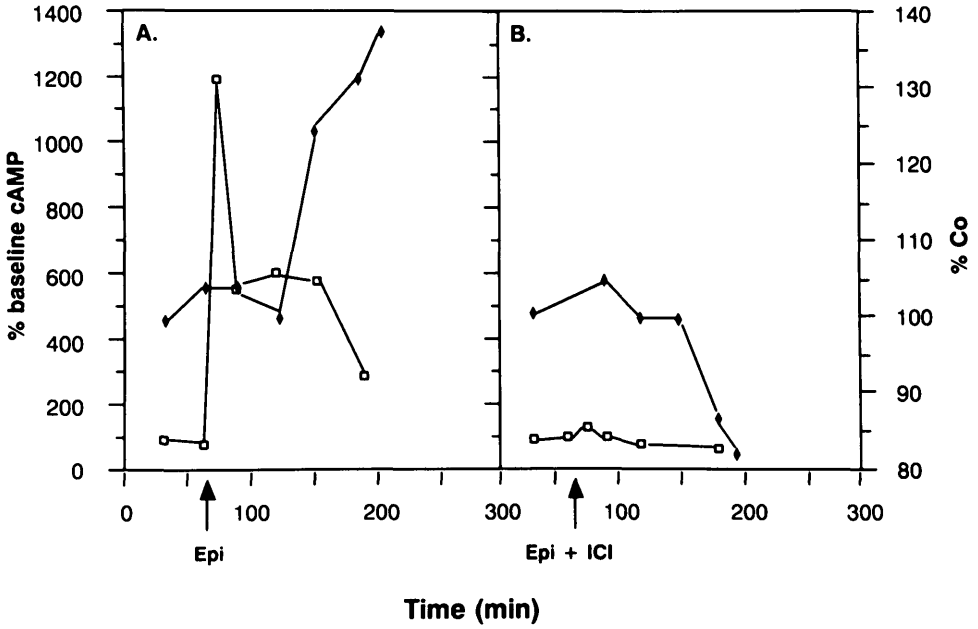

Fig. 2. Outflow facility and cyclic adenosine monophosphate (AMP) after epinephrine with and without ICI118, 551. Outflow facility was determined in a pair of human ocular anterior segments, as described in Materials and Methods. After a baseline facility was determined (C_o), 10^{-5} mol/l epinephrine (Epi) was added to the perfusion solution of one eye (**A**), while the fellow eye received 10^{-5} mol/l epinephrine and 2×10^{-7} mol/l ICI118, 551 (Epi + ICI) (**B**). Facility measurements continued. During the experiment, perfusate was removed at regular intervals from the well formed by the angle between the external sclera and the clamping ring. Cyclic AMP determinations were made by radioimmunoassay, as described in Materials and Methods. Data are expressed as the percent of starting facility (% C_o; filled diamonds) and fmol cyclic AMP per sample (% baseline cAMP; open squares) at the indicated times.

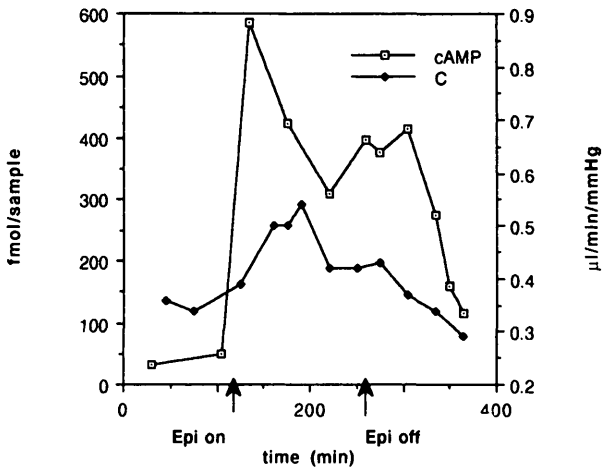

Fig. 3. Correlation between epinephrine-induced outflow facility and cyclic adenosine monophosphate (AMP) accumulation. Outflow facility (µl/min/mmHg) and perfusate cyclic AMP accumulation (fmol per sample) were determined in the perfused ocular anterior segment from a 66-year-old female, as described in Materials and Methods. Epinephrine (10^{-5} mol/l) was added to (Epi on) and removed from (Epi off) the perfusion medium by exchange at the indicated time points.

(Figs. 2a, 3). Figure 3 also shows an example in which, after facility and cyclic AMP were elevated in response to epinephrine, cessation of the epinephrine infusion was followed by a return of both measures to baseline.

In other biochemical studies of intact outflow tissue, excised trabecular meshwork cultured under conditions similar to those employed in the perfused eye studies showed a substantial increase in cyclic AMP content in response to epinephrine (Table 3).

Discussion

The results of this study, using isolated cultured outflow tissues, demonstrate that epinephrine increases outflow facility in the human eye in a dose-dependent manner. The ED_{50} for the facility effect in the present studies was 8×10^{-6} mol/l, with a maximal facility increase of 44% occurring at 2.5×10^{-5} mol/l. Previous biochemical studies of β-adrenergic-sensitive adenylate cyclase in the isolated rabbit ciliary processes[25] and in cultured human trabecular meshwork cells[29] have yielded a K_a for epinephrine-induced cAMP stimulation of 3×10^{-6} mol/l and 10^{-6} mol/l, respectively. This is the same order of magnitude as the estimated ED_{50} in the present study.

The epinephrine-induced increase in outflow facility that we observed appears to be mediated by β-adrenergic receptors. In studies with selective antagonists, we found that blockade of α-adrenergic receptors with phentolamine had no effect on the epinephrine-mediated facility response. In contrast, timolol completely blocked epinephrine's effect on facility. Our result is consistent with results obtained in previous studies using in vivo anterior chamber perfusion of whole eyes from subhuman species[9,11-16] and cell culture models,[2,4,29] which support involvement of β-adrenergic receptors in the effects of epinephrine on outflow facility and cAMP accumulation in trabecular meshwork cells, respectively.

Based on blocking studies with ICI118,551, we further conclude that epinephrine's effects are mediated, primarily by an action of β-2 adrenergic receptors. This physiologic observation is consistent with prior biochemical studies that show a predominance of β-2 receptors in intact[5] and cultured[4] trabecular meshwork. This observation also is consistent with clinical observations using noninvasive techniques,[30] suggesting a role for β-2 receptors in epinephrine's effect on facility. The present study is the first to use a highly subtype-specific antagonist to directly demonstrate β-2 involvement in the epinephrine-mediated facility effect. The use of the perfused anterior segment in the present study further localizes the effect on facility to the tissues of the outflow system. Although effects of epinephrine have been documented in animal eyes, prior attempts to demonstrate effects on outflow facility in isolated human eyes have been unsuccessful. Lack of responsiveness to epinephrine in the intact enucleated human eye may be a result of the rapid onset of postmortem tissue autolysis. With the model system used in the present study, highly vascularized tissue is removed and the outflow tissue is preserved in culture medium soon after death. This probably accounts for the maintenance of tissue viability and hormone responsiveness for several days under our culture conditions.[20]

The simultaneous measurement of outflow facility and analysis of perfusate samples at timed intervals after exposure to epinephrine demonstrates that epinephrine-induced increases in facility are associated with epinephrine-induced increases in cyclic AMP.

Table 3. Cyclic AMP accumulation in excised trabecular meshwork after exposure to 10^{-5} mol/l epinephrine

	Cyclic AMP accumulation after			
	10 min		*90 min*	
	(pmol/mg)	*(epi/control)*	*(pmol/mg)*	*(epi/control)*
Control	1.19 ± .08	—	1.22 ± .06	—
Epi	3.2 ± .18	2.7	5.12 ± .25	4.2

Pieces of trabecular meshwork (1–3 mm) from a pair of human eyes were excised and incubated in culture medium alone (control) or in culture medium containing 10^{-5} mol/l epinephrine (epi) for the indicated period of time. Cyclic adenosine monophosphate (AMP; pmol/mg protein) was measured by radioimmunoassay, as described in Materials and Methods. Data are means ± SEM of triplicate determinations.

These changes in total cyclic AMP were measured in the effluent from perfused anterior segments. Prior studies in a variety of cell culture systems have shown that, under basal and hormone-stimulated conditions, substantial amounts of cyclic AMP rapidly appear outside of cells in the extracellular medium. This extracellular cyclic AMP, which represents cyclic nucleotide transported out of cells, also has been observed in superfused tissues such as superior cervical ganglia.[31] Because changes in media or superfusant cyclic AMP concentrations have been found to be correlated with alterations in intracellular cyclic AMP levels, this extracellular cyclic AMP can be used, as in the present studies, as a marker for changes in intracellular cyclic AMP. Our direct measurement of intact trabecular meshwork pieces shows that under culture conditions similar to those of the perfused anterior segment, epinephrine increases intracellular cyclic AMP in this outflow tissue. Therefore, it is likely that our measurements in the perfusate (which potentially could also include some cyclic AMP generated by corneal endothelial cells and other nontrabecular cells) reflect intracellular events in the outflow pathway cells.

The observed elevation in cyclic AMP occurred within the first half hour of perfusion with epinephrine, whereas changes in outflow facility did not occur for about 90 min. Although this dissociation in time raises the question of whether the facility change is unrelated to cyclic AMP accumulation, published reports of increased outflow facility induced by analogues of cyclic AMP[11,12,14,16] strongly implicate the role of cyclic AMP in mediating epinephrine's effect on facility. Furthermore, in our studies with antagonists, we observed a strong association between increases in cyclic AMP and subsequent changes in facility. The delay in facility increase raises the possibility of a requirement for an alteration in intracellular events downstream from the second messenger cascade or possibly even subsequent extracellular events. Regarding this latter possibility, it should be noted that a second messenger role for cyclic AMP in mediating epinephrine's effects on outflow facility does not rule out an involvement of prostaglandins. A β-adrenergic stimulation of cyclic AMP leading (directly or indirectly) to a release of prostaglandins would be consistent with reports that indomethacin can block epinephrine-induced IOP reductions in the rabbit[32,33] and in the human eye.[34]

Future studies[35] hopefully will determine whether epinephrine's effects on outflow facility also involve the production or release of prostaglandins.

Key words: outflow facility, human eye, epinephrine, glaucoma, cyclic AMP

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