Pergamon

PII: S0042-6989(96)00106-X

Vision Res., Vol. 37, No. 1, pp. 9–15, 1997

Printed in Great Britain
0042-6989/97 $17.00 + 0.00

Facility Changes Mediated by cAMP in the Bovine Anterior Segment *In Vitro*

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Received 27 September 1995

The aim of this study was to investigate the influence of substances that increase intracellular cAMP levels on the aqueous humor outflow facility (*C*) of isolated bovine anterior segments.

Anterior segments were perfused *in vitro* at a constant pressure of 10 mmHg for 270 min with a general protocol as follows: 90 min control perfusion with DMEM, 90 min of experimental perfusion with DMEM containing the test drug(s), and 90 min of postdrug-perfusion with DMEM. *C* was calculated as the ratio between the rate of medium inflow (μl/min) and the perfusion pressure (mmHg).

Anterior segments can be perfused *in vitro* for up to 5 hr without significantly modifying their *C*. The addition of epinephrine, forskolin, dibutyryl-cAMP or isobutylmethylxanthine to the control perfusion medium elicited a significant increase of *C*. If, during isobutylmethylxanthine perfusion, forskolin or epinephrine was added, *C* increased significantly. Finally, perfusion with indomethacin prior to addition of epinephrine prevented the increase of *C* induced by epinephrine.

Epinephrine, the adenylate cyclase activator forskolin, the cAMP analog dibutyryl-cAMP, and the phosphodiesterase inhibitor isobutylmethylxanthine all increase aqueous facility. It seems reasonable to suspect that the cAMP system is involved in epinephrine's effects on bovine trabecular meshwork cells. Moreover, the complete inhibition by indomethacin of the outflow facility increase induced by epinephrine suggests that prostaglandins may be involved in the outflow facility mechanisms related to adrenoreceptor stimulation of trabecular meshwork cells. Copyright © 1996 Elsevier Science Ltd.

Trabecular meshwork Epinephrine cAMP Indomethacin Glaucoma

INTRODUCTION

The ocular hypotensive effects of catecholamines have been associated with activation of receptors coupled to adenylate cyclase (AC), an enzyme that induces a rise in cyclic adenosine 3′,5′-monophosphate (cAMP) levels (Sears & Neufeld, 1973; Gregory *et al.*, 1981; Caprioli *et al.*, 1984; Hoyng *et al.*, 1991). cAMP analogs and AC activators have been shown to reduce intraocular pressure (IOP) (Neufeld *et al.*, 1973; Potter & Rowland, 1981; Anderson & Williams, 1990). This hypotensive action of catecholamines and cAMP appears to be due, at least in part, to a decrease in aqueous humor secretion (Wax & Barrett, 1993; Bhattacherjee *et al.*, 1993; Mittag *et al.*, 1993). Nevertheless, biochemical and immunocytochemical studies have reported the presence of AC in trabecular meshwork tissue of bovine, monkey, and human eyes (Bartels, 1988; Crawford *et al.*, 1991; Hoyng & Busch, 1992; Erickson-Lamy & Nathanson, 1992; Busch *et al.*, 1993). Therefore, the possibility exists that cAMP is also implicated in the modulation of aqueous humor outflow facility (*C*) (Neufeld & Sears, 1975; Bartels *et al.*, 1981).

Aqueous humor drainage through conventional pathways (i.e., via the trabecular meshwork and Schlemm's canal) can be studied in an isolated anterior segment perfused *in vitro*, a preparation in which the contributions to *C* from drainage through uveoscleral routes, from changes in choroidal volume, or from pseudofacility are excluded (Erickson-Lamy *et al.*, 1988, 1991).

The aim of the present work was to investigate the influence of substances that increase intracellular cAMP levels on the aqueous humor *C* of the isolated anterior segment of bovine eyes. Preliminary results have been reported elsewhere (Gilabert *et al.*, 1994a,b).

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MATERIALS AND METHODS

Eyes

Eyes from cows 3–6 months old were obtained in the local abattoir. Enucleation was made between 2 and 4 hr after death. Eyes were immediately submerged in cold (4°C) phosphate buffered saline (PBS) at pH 7.42, supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin, and 5 μg/ml amphotericin B.

Perfusion

The technique for perfusion of the anterior segment described by Erickson-Lamy *et al.* (1988, 1991) was employed. In brief, 30–40 min after enucleation, eyes were bisected along the equator. Anterior segments were placed in a specially designed chamber (Erickson-Lamy *et al.*, 1988) and perfused with Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 μg/ml glucose (Gibco), 50 IU/ml penicillin (Seromed-Biochrom), 50 μg/ml streptomycin (Seromed-Biochrom), and 5 μg/ml amphotericin B (Boehringer Mannheim). The pH of the perfusion medium was adjusted to 7.42 at a temperature of 36°C. The osmolarity of the solution was 303.2 $\pm$ 4.2 mOs/kg (mean $\pm$ SD). Perfusion of the anterior segment was carried out in an incubator (Selecta, 4000602, Spain), maintained at a temperature of 36°C in an atmosphere of 5% CO_2 in air. The perfusion pressure was kept constant at 10 mmHg. The inflow rate was determined by continuous weighing of the perfusion medium reservoir with a force-displacement transducer (Scaime-GM3, France) connected to a chart recorder (Yew, 3021, Japan).

Outflow facility measurements

C was calculated as the ratio (μl/min/mmHg) between the rate of medium inflow (μl/min) and the perfusion pressure (mmHg). Outflow facility values were expressed as the average of 15 min facility determinations. Baseline facility (C_0) was calculated at the beginning of the experiment, using the first 90 min period of stable inflow of medium. Facility values after administration of a drug (C_d) were determined when the inflow of medium following the application of the drug had reached a new steady-state level.

Drugs

Epinephrine (EPI), forskolin (FSK), dibutyryl-cAMP (db-cAMP) and isobutylmethylxanthine (IBMX) were obtained from Sigma Chemical Co. (Madrid, Spain). Indomethacin (INDO) was obtained from Merck, Sharp & Dohme (Inacid® IM, Spain); Inacid powder was diluted in 2 ml DMEM just before each experiment. Stock solutions of FSK (2×10^{-2} M) and db-cAMP (5×10^{-2} M) were made in DMSO (Sigma, Spain). Stock solutions of IBMX were prepared in 60% ethanol (Sigma, Madrid, Spain) in PBS (Gibco). Stock solutions were stored at -20°C. Solutions of EPI and INDO were prepared in DMEM immediately before perfusion. Just before perfusion, drugs were dissolved in DMEM to final concentrations of: EPI, 1 μM; FSK, 1–10 μM; db-cAMP, 0.1–1 μM; IBMX, 1 μM; and INDO, 1 μM. Final concentrations of ethanol and DMSO in the DMEM were under 1%.

Experimental protocol

After the onset of perfusion with control medium (DMEM), C_0 was determined for 90 min. Thereafter, the control perfusion medium was substituted by the perfusion medium containing the drug. Following a 90 min perfusion period with the drug, C_d was determined. Finally, perfusion was resumed with control DMEM for 90 min, and the post-drug outflow facility (C_{pd}) calculated.

A separate group of 13 anterior segments was perfused only with control medium for 5 hr, to determine the variation of outflow facility with time. Also, two groups of six anterior segments were respectively perfused with 1% ethanol and 1% DMSO as a control for those experiments employing experimental drugs which require them for solubilization (Gilabert *et al.*, 1994b).

Data analysis

Drug effects were calculated as the ratio between C during or after drug application and baseline facility (C_d /C_0 or C_{pd} /C_0). Results were expressed as mean $\pm$ SEM. C at each time vs baseline facility at 60 min was compared in all protocols using Student's paired t-test with Bonferroni correction. Comparisons between the C_d in one protocol vs the C_d in another protocol at the same time were made using Student's unpaired t-test with Bonferroni correction. Significance in either case was set at $P < 0.05$ (*), $P < 0.01$ (**), or $p < 0.001$ (***).

RESULTS

Table 1 summarizes the modifications of C induced by DMEM, vehicles, and the different cAMP-related drugs employed in this study.

Facility was not significantly modified following 4.5 hr of perfusion with DMEM ($P > 0.37$) as indicated the ratio of $C_d/C_0 = 0.97 \pm 0.05$ (Table 1). Also, perfusion with DMEM containing 1% ethanol or 1% DMSO, the vehicles used to solubilize the drugs in this study, did not produce any significant ($P > 0.29$ or $P > 0.80$) changes in C.

Epinephrine

C increased significantly over baseline ($P < 0.01$, $n = 9$) 30 min after beginning perfusion with 1 μM EPI, reaching a maximum between 30 and 60 min after the onset of EPI perfusion. C_{pd} returned to initial values after restoration of perfusion with DMEM control medium [Table 1 and Fig. 1(A)]. For comparison, Fig. 1(A) also depicts the outflow facility ratio during perfusion of anterior segments with DMEM control solution during an equivalent period of time (270 min).

Forskolin

Perfusion with FSK (1 and 10 μM) induced a dose-related increase in C. With 10 μM FSK, C_d increased significantly over C_0 30 min after the onset of perfusion

TABLE 1. Outflow facility of the perfused bovine anterior segment during perfusion with DMEM, vehicles and different drugs and concentrations

	Protocol	n	C_0		C_d	C_d/C_0	C_{pd}/C_0
Control	DMEM	13	1.15 ± 0.20	1.05 ± 0.17	0.91 ± 0.18	0.97 ± 0.05	0.92 ± 0.05
Vehicles	Ethanol 1%	6	0.78 ± 0.32	0.76 ± 0.33	0.84 ± 0.34	0.96 ± 0.02	1.05 ± 0.05
	DMSO 1%	5	0.74 ± 0.21	0.73 ± 0.20	0.70 ± 0.27	1.01 ± 0.05	1.02 ± 0.08
Drugs	EPI 1 μM	9	1.11 ± 0.19	1.36 ± 0.21***	1.20 ± 0.18**	1.26 ± 0.05†††	1.11 ± 0.08
	FSK 1 μM	6	1.15 ± 0.23	1.32 ± 0.27*	1.15 ± 0.29	1.17 ± 0.04†	0.95 ± 0.09
	FSK 10 μM	6	0.89 ± 0.18	1.24 ± 0.20**	0.97 ± 0.21	1.43 ± 0.11†††	1.05 ± 0.15
	IBMX 1 μM	8	0.63 ± 0.15	0.73 ± 0.16**	0.73 ± 0.15**	1.20 ± 0.05††	1.23 ± 0.07††
	db-cAMP 0.1 μM	6	0.85 ± 0.13	1.20 ± 0.19*	1.05 ± 0.15	1.41 ± 0.07†††	1.26 ± 0.12††
	db-cAMP 1 μM	6	0.87 ± 0.19	1.27 ± 0.30**	1.29 ± 0.31	1.41 ± 0.12††	1.48 ± 0.10†††
	INDO 1 μM	8	0.55 ± 0.09	0.56 ± 0.11	0.59 ± 0.10	1.00 ± 0.06	1.06 ± 0.08

C_0, baseline facility; C_d, average of facility during drug perfusion; C_{pd}, average of facility after drug perfusion obtained in DMEM; EPI, epinephrine; FSK, forskolin; IBMX, isobutylmethylxanthine; db-cAMP, dibutyryl-cAMP; INDO, indomethacin. Data are mean ± SEM. n, number of eyes. Comparisons of this table have been performed using paired (*, C_x vs their C_0) or unpaired (†, C_x/C_0 vs C_x/C_0 control DMEM group) Student's t-test. Significances are as indicated in the data analysis section of Methods.

($P < 0.05$, $n = 6$). When a lower concentration (1 μM FSK) was applied, the increase in C_d was not statistically significant ($P > 0.05$). In both cases, C_{pd} recovered to its initial values on returning to perfusion with control medium [Table 1 and Fig. 1(B)].

Dibutyryl cyclic-AMP

A significant increase of C_d was obtained 60 min after perfusion with either 0.1 μM ($P < 0.01$) or 1 μM db-cAMP ($P < 0.05$) [Table 1 and Fig. 1(C)]. The time course of the change in C_d [Fig. 1(C)] shows that perfusion with both concentrations of db-cAMP increased C after 60 min of perfusion. After returning to perfusion with DMEM control solution, C_{pd} recovered towards baseline values following perfusion with the lower concentration of db-cAMP (0.1 μM). However, C_{pd} remained elevated throughout the rest of the experiment when 1 μM db-cAMP was perfused.

Isobutylmethylxanthine

Perfusion with IBMX 1 μM significantly increased C_d after 60 min ($P < 0.05$) [Table 1 and Fig. 2(A)].

When 1 μM EPI was added to the perfusion medium 30 min after beginning perfusion with 1 μM IBMX (EPI–IBMX), a significant ($P < 0.001$) increase of C_d over the post-IBMX values was observed [Fig. 2(B)]. This C_d elevation at 60 min was higher compared to the value obtained with 1 μM EPI alone at the same time. Moreover, addition of 10 μM FSK (FSK–IBMX) 30 min after the onset of perfusion with 1 μM IBMX evoked a significant ($P < 0.05$) additional increase in C [Fig. 2(C)]. Both increases induced by EPI–IBMX or FSK–IBMX were higher than the increases induced by either EPI or IBMX alone and FSK or IBMX alone and appeared to be additive.

In any condition in which IBMX was perfused, either alone or with EPI or FSK, C_{pd} did not recover to baseline values, instead it remained high in comparison with DMEM control for the 90 min post-drug perfusion time ($P < 0.001$).

For the purpose of comparison, the plot of EPI alone [Fig. 2(B)] and FSK alone [Fig. 2(C)] has been normalized by shifting it 30 min to the right so that the start of the perfusion of EPI or FSK in all plots in each figure coincides.

Indomethacin

As shown in Fig. 3, perfusion with INDO 1 μM for 90 min did not induce any significant ($P > 0.89$) change in C during the entire protocol (C_d and C_{pd}). Note that Fig. 3 shows that the addition of EPI 30 min after beginning INDO perfusion (EPI–INDO protocol) did not cause any further modification of C_d ($P > 0.5$). Figure 3 also depicts the outflow facility ratio C_d during perfusion with only EPI (1 μM) during an equivalent period of time [see Fig. 1(A)]. For the purpose of comparison, the plot of EPI alone has been normalized by shifting it 30 min to the right so that the initiation of the perfusion of EPI plus INDO and of EPI alone have been set to coincide.

DISCUSSION

In acutely cultured anterior segments of young cow eyes, aqueous humor outflow facility remains stable during long perfusion periods (up to 4.5 hr) and responds consistently to various drugs. This observation confirms the usefulness of cultured outflow tissues as a simple model to analyze the effects of drugs on aqueous drainage through conventional pathways.

Adrenergic influences on IOP are well documented. Sympathetic stimulation or topical administration of adrenergic agonists decrease IOP (Eakins & Ryan, 1964; Langham & Rosenthal, 1966; Bill, 1969). However, ocular hypotension can also be obtained by administration of either alpha or beta-blocking drugs (Vareilles *et al.*, 1977; Boas *et al.*, 1981; Öhrstroöm, 1981; Trope & Rumley, 1985). Blockade of adrenergic receptors in the ciliary epithelium, leading to the inhibition of aqueous inflow, has been hypothesized to be responsible for these hypotensive effects (Zimmerman *et al.*, 1977). However, the possibility that aqueous outflow is influenced by adrenergic stimuli in the trabecular meshwork has received less attention (Langham & Diggs, 1974; Erickson-Lamy & Nathanson, 1992; Tripathi *et al.*,

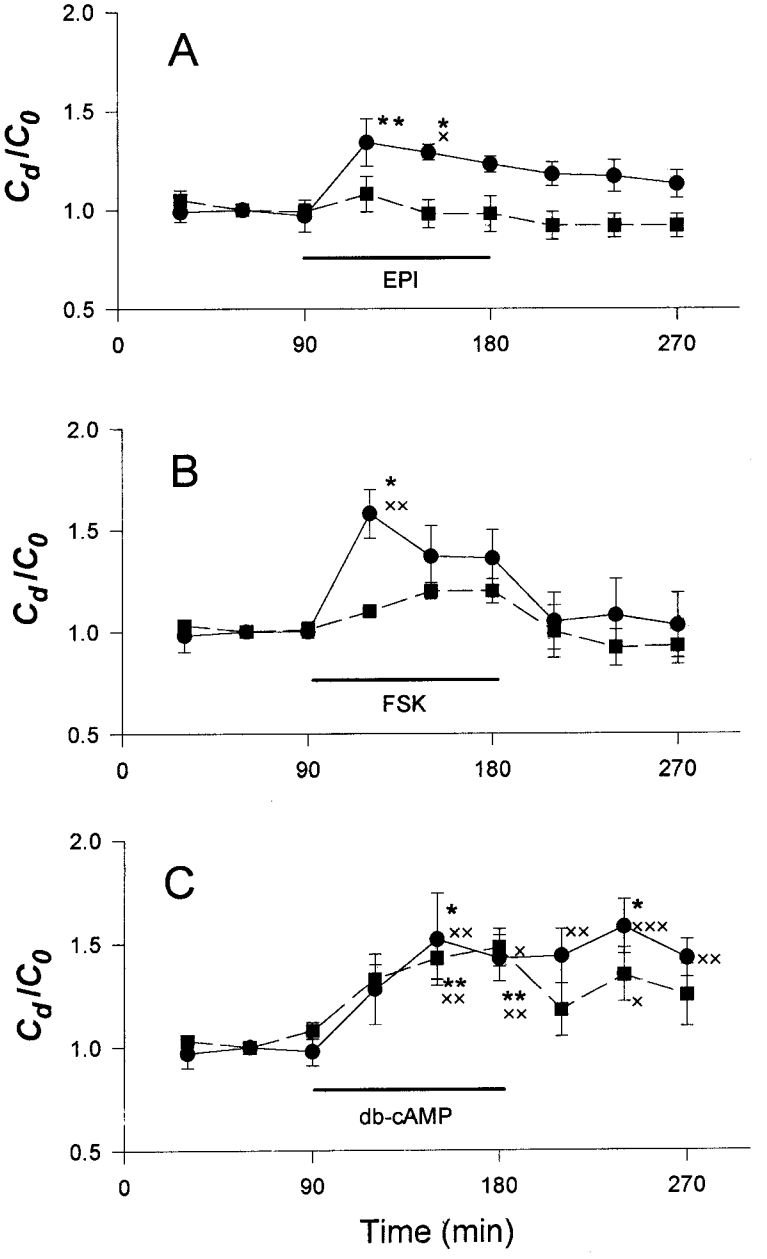

FIGURE 1. Effects of perfusion with DMEM and epinephrine (EPI) (A), forskolin (FSK) (B) and dibutyryl cyclic-AMP (db-cAMP) (C) on outflow facility in bovine anterior segments. (A) Perfusion with DMEM for 4.5 hr (squares and broken line, $n = 13$) and perfusion with 1 μM EPI (dots and solid line, $n = 9$). (B) Perfusion with FSK 1 μM (squares and broken line, $n = 6$) and FSK 10 μM (dots and solid line, $n = 6$). (C) Perfusion with db-cAMP 0.1 μM (squares and broken line, $n = 6$) and db-cAMP 1 μM (dots and solid line, $n = 6$). The solid horizontal bars represent the period of perfusion with the drug. Data are expressed as mean $\pm$ SEM (n = number of eyes) of the ratio (C_d/C_0) of the drug-treated facility to the baseline facility. The statistical significance of paired comparisons between C at each time vs baseline C at 60 min for each protocol are indicated by (*), while the statistical significance of unpaired comparisons between the C vs control C in the DMEM protocol at the same time are indicated by multiplication signs (×). Student's t-tests with Bonferroni correction were used in both cases.

1992). The present study shows that, under our experimental conditions, the mixed α and β adrenoceptor agonist, EPI, increased true outflow facility. Based on the abundant evidence obtained in other systems, it seems reasonable to suspect that the outflow facility increase induced by EPI is associated with AC activation and with elevated intracellular cAMP levels in trabecular cells. This assumption was supported by the observation that FSK, a direct stimulator of the catalytic unit of AC, evoked a dose-dependent increase on outflow facility. AC is present in the membrane fraction and whole tissue homogenates of bovine trabecular meshwork (Busch *et al.*, 1993). Moreover, perfusion with the cAMP analog, db-cAMP, markedly increased outflow facility. This increase persisted 90 min after cessation of perfusion, in agreement with previous evidence showing a long-lasting IOP reduction after intravitreal injection of cAMP analogs in rabbits (Bartels *et al.*, 1981).

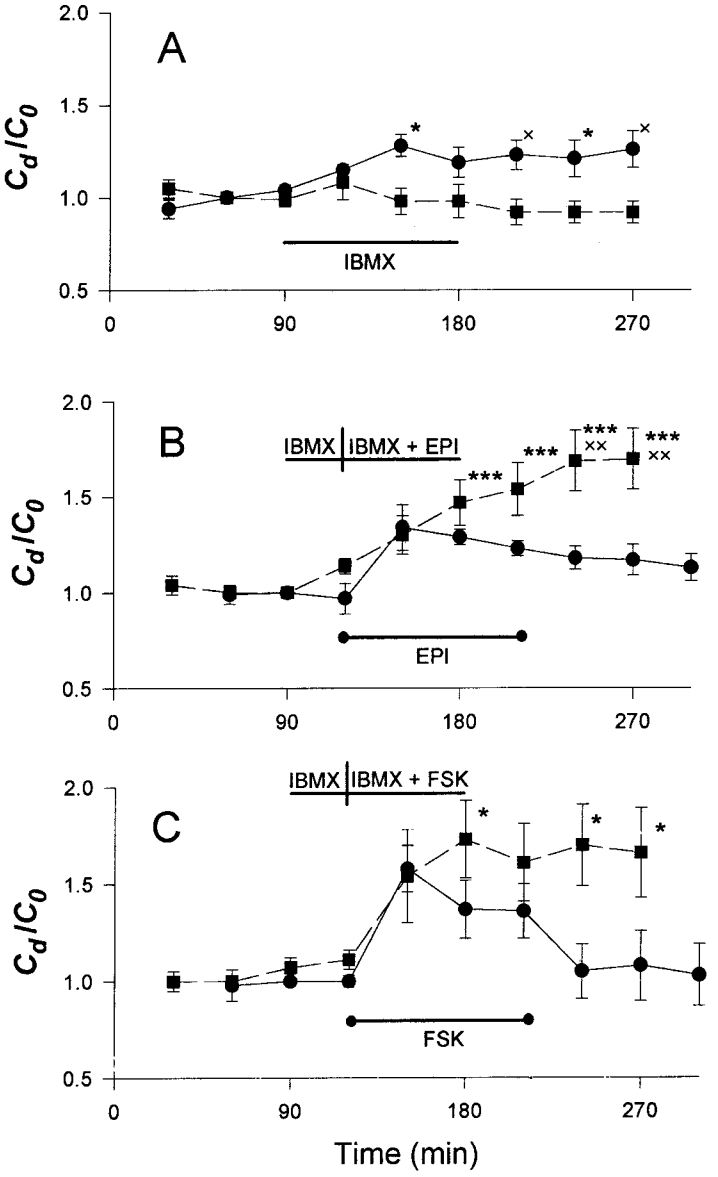

FIGURE 2. Effects of perfusion with isobutylmethylxanthine (IBMX) alone (A), combined with epinephrine (EPI) (B) or with forskolin (FSK) (C) on outflow facility in anterior segments. (A) shows perfusions with 1 μM IBMX (dots and solid line, $n = 8$) and DMEM (squares and broken line, $n = 13$). (B) shows perfusion with 1 μM IBMX plus 1 μM EPI (squares and broken line, $n = 6$) and 1 μM EPI (dots and solid line, $n = 9$) alone. (C) shows 1 μM IBMX plus 10 μM FSK (squares and broken line, $n = 6$), and 10 μM FSK alone. For purposes of comparison, the plots of EPI alone (B) and FSK (C) have been normalized by shifting it 30 min to the right, so that the start of perfusion with EPI or FSK plus IBMX and EPI or FSK alone has been set to coincide. The solid horizontal bars represent the period of perfusion with the drug(s). Data are expressed as mean $\pm$ SEM (n = number of eyes) of the ratio (C_d/C_0) of the drug-treated facility to the baseline facility. The statistical significance of the paired comparisons between C at each time vs baseline C at 60 min for each protocol are indicated by (*), while statistical significance of the unpaired comparisons between C vs C of the control DMEM protocol at the same time are indicated by multiplication signs (×). Student's t-tests with Bonferroni correction were used in both cases.

The intracellular catabolism of cAMP is mediated by phosphodiesterase and is diminished by phosphodiesterase inhibitors (Beavo *et al.*, 1970). In our preparation, IBMX perfusion was associated with a slight but statistically significant increase in outflow facility. Moreover, simultaneous perfusion of EPI or FSK and IBMX increased outflow facility more than when EPI or FSK were applied alone, and the increase appeared to be additive to the increase due to IBMX. These additive effects are consistent with blockade of intracellular cAMP degradation by IBMX, and their time course appeared similar to the irreversible facility increase elicited by 1 μM db-cAMP.

It should be considered that the effect of EPI on outflow facility might not be mediated exclusively by stimulation of AC through β_2-adrenoceptors. For instance, isoproterenol does not activate AC on bovine trabecular cells (Busch *et al.*, 1993). However, an increase in the content of cAMP in aqueous humor after sympathetic nerve stimulation (Gallar & Liu, 1993) or perfusion with norepinephrine or EPI has been reported (Norton & Viernstein, 1972; Erickson-Lamy & Nathan-

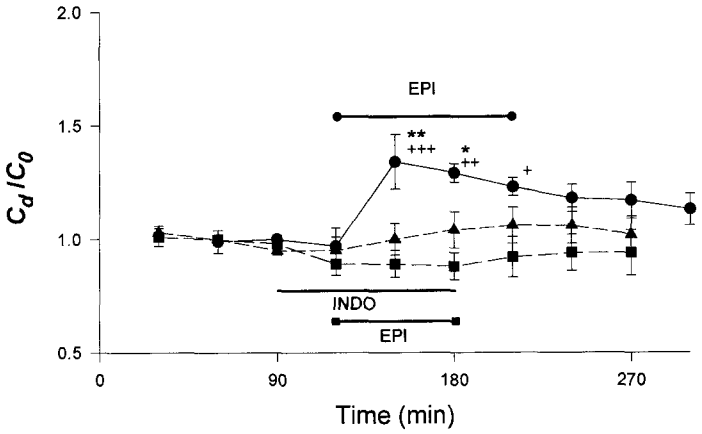

FIGURE 3. Effects of perfusion with indomethacin (INDO) alone or in combination with EPI on outflow facility in anterior segments. 1 μM INDO (triangles and broken line, $n = 7$). 1 μM INDO plus 1 μM EPI (squares and broken line, $n = 6$). 1 μM EPI (dots and solid line, $n = 9$). For purposes of comparison, the plot of EPI alone has been normalized by shifting it 30 min to the right, so that the start of perfusion with EPI plus INDO, and EPI alone has been set to coincide. The solid horizontal bars represent the time of perfusion with the drug(s). Data are expressed as mean $\pm$ SEM (n = number of eyes) of the ratio (C_d/C_0) of the drug-treated facility to the baseline facility. The statistical significance of paired comparisons between C at each time vs baseline C at 60 min for each protocol are indicated by (*), while the statistical significance of the unpaired comparisons between C of the EPI protocol vs C of the INDO plus EPI protocol are indicated by (+). Student's t-tests with Bonferroni correction were used in both cases.

son, 1992). Thus, adrenergic stimulation might affect intracellular events downstream from the second messenger cascade.

However, it should be noted that the cAMP elevation was not correlated in time with the IOP reduction and outflow facility evoked by these maneuvers (Norton & Viernstein, 1972; Erickson-Lamy & Nathanson, 1992). An alternative explanation for the effects of IBMX is that they were due to the action of methylxanthines as competitive inhibitors of adenosine (Fredholm, 1980). Endogenous adenosine antagonizes some of the effects of adrenergic agents (e.g., inhibition of AC), and adenosine receptor agonists have been shown to increase IOP in rabbits (Hirschfield *et al.*, 1986).

In our experiments, INDO effectively blocked the EPI induced increases in outflow facility. This suggests an involvement of prostaglandins (PGs) in the facility effects of this catecholamine. It has been reported that PGE_1 and PGE_2 elevate cAMP in bovine and human trabecular tissue and in iris-ciliary body, via AC activation (Bhattacherjee *et al.*, 1993; Busch *et al.*, 1993). Moreover, effects of PGs on outflow and IOP modulation have been reported (Bhattacherjee & Hammond, 1977; Camras *et al.*, 1985; Nebigil & Malik, 1993; Poyer *et al.*, 1992) albeit via a non-trabecular mechanism of action. Thus, the possibility exists that EPI modifies outflow facility or conventional pathways via a mechanism involving cyclo-oxygenase products. It should be noted that, although the concentration of INDO used (10^{-6} M) is that commonly employed in organ culture (Erickson *et al.*, 1994), at this high a concentration, INDO could have other actions besides inhibition of fatty acid cyclo-oxygenase (e.g., inhibition of phosphodiesterase (Insel, 1990)).

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Acknowledgements—The study was supported in part by FISss grant 94/1180, Spain. RGV was supported by *Fundació CLÍNIC* fellowship from Cusí, SA.