Effects of Al³⁺ and Be²⁺ Ions Combined With NaF on Ciliary Process Adenylyl Cyclase Activity and Aqueous Humor Dynamics in the Rabbit Eye

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Purpose. The activity of Al³⁺, Ga³⁺, and Be²⁺ ions in the presence of NaF to directly activate G-proteins was investigated by their potentiative effect on forskolin (FSK)-activated adenylyl cyclase in rabbit ciliary process membranes and their effects on aqueous humor dynamics in vivo.

Methods. Adenylyl cyclase (AC) was determined by radiometric conversion of ATP to cAMP by the particulate fraction of rabbit ciliary processes. Intravitreal injections of sterile solutions of analytical grade salts were made into the center of the vitreous in a volume of 20 μl. Intraocular pressure, aqueous humor flow, and uveoscleral outflow measurements were made by pneumatonometry, fluorophotometry, and fluorescein-dextran method, respectively. Outflow facility was determined by tonography in the intact eyes and by two-level constant pressure perfusion in cannulated eyes.

Results. Both Al³⁺ (EC$_{50}$, 40 μmol/l) and Be²⁺ (EC$_{50}$, 11 μmol/l) in the presence of 0.5–2 mM NaF activated the stimulatory G-protein G$_s$. Ga³⁺ was ineffective and did not antagonize the activation by Al³⁺. Intravitreal injections of Al³⁺ (1 μmol/eye) or Be²⁺ (0.5 or 1 μmol/eye) had no significant intraocular pressure (IOP) effect, nor did 1.5 or 3 μmol/eye of NaF, but when either cation was injected together with NaF, IOP decreased by up to 40% for up to 140 hr. At the time of maximum IOP effect (72 hr) aqueous humor flow determined by fluorophotometry was decreased in BeCl₂+NaF-treated eyes by 40% relative to BeCl₂-treated eyes; however, tonographic facility of outflow was unaffected. Uveoscleral flow was also decreased by 38% in BeCl₂+NaF treated eyes.

Conclusions. These findings support the hypothesis that G$_s$ activation of ciliary process adenylyl cyclase decreases aqueous humor formation rate in rabbit eyes, and that activation of G-proteins mediates contraction of ciliary muscles causing a decrease of aqueous humor outflow via the uveoscleral route. The results suggest that G-proteins putatively involved in trabecular facility changes are less sensitive to activation by BeF₃⁻ than are other parameters of aqueous humor dynamics. Invest Ophthalmol Vis Sci. 1993;34:606–612.

Guanine nucleotide binding proteins (G-proteins) are important intermediates in the regulation of cellular responses by external stimuli. The heterotrimeric class of G-proteins function as signal transducers by

coupling a wide range of receptors located on cell membranes to their intracellular effector systems.[1] Sternweis and Gilman[2] showed that the well-known stimulatory effect of fluoride on adenylyl cyclase resulted from direct activation of G_s by the fluoroaluminate anion (AlF_4^-). This activation is highly specific; of 30 divalent and trivalent metal cations tested, only Al^{3+} and Be^{2+} activated G_s in the presence of F^-. Activation by the fluoride complexes of these two cations appears to be a general response of heterotrimeric G-proteins, such as transducin,[3] and G-proteins that couple receptors to inositol phosphate metabolism and calcium-mediated cellular responses.[4]

The major clinical drugs used for glaucoma therapy—adrenergic blockers, epinephrine, and pilocarpine—act via receptors coupled to G-proteins. Receptor coupling via G-proteins also applies to other, less common clinical or experimental agents that affect intraocular pressure (IOP), including α_2-adrenergic agonists[5,6] and a variety of peptides (eg, VIP[7] and angiotensin[8]). There is still considerable uncertainty about the mechanism of action of many of these agents, both at the cellular level[9] and the extent to which aqueous humor inflow, trabecular outflow, or uveoscleral outflow contribute to the in vivo IOP lowering effect. In this study, we report on experiments aimed at bypassing the receptor level and activating G-proteins directly by AlF_4^- and BeF_3^- in rabbit ciliary processes. The effect of these agents was studied biochemically on the adenylyl cyclase enzyme and the major components of aqueous humor dynamics: aqueous humor formation rate, uveoscleral outflow, and trabecular outflow in the albino rabbit eye.

METHODS

Materials and methods were the same as used in previous publications, as outlined below.

Adenylyl cyclases assays were done on the particulate fraction from rabbit ciliary processes homogenized in a low-salt isotonic, chelating buffer (EDTA and EGTA) containing an antioxidant, (dithiothreitol), antiprotease (leupeptin), and antiphospholipase (indomethacin), as previously described.[7,10] Adenylyl cyclase activity was determined by the radiometric assay[11] and sample tubes were run with 3 mM Mg^{2+} present for 3 min before column separation of ^{32}P-cAMP, as previously described.[7,10,12]

Intravitreal Injections and Intraocular Pressure (IOP) Determinations

Analytical grade salts for contralateral control-eye injections ($NaCl$, Na_2SO_4, $NaNO_3$) and for experimental or control-eye injections (NaF, $Ga_2[SO_4]_3$, $Al_2[SO_4]_3$, $BeNO_3$, $BeCl_2$) were made up in sterile H_2O and filtered through a 0.22-μm Millipore filter before use.

All injections were 20 l and were delivered into the center of the vitreous cavity by sterile syringe (26-gauge needle) in rabbits under systemic (12.5 mg/kg) ketamine and local topical ocular 0.5% proparacaine anesthesia, as previously described.[12] Intraocular pressure measurements were made at intervals for up to 6 days postinjection by pneumatonograph, as previously described.[12] Intraocular pressure responses of experimental eyes were evaluated as the percent change relative to the contralateral (control) eye IOP measured at the same time ±SEM in groups of six rabbits for each IOP/time curve shown in Figures 4, 5, and 6.

Uveoscleral Outflow, Aqueous Humor Flow, and Outflow Facility Determinations

Fluorophotometric measurements of aqueous flow were made on rabbit eyes pretreated with 4×50 μl topical fluorescein 16 hr before measurements exactly as previously reported.[13] Fluorescein-dextran (MWt 75,000) was used for uveoscleral outflow determinations in eyes perfused at 20 mmHg constant pressure,[14] and tonography was used for conventional (trabecular) outflow facility measurement.[15] Outflow facility was also determined in cannulated eyes of albino rabbits (2–3 kg bodyweight) anesthetized with urethane (400 mg/kg IV) by two-level constant pressure (30 and 40 mmHg) perfusion according to the method of Barany.[16] $BeCl_2$+ NaF at 1+3 and 2+6 mM was included in the perfusion medium of the experimental eyes with contralateral control eyes receiving normal Barany's solution. Pressure changes were made every 10 min over a 2-hr period and fluid flow measured for 8 min, starting 2 min after the pressure change, in each period.

This investigation adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.
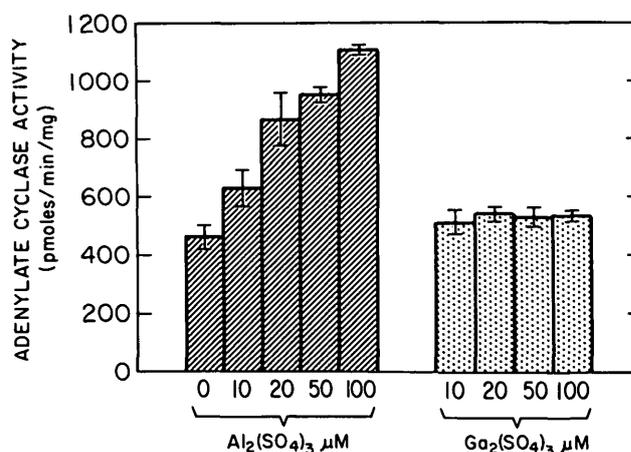

FIGURE 1. Comparison of $Al_2(SO_4)_3$ and $Ga_2(SO_4)_3$, 0-100 μmol/l together with 500 μmol/l NaF on activation of ciliary process membrane AC by 3.3 μmol/l FSK.
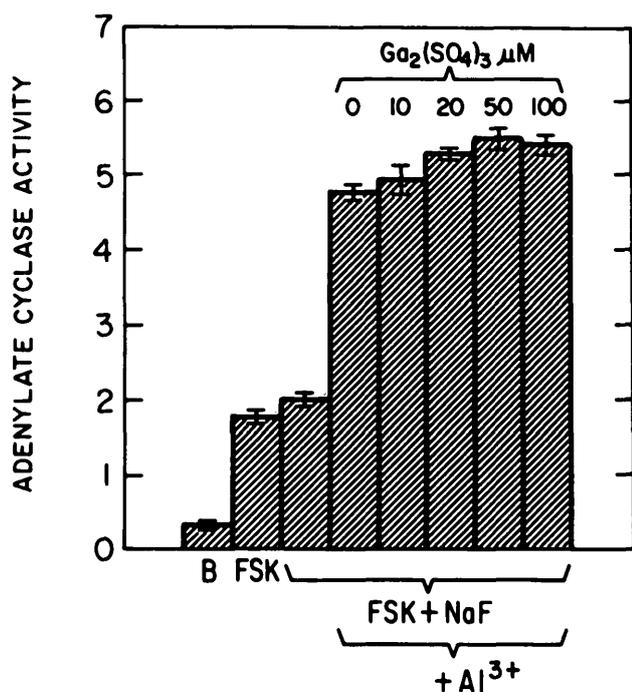
FIGURE 2. Lack of inhibition by $Ga_2(SO_4)_3$, 10–100 $\mu mol/l$, on stimulation by 100 $\mu mol/l$ $Al_2(SO_4)_3$+500 $\mu mol/l$ NaF of ciliary process AC activated by 3.3 $\mu mol/l$ FSK. Enzyme activity units on y-axis given in nmol/mg protein/10 min.

RESULTS

Biochemical Experiments

The ability of Al^{3+} and Be^{2+} to activate G-proteins was tested on G_s activation of adenylyl cyclase in ciliary processes by comparison to Ga^{3+}, a trivalent ion with chemistry related to that of Al^{3+}.

Figure 1 shows the AC response of ciliary process membranes in the presence of 0.5 mM NaF and 3.3 $\mu mol/l$ forskolin when $Al_2(SO_4)_3$ or $Ga_2(SO_4)_3$ was
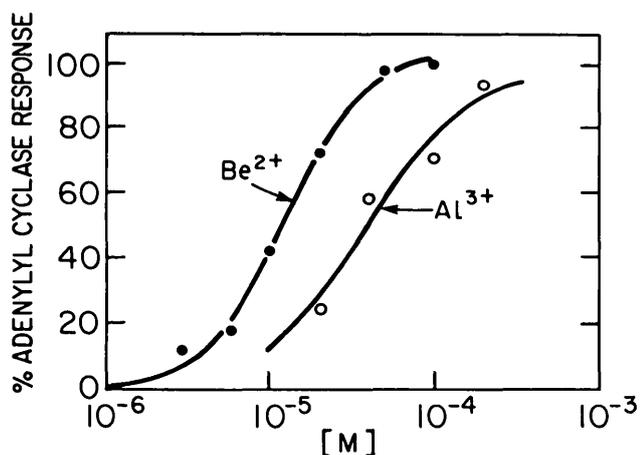

FIGURE 3. Normalized dose-response curves for Al^{3+} and Be^{2+} activation of the ciliary process AC response to 3.3 $\mu mol/l$ FSK in the presence of 500 $\mu mol/l$ NaF. EC_{50} for Be^{2+}, ~11 $\mu mol/l$, for Al^{3+}, ~38 $\mu mol/l$.
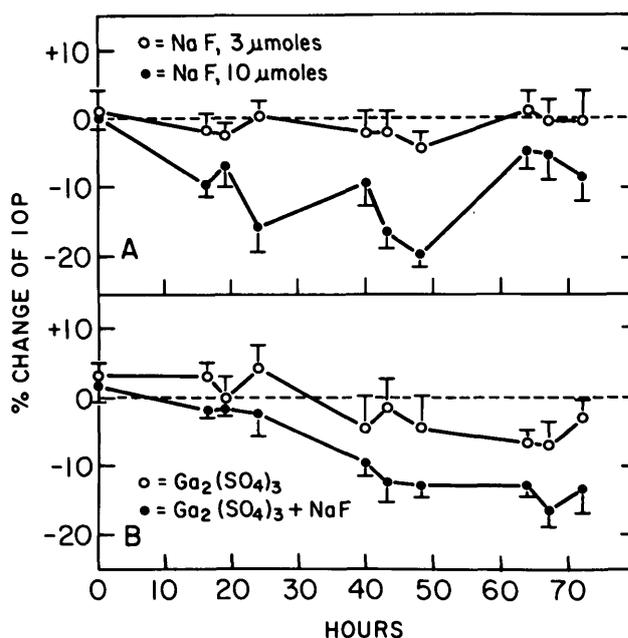

FIGURE 4. (A) Effect on IOP in the rabbit eye of intravitreal injection of 3 (open symbols) or 10 (closed symbols) $\mu mol/$ eye of NaF relative to 3 or 10 μmol of NaCl injected into contralateral control eyes. Error bars represent SEM for percent change relative to control eyes in six rabbits. (B) Intraocular pressure effect (% change) of intravitreal injection of 0.5 $\mu mol/$eye $Ga_2(SO_4)_3$ relative to injection of Na_2SO_4 (2 $\mu mol/$eye) in contralateral control eyes (open symbols), and to injection of 0.5 μmol $Ga_2(SO_4)_3$+3 μmol NaF relative to Na_2SO_4 (2 μmol)+3 μmol NaF in contralateral control eyes (closed symbols). Error bars represent SEM for n=6.
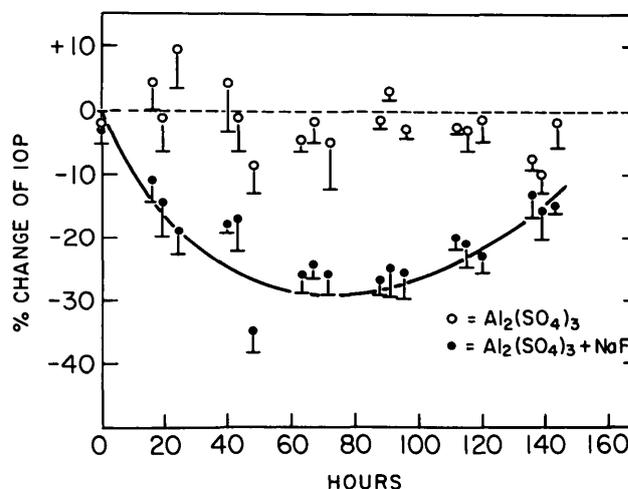
FIGURE 5. Percentage change of IOP in eyes of six rabbits given intravitreal injections of $Al_2(SO_4)_3$ (0.5 $\mu mol/$eye) relative to contralateral control eyes injected with 2 μmol Na_2SO_4 (open symbols). Curve with closed symbols shows percent change of IOP in eyes given intravitreal injections of 0.5 μmol $Al_2(SO_4)_3$+3 μmol NaF relative to contralateral eyes given intravitreal injections of Na_2SO_4 (2 μmol)+NaF (3 μmol). Bars represent SEM.
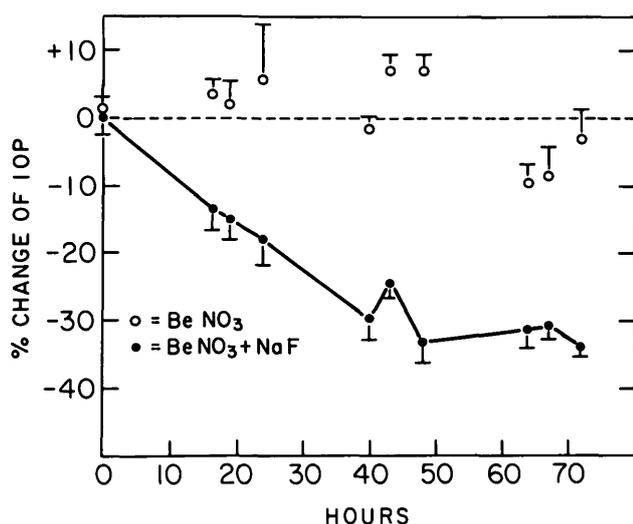
FIGURE 6. Percent change of IOP in eyes of six rabbits given intravitreal injection of 1 μmol Be(NO$_3$)$_2$ (open symbols) or Be(NO$_3$)$_2$ (1μmol)+NaF (3 μmol) relative to control eyes given intravitreal injections of Na$_2$ NO$_3$ (2 μmol). Bars represent SEM.

added in varying amounts. Fluoroaluminate potentiated the response of forskolin, showing a dose-dependent activation of G$_s$, while the corresponding fluorogallate (GaF$_4^-$) is inactive. The data shown in Figure 2 indicates that GaF$_4^-$ also does not antagonize the stimulation of AC by AlF$_4^-$, and, therefore, does not bind to the site on G$_s$ responsible for activation by fluoroaluminate. Be^{2+}, the only other cation similarly reported to activate G$_s$ in the presence of F$^-$, is about fourfold more active on a molar basis than is Al^{3+} (Fig. 3).

Biologic Experiments

Because of the ionic nature of these agents, they were administered to rabbit eyes by intravitreal injection. The initial experimental design was to determine a threshold dose of NaF which did not affect intraocular pressure by itself. Sodium fluoride at 10 μmol per eye had a small effect on IOP, but the injection of 3 μmol per eye was not significantly different from the NaCl-injected contralateral eyes, as shown in Figure 4a. In the next experiments, we determined the intraocular effect of intravitreally injected Al^{3+}, Ga^{3+}, or Be^{2+} alone or when combined with sodium fluoride in the ratio of approximately 1:3. Injection of 1 μmol Ga^{3+} (as Ga$_2$ [SO$_4$]$_3$) alone gave no response, but when given together with 3 μmol of F$^-$ there was a modest decline that barely reached statistical significance at only two time points during the 72-hr period of measurement relative to eyes given 1 μmol Ga^{3+} only (Fig. 4b). A similar experiment using the same dose of Al$_2$ (SO$_4$)$_3$ caused a prolonged fall in IOP lasting 6 days (Fig. 5). The same response was obtained when 1 μmol of Be^{2+} was introduced into eyes together with 3 μmol of F$^-$ (Fig. 6).

The mechanism for the decreased IOP was examined in additional groups of rabbit eyes (Table 1) injected with 0.5 μmol BeCl$_2$+1.5 μmol NaF (experimental eyes, X) and compared to contralateral eyes receiving 0.5 μmol BeCl$_2$ only (control, C). Tonographic measurements of outflow facility, fluorophotometric measurements for aqueous humor formation rate, and uveoscleral outflow determinations were done at 72 hr after injection, the time for maximal IOP response, as indicated in Figures 5 and 6. For these experiments,

TABLE 1. Intraocular Pressure (IOP), Aqueous Formation Rate (flow), Outflow Facility, and Uveoscleral Flow Determined 72 hr After Intravitreous Injection of BeCl$_2$+NaF (X eyes) or BeCl$_2$ (C eyes)

IOP ($\pm$SEM; mmHg)*		Aqueous Flow ($\pm$SEM; μl/min)		
C	X	C	X	Change
17.1 $\pm$ 0.86	14.6 $\pm$ 0.41†	2.00 $\pm$ 0.29	1.2 $\pm$ 0.25†	−40%
		Outflow Facility ($\pm$SEM; μl/min/mmHg)		
17.5 $\pm$ 0.96	12.6 $\pm$ 0.89***	0.193 $\pm$ 0.012	0.208 $\pm$ 0.012	NS
		Uveoscleral Outflow§ ($\pm$SEM; μl/min)		
18.8 $\pm$ 0.53	14.7 $\pm$ 0.68‡	0.42 $\pm$ 0.053‖	0.26 $\pm$ 0.064†	−38%

* Mean IOP for 24 hr (four measurements) preceding determination of aqueous or uveoscleral flow or facility.
† n = 6, $P < 0.05$.
‡ n = 7, $P < 0.01$.
§ Absolute values for uveoscleral flow are higher than literature values and varied according to batch of fluorescein-dextran.
‖ Not significantly different from saline-injected normal rabbit eye value of 0.38 $\pm$ 0.024 μl/min (n = 7) using same batch of fluorescein-dextran.
*** n = 8, $P < 0.01$.

the dosage of Be^{2+} and F^- was halved (0.5 μmol $BeCl_2$, 1.5 μmol NaF per eye) and the more physiologic chloride salt of Be^{2+} was used instead of beryllium nitrate, as used in the experiments shown in Figure 6. In these experiments, the fall in intraocular pressure at 72 hr was in the range of 2.5–4 mmHg, compared with the 8–10 mmHg fall of IOP obtained with 1 μmol per eye injections of Al^{3+} or Be^{2+} together with NaF. The results in Table 1 show that uveoscleral outflow and aqueous humor flow rate were both decreased by about 40%, but there was no change in conventional (tonographic) outflow facility compared with experimental eyes receiving $BeCl_2$ only. The absence of a trabecular effect was confirmed by constant pressure facility measurements in cannulated rabbit eyes. In eyes contralateral to those that received 1+3 mM or 2+6 mM BeCl and NaF, the control facilities were 0.171±0.09 and 0.176±0.012 SEM, respectively. The corresponding experimental eyes perfused with BeF_3^- had facilities of 0.170±0.016 and 0.175±0.012 μl/min/mmHg.

DISCUSSION

The biochemical experiments using the adenylyl cyclase/G_s system in ciliary processes established the effectiveness of Al^{3+} and Be^{2+} to activate heterotrimeric G-proteins (in this instance, G_s) in ocular tissues in the presence of fluoride ions. This is manifested by the AC response to forskolin that is synergistic with activated G_s, thus giving potentiated responses,[6] as seen in Figures 1 and 3. These experiments also showed that the Ga^{3+} ion, which has similar chemistry to Al^{3+}, is neither an activator nor an inhibitor of G-protein activation (Figs. 1, 2). This lack of activity for Ga^{3+} provided the rationale to use Ga^{3+} as a control in subsequent in vivo experiments (Fig. 4) to account for possible nonspecific effects of injecting trivalent cations into the eye.

Studies on the mechanism of action of Al^{3+} and Be^{2+} cations in the presence of F^- suggest that the active species is AlF_4^- or BeF_3^- H_2O formed in solution. Both of these ionic species have tetrahedral geometry that mimic the phosphate anion.[17] It has been proposed that these agents bind to unactivated G-proteins at the site where GDP is bound, forming a $GDP.AlF_4^-$ complex that mimics GTP and causes activation of G-proteins independent of receptors.[18] In the continued presence of fluoroaluminate, the active form of the G-protein, α_s, persists because the normal termination mechanism (the GTP-ase reaction) cannot occur. At much higher concentrations, fluoroaluminate can also ligand with bound ADP in proteins that have ATP binding sites.[19]

Although fluoroaluminate has the potential to interfere with many nucleotide binding proteins, the bio-

logical effects of AlF_4^- in intact cells, although more complex than on isolated proteins, are ascribed to activation of G-proteins. For example, in some cells, fluoroaluminate inhibits the stimulation of AC by agonists whose receptors are coupled to G_s, but appears to have little effect by itself on cAMP levels.[20] These responses were interpreted as a preferential activation of G_i, which, in cells, appears to be more sensitive to fluoroaluminate than is G_s.[21] However, in other cells, the effect of fluoroaluminate on ion transport[22] is consistent with secretagogue responses and with activation of G_s. These divergent findings illustrate the point that the final cellular response to fluoroaluminate may be the resultant of multiple or opposing G-protein-regulated mechanisms and can, therefore, depend on the relative amounts and types of G-protein in each specific tissue. We attribute the IOP effect of intraocular fluoroaluminate or BeF_3^- to the 40% decrease in aqueous humor formation rate (Table 1). Although ciliary processes contain the inhibitory G_i protein,[23] this G-protein interaction with AC is greatly exceeded by G_s and, thus, only a partial degree of AC inhibition can be elicited in intact tissues,[24,25] and even less in membranes.[26]

The IOP effect caused by AlF_4^- or BeF_3^- (Figs. 5, 6) is similar to results using forskolin,[27] a direct activator of AC, and supports the concept that increased levels of cAMP decreases net secretion of aqueous humor by ciliary processes in the rabbit eye.[28] In many fluid-secreting cells, the activity of ion channels can be an important rate-limiting step in ion transport.[29] Based on the widespread transport effects of secretagogues coupled to G_s, it appears that cAMP regulates many types of anion channel,[30] either by activating silent channels or by increasing their conductive state. This concept applied to ciliary processes or choroid plexus in experimental animals suggests the hypothesis that in these epithelia secretagogues that increase cAMP activate anion channels, and cAMP may thus promote fluid transport toward the stroma, reducing the net rate of secretion of cerebrospinal fluid[31] or aqueous humor.[32]

The decrease in uveoscleral flow observed in these experiments (Table 1) is also in accord with the G-protein mechanism for the effect of fluoroaluminate. The predominant G-protein in ciliary muscle cells is likely to be coupled to inositol phosphate metabolism, increased intracellular calcium levels, and contraction of the muscle. However, in the rabbit ciliary muscle, the contraction response does not affect trabecular outflow because IOP in the rabbit eye is unresponsive to pilocarpine. Thus, no outflow effect was seen in the present experiments, although the uveoscleral results indicate contraction of ciliary muscle fibers.

One of the important objectives of this study was to determine whether G-proteins are involved in con-

trolling trabecular outflow. A role for cAMP in facility increase is not yet firmly established. Some earlier studies using forskolin, which also bypasses receptors, showed effects on facility,[33] but several more recent investigations concluded that most of the IOP response to topical forskolin was the result of a decrease in aqueous humor formation rate.[34,35] Forskolin has been found to activate cAMP-dependent responses in all cells and tissues tested and, therefore, the absence of a definitive effect of topical forskolin on facility in experimental studies is puzzling. More recently, human eyes perfused in vitro with epinephrine showed increased facility and cAMP levels in the perfusate.[36] This strongly supports the involvement of β-receptors in the facility response, but does not yet prove that cAMP mediates the facility change (the cAMP could arise from extraneous cells in the eye preparation). In recent experiments on bovine and human trabecular meshwork,[37] we found that the AC enzyme in trabecular tissue homogenates is similarly stimulated by forskolin and by AlF_4^- as shown here for the AC enzyme in ciliary processes. For these reasons, the in vivo demonstration of G-protein involvement in facility would be an important first step in understanding the intracellular effector system in trabecular outflow regulation. The site of uveoscleral outflow is the anterior iris root, near the site where fluid leaves the eye by trabecular outflow. Because BeF_3^- caused the expected change in uveoscleral flow, one might suppose that active concentrations of this agent, having reached the anterior iris root, should then enter the adjacent trabecular outflow pathway. However, the absence of an effect on trabecular outflow after intravitreal injection of $BeCl_2 +$ NaF could be due to too low a dose reaching the anterior chamber. (With intravitreal injection, higher concentrations of BeF_3^- are likely to occur in the posterior chamber than in the anterior chamber or trabecular outflow pathway). Therefore, the selective effect on ciliary processes (aqueous humor formation rate) and on ciliary muscle (uveoscleral outflow) could be the result of an effective concentration of BeF_3^- being reached only in the posterior chamber and affecting these tissues from that locus. To address this question, we used the perfused albino rabbit eye preparation to determine whether $BeCl_2 +$ NaF alters outflow facility directly. When these agents at either 1+3 or 2+6 mM were included in Baranys perfusion medium, there was no significant difference in facility compared with the contralateral eye perfused with normal Baranys solution over a 2-hr period. Thus, we conclude that the acute general activation of G-proteins by BeF_3^- does not play a significant role in trabecular facility of the rabbit eye. An alternative explanation for the lack of a trabecular effect may be species related. It is possible that G-proteins involved in trabecular facility changes may be much less sensitive to

activation by extracellularly administered BeF_3^- than are other parameters of aqueous humor dynamics in the rabbit compared to the primate eye.

Key Words

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