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The Adenylate Cyclase Receptor Complex and Aqueous Humor Formation

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The secretory tissue of the eye, the ciliary processes, contains an enzyme receptor complex, composed of membrane proteins, the catalytic moiety of the enzyme adenylate cyclase, a guanyl nucleotide regulatory protein (or N protein), and other features. The enzyme can be activated by well-known neurohumoral or humoral agents, catecholamines, glycoprotein hormones produced by the hypothalamic pituitary axis, and other related compounds, including placental gonadotropin, organic fluorides, and forskolin, a diterpene. These compounds cause the ciliary epithelia to produce cyclic AMP at an accelerated rate. Cyclic AMP, as a second messenger, causes, either directly or indirectly, a decrease in the net rate of aqueous humor inflow that may be modulated by cofactors. Clinical syndromes fit the experimental data so that an integrated explanation can be given for the reduced intraocular pressure witnessed under certain central nervous system and adrenergic influences. The molecular biology of this concept provides important leads for future investigations that bear directly both upon the regulation of intraocular pressure and upon glaucoma.

INTRODUCTION

The mechanisms responsible for the regulation of intraocular pressure (IOP) represent a significant but largely unanswered problem. There is virtually no evidence in favor but there is evidence against instantaneous regulation or feedback control of IOP [1]. There are considerable data, however, suggesting that humoral or neurohumoral pathways can influence the steady-state level of IOP by altering the rate of aqueous inflow. The introduction of beta blockers for the treatment of glaucoma prompted a renewed and intense interest in the adrenergic aspects of IOP control. Studies of the adrenergic system, and other studies as well, have led to the hypothesis, for which a great deal of evidence has accumulated, that the adenylate cyclase receptor complex in the ciliary epithelia plays a central role in the maintenance and regulation of IOP. The ability to make measurements of gross outflow facility relatively easily encouraged a number of investigators to study its role in eye pressure regulation [1-4]. Interest in the outflow pathways as a regulatory modum for IOP was further kindled by the finding that increased cyclic AMP levels in aqueous after adrenergic agents were associated with decreases in IOP and increases in outflow facility [5-8]. The evidence indicated that even pharmacologic doses of

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those adrenergics increasing outflow facility caused only modest decreases in IOP. These relatively small changes in IOP modulated by changes in outflow facility did not approach the larger fluctuations that are encountered with endogenous changes in IOP such as circadian variation. Furthermore, inaccuracies in the manometric techniques for measurements of outflow shifted emphasis to inflow as an equally, if not more important, determinant of steady-state levels of IOP. The diurnal variation in IOP is most likely the result of changes in net aqueous humor inflow, changes that more easily account for the magnitude of the fluctuations in pressure. These more prominent changes in IOP have prompted a search for a pathway for both endogenous and exogenous factors that could be mediated by modulation of aqueous humor inflow.

HISTORICAL BACKGROUND

Sidler-Hugenin was the first to report a diurnal rhythm in IOP by tactile measurements of eye pressure in 1899 [9]. In 1904 Maslenikow confirmed these findings using applanation tonometry [10]. Twenty years later, Thiel [11] reported an early morning peak in diurnal curve. In 1951 Langley and Swanljung identified a number of patterns of diurnal variation [12]. Later, Drance [13], DeVenecia and Davis [14], and Katavisto [15] reported on the characteristics of diurnal variations in both normal and glaucomatous populations.

The physiologic and biochemical bases for these daily swings in IOP have been elusive. Ericson [16] reported that variations in aqueous inflow were responsible for the changes in IOP and found that aqueous production decreased at night, corresponding to the decrease in IOP usually seen at this time. Schmerl et al. [17] postulated that day-night cycles may lead to the production of neurohumoral factors which circulate to the eye and regulate IOP. They isolated from the cerebrospinal fluid of rabbits two compounds, hyperpiesin and myopiesin, which respectively caused an increase and a decrease in the IOP. Hyperpiesin was produced in rabbits exposed to light and was later converted to myopiesin during darkness.

The large swings in IOP related to diurnal variation implies the existence of central regulation of aqueous humor formation. This concept would require a locus for regulation, neural and/or humoral mediators, and specific receptors on the target ciliary epithelia. Duke-Elder [18], Elwyn [19], Hess [20], Magitot [21], and Schmerl and Steinberg [22] have all hypothesized the existence of one or more centers in the hypothalamus or diencephalon responsible for the regulation of IOP. Gloster and Greaves [23] and von Sallman and Lowenstein [24] electrically stimulated the hypothalamus to induce changes in IOP. These changes were small and transient. In 1951 Nagai et al. [25] reported on changes in IOP induced by electrical stimulation of the hypothalamus. Many, if not all, of these changes could not be disentangled from simultaneous changes in systemic blood pressure or blood flow. Recently, attempts have been made to relate efferent fibers in the optic nerve to osmotic alterations of IOP, perhaps mediated by the hypothalamus [26,27]. The search for actual mediators and receptors of aqueous humor formation in the ciliary body seems more promising. The ciliary processes themselves contain an enzyme receptor complex, the catalytic component of which is adenylate cyclase, the enzyme responsible for the formation of cyclic AMP, a second messenger [28–30]. This receptor complex is ubiquitous in cell membranes of the tissues of many organisms and is responsible for many regulatory functions. Evidence implicating a central role for adenylate cyclase in the regulation of aqueous humor formation is rapidly increasing. Stimulation of

this enzyme and subsequent increases in intracellular cyclic AMP produced by several endogenous or exogenous factors decrease net aqueous humor flow and lower IOP [29,31-33]. The following studies were conducted in order to investigate the role of cyclic AMP in the maintenance of eye pressure by changes in net inflow, and bear on molecular and physiologic mechanisms involved not only in regulation of IOP but also upon the clinical treatment of elevated pressure in glaucoma.

RECENT STUDIES

The approach to the problem of the molecular mechanisms involved in the control of IOP is best done with techniques from several disciplines. In order to link chemical events occurring at a subcellular level to resultant decreases in IOP, biochemical, physiological, pharmacological, and anatomical studies were conducted. The identification and quantification of responses of ciliary adenylate cyclase to chemical stimuli were studied *in vitro*. The physiological perturbations in blood flow, aqueous flow, outflow resistance, and IOP caused by stimulation of adenylate cyclase by various agents were studied. Anatomical changes in the ciliary processes after treatment with stimulators of adenylate cyclase are also consistent with the idea that ciliary cyclic AMP plays a central role in the regulation of IOP by changes in aqueous flow.

Several agents known to stimulate cyclic AMP production by different molecular pathways were used. Cholera toxin is a potent irreversible activator of adenylate cyclase which binds to cell membrane gangliosides. The gonadotropins (especially hCG and FSH) are glycoprotein hormones which stimulate cyclase by binding to specific cell surface receptors. Forskolin, a diterpene derivative of the plant *Coleus forskohlii,* penetrates directly to the catalytic unit and stimulates cyclic AMP production without interaction with cell surface receptors [34-37]. The classic beta receptor agonist, isoproterenol, was used in some *in vitro* studies.

In Vitro *Studies*

Activation of adenylate cyclase was demonstrated directly by measuring the rate of conversion of $\pm a^{32}$Pl ATP to $\pm^{32}$Pl cyclic AMP in the particulate fraction of a broken cell preparation, or indirectly, by measuring cyclic AMP produced by intact processes [28]. Cholera toxin is a potent irreversible stimulator of adenylate cyclase. Incubation of intact ciliary processes with cholera toxin significantly increases intracellular cyclic AMP production in concentrations as low as 10^{-10}M (Fig. 1). In ad-

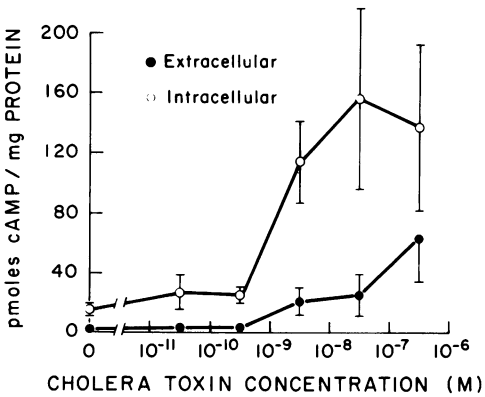

FIG. 1. Dose-response relationship between cholera toxin concentration and cyclic AMP production by intact ciliary processes (n = 3). Ciliary processes were incubated in Hanks' balanced salt solution at 37°C with the indicated concentrations of cholera toxin for five hours. Then theophylline was added to a final concentration of 10 mM. The processes were incubated an additional 10 minutes, an aliquot of the medium was removed, 100 percent (w/v) TCA was added to the tissue suspensions to a final concentration of 6 percent, and the tissue was homogenized. Intracellular and extracellular cyclic AMP were assayed (From Gregory et al. [33]).

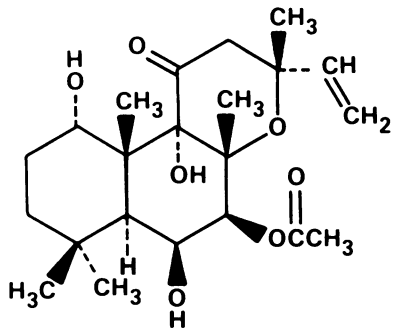

FIG. 2. Structure of forskolin.

dition, production of cyclic AMP in intact ciliary processes from human eyes was increased tenfold with either isoproterenol or cholera toxin [29].

Forskolin (Fig. 2) dramatically increased adenylate cyclase activity in rabbit and human ciliary epithelial preparations. Comparisons of forskolin with other cyclase stimulators are shown in Fig. 3. Beta blockade in the form of timolol did not alter this dose-response curve, supporting the concept that forskolin acts directly on the adenylate cyclase complex independently of cell surface reception. Maximal stimulation of human adenylate cyclase activity from cultured epithelial cells using a forskolin concentration, limited by its solubility of 6×10^{-4}M, was 57-fold compared to basal activity.

The effect of isoproterenol stimulation of adenylate cyclase is greatly potentiated by the presence of forskolin (Table 1). The stimulation of cyclic AMP production in the presence of forskolin and isoproterenol together is significantly greater than the sum of the stimulation using either agent alone, indicating that forskolin and isoproterenol have a synergistic effect on adenylate cyclase stimulation.

The effects of human chorionic gonadotropin (hCG) on adenylate cyclase production in crude membrane preparations of ciliary epithelium were investigated. Significant increases compared to basal activity were easily detected at micromolar concentrations of hCG (Table 2).

Effect of Adenylate Cyclase Stimulation on Intraocular Pressure

Intravitreal injections of cholera toxin in concentrations as low as 0.015 μg into rabbit eyes significantly lowered IOP (Fig. 4). Delivery of 2.1 μg of cholera toxin by close arterial injection via the internal maxillary artery in rabbits also significantly decreased IOP (Fig. 5).

The effects of both intravitreal and topical forskolin on IOP were investigated.

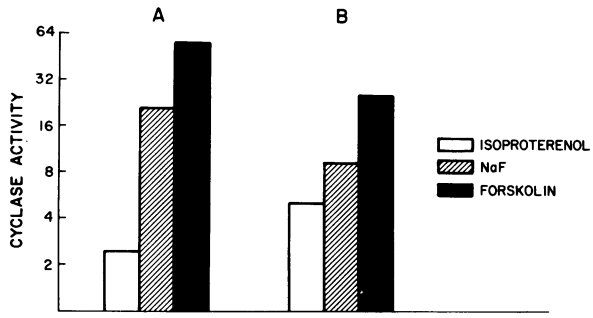

FIG. 3. Stimulation of human ciliary adenylate cyclase *in vitro*. Numbers represent ratios of activity in the presence of agent to basal activity. Activation by forskolin was significantly greater than isoproterenol or NaF. Left panel represents cultured fetal human ciliary epithelia. Right panel represents freshly excised human ciliary processes.

TABLE 1
Synergism Between Forskolin and Isoproterenol

Forskolin (μM)	Cyclase Activity (pmol/min/mg protein)	Isoproterenol Stimulation ** (pmol/min/mg protein)
0	49.7 ± 2.3 (3)*	13.7 ± 1.5 (3)
0.2	124 ± 12 (3)	44.7 ± 6.7 (3)†
2.0	292 ± 18 (3)	93.7 ± 27.4 (3)†
20.0	616 ± 51 (3)	102 ± 23 (3)†

* The value is basal cyclase activity. All values reported are mean ± S.E.M.(n).

** Isoproterenol stimulation is adenylate cyclase activity with 100 μM isoproterenol minus the activity in the absence of isoproterenol for each forskolin concentration.

† Isoproterenol stimulation in the presence of forskolin is greater than the stimulation in the absence of forskolin ($p < 0.05$).

An intravitreal dose as low as 0.16 μg significantly reduced IOP three to nine hours after drug delivery (Fig. 6). Topical forskolin, delivered as a suspension in concentrations of 0.1 percent, 0.5 percent, 1.0 percent, and 4.0 percent, dramatically reduced rabbit IOP with the duration of action being dose-dependent (Fig. 7). The 1.0 percent topical suspension was also tested in ten monkeys and ten normal human volunteers. In both cases IOP was significantly reduced for no less than five hours after a single drop of the suspension (Fig. 8).

Commercial preparations of the gonadotropins hCG and FSH, when delivered intravitreally in eyes of normal and oophorectomized rabbits, significantly reduced

TABLE 2
Comparison of hCG Effect on Ciliary Process Adenylate Cyclase with Effects of Known Activators

Agent	Activation[a]
hCG (1 μM)	1.36 ± .08 (5) [b]
ℓ-epinephrine (0.1 mM)	1.82 ± .22 (5) [c]
sodium fluoride (4 mM)	7.48 ± .92 (4) [b]
GTP (10 μM)	1.69 ± .08 (3) [d]

[a] The values given represent the mean ± S.E.M. (n) of the ratios of enzyme activity in the presence of agent to the activity in the absence of agent.

[b] $p < .005$ [c] $p < .025$ [d] $p < .01$

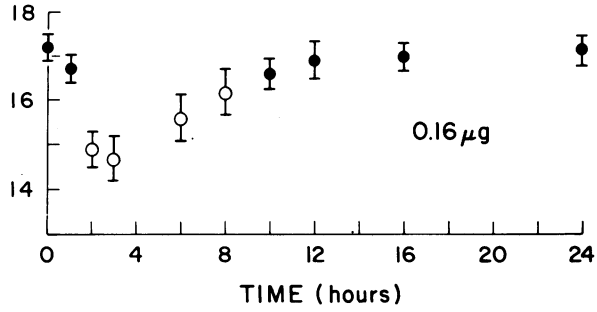

FIG. 4. Intraocular pressure (mean ± S.E.M.) versus time after intravitreal injection of 0.16 μg (n = 20). Open circles represent significant departures from baseline ($p < 0.01$) (Modified from Caprioli et al. [45]).

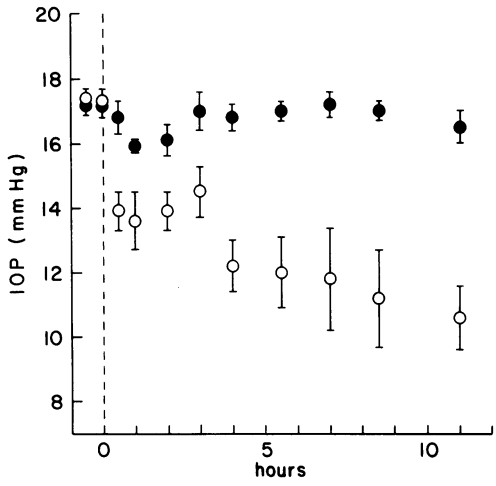

FIG. 5. Cholera toxin (2.1 μg) was infused via the right internal maxillary artery of six rabbits and IOP was recorded by applanation. Mean ± S.E.M. (6) o controls; o treated (Modified from Gregory et al. [33]).

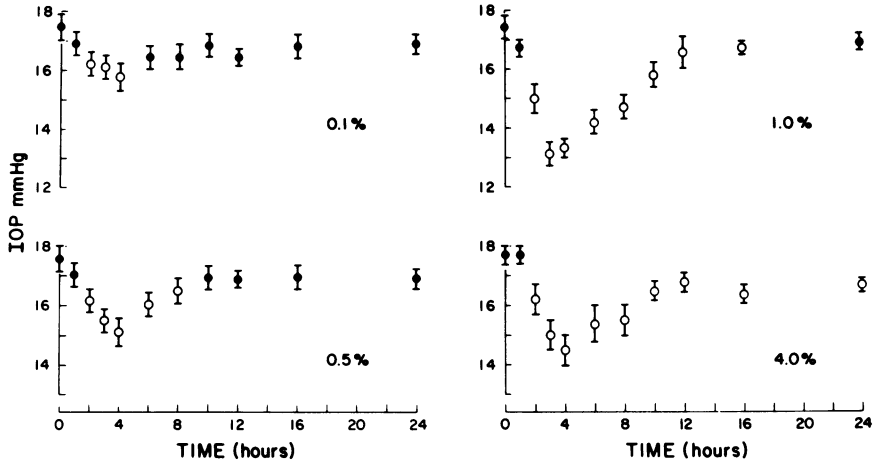

FIG. 6. Intraocular pressure (mean ± S.E.M.) versus time after topical delivery of forskolin suspension 0.1 percent (n = 20), 0.5 percent (n = 20), 1.0 percent (n = 20), and 4.0 percent (n = 20) in rabbits. Open circles represent significant departures from baseline ($p < 0.01$).

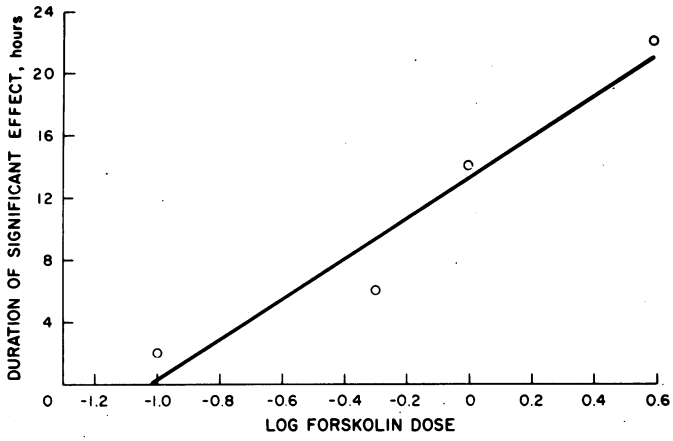

FIG. 7. Linear regression of the duration of statistically significant effect as a function of the logarithm of toical forskolin concentration (percentage suspension) in rabbits (5 = 0.97, p = 0.0002). X intercept indicates least effective dose.

IOP compared to control eyes in a dose-dependent fashion. The data are represented as percentage decrease in outflow pressure, assuming an episcleral venous pressure of 9.0 mm Hg (Fig. 9).

Interestingly, progesterone and quingestanol given in large doses (10^{-7} to 10^{-4}M) into the vitreous in rabbit eyes did not reduce IOP. This suggests that the hypotony commonly seen with pregnancy and previously reported to be secondary to elevated progesterone levels [38] is not caused by progesterone but rather due to high plasma levels of gonadotropins, particularly hCG (10^{-7}M)[32]. The effect can be reproduced by systemic injection [32].

Aqueous Flow

Aqueous flow studies were performed after close arterial delivery of cholera toxin to rabbit eyes [33]. Compared to control eyes, an approximate aqueous flow reduction of 50 percent was realized in the eyes treated by close arterial infusion of cholera toxin.

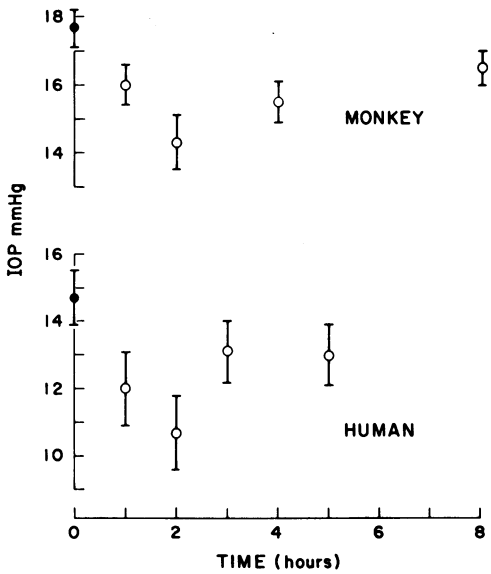

FIG. 8. Intraocular pressure (mean ± S.E.M.) versus time after administration of 1.0 percent forskolin topically in monkeys (n = 10) and humans (n = 10). Open circles represent significant departures from baseline (p F 0.01).

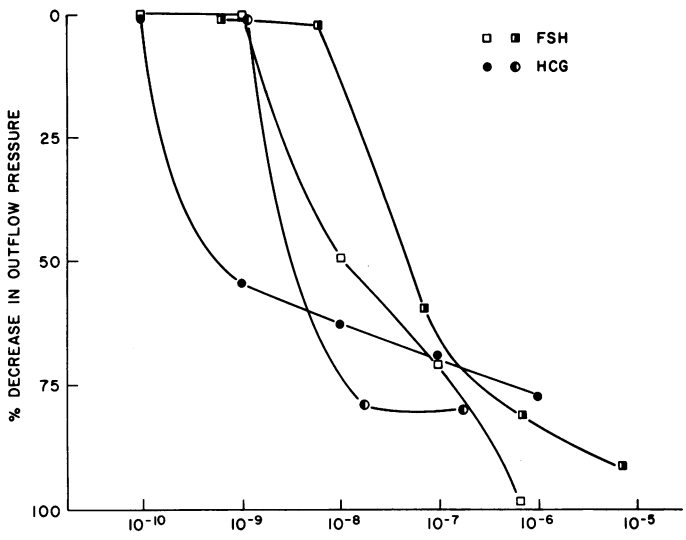

FIG. 9. A summary dose-response curve showing the effect of commercial hCG and FSH preparations upon outflow pressure in the rabbit eye reflecting the time of peak response, usually about 16 hours. Each point represents the mean of five to eight eyes (Modified from Sears and Mead [32]).

The method of intravitreal injections of fluorescein-dextran as described by Maurice [39] was also used to investigate the effects of aqueous flow by a percent suspension of topical forskolin. Two weeks prior to the experiment, six rabbits were injected intravitreally bilaterally with 10 μl of a 10 percent fluorescein-dextran solution (MW 40,000). 50 μl of a 1 percent forskolin suspension was applied unilaterally to four rabbits, and two other rabbits received acetazolamide 25 mg/kg. Three hours later anterior chamber paracenteses (100 μl) were performed in all animals and the concentrations of fluorescein determined spectrophotofluorometrically. Comparison of treated, control acetazolamide, and untreated eyes revealed an aqueous flow decrease in the forskolin-treated eyes of 46 ± 8 percent, and in the acetazolamide-treated eyes of 44 ± 10 percent, relative to control normal eyes. This confirmed our previous determinations of aqueous flow after topical forskolin, using an intravenous fluorescein technique, in which a 50 percent decrease in aqueous flow relative to the control eyes was found [31]. Importantly the decrease in outflow pressure and the decrease in net flow were comparable, indicating that the decrease in IOP can be entirely accounted for by a reduction in aqueous flow. Outflow facility did not change [33].

Blood Flow

Blood flow measurements were performed using the radioactively labeled microsphere technique previously described by Alm and Bill [40]. After a close arterial infusion of 2.1 μl of cholera toxin into the right internal maxillary artery in rabbits, blood flow increased and reached a peak of approximately 2.2 times the control eye at 8 to 13 hours after the infusion [33].

Regional ocular blood flow was also investigated after topical delivery of 50–100 μl of a 1 percent forskolin suspension in rabbits (Fig. 10). Ciliary body blood flow increased approximately 2.5-fold relative to basal values at approximately one hour after drug delivery and returned to baseline in approximately four hours. IOP in the same eyes decreased with lowest IOP levels seen at approximately four hours. Blood

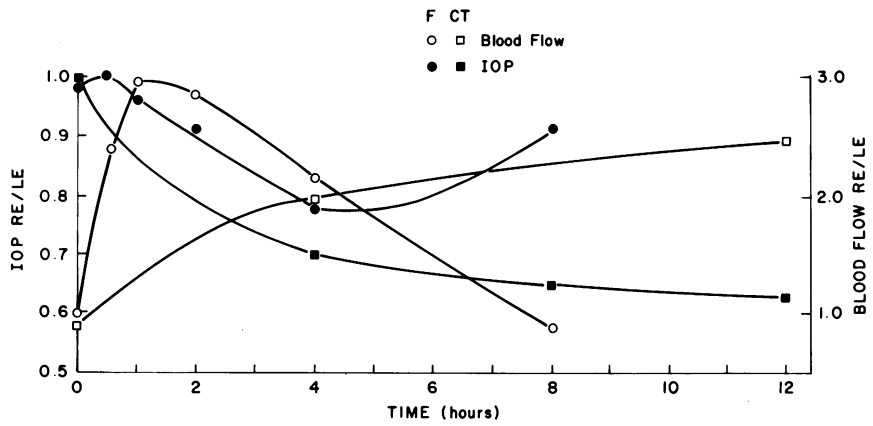

FIG. 10. Forskolin or cholera toxin increases ciliary body blood flow and decreases intraocular pressure. Results plotted as ratio of treated/untreated, RE/LE; left ordinate, IOP; right ordinate, blood flow (Modified from Sears et al. [29]).

flow to the iris, choroid, retina, and optic nerve did not significantly change compared to the contralateral untreated eye. The onset, peak effects, and duration of the alteration in blood flow and IOP are run on different time courses. Interestingly, blood flow to structures other than the ciliary body, e.g., choroid, was found unaltered. The effect of forskolin on blood flow is compared to the effect after cholera toxin (Fig. 10).

Fine Structural Studies

The apices of the nonpigmented epithelium and the pigmented epithelium of the ciliary processes face each other as a result of the invagination of the optic vesicle during embryologic development. These apical surfaces are lined by numerous microvilli which interdigitate and form a large surface area between these two cell surfaces. Between these surfaces, a potential space exists, the "ciliary channels," into which water and metabolites may be secreted. These channels are evident in the ciliary processes of both normal rabbit and man [41]. In order to study any changes in fluid flux that may occur as a result of stimulation of adenylate cyclase by some of the previously mentioned agents, these ciliary channels were investigated with the aid of electron microscopy. As many as 15 ciliary processes from different regions of five groups of rabbits were studied for any changes in these ciliary channels. Rabbits received either intravitreal cholera toxin, topical isoproterenol, topical forskolin, acetazolamide, topical talc, or no treatment as a control. Those eyes treated with cholera toxin, isoproterenol, or forskolin, all activators of adenylate cyclase, showed progressive enlargement of the ciliary channels, with the most dramatic effects evident with cholera toxin and forskolin (Fig. 11A,B,C,D), at a time preceding any change in IOP. The untreated eyes and the eyes of acetazolamide- or talc-treated animals revealed ciliary channels ordinary in appearance (Fig. 11E,F). These findings indicate a movement of fluid into the expandable apical space between the nonpigmented and pigmented epithelial cells. The gap, desmosomal and tight junctions were all intact.

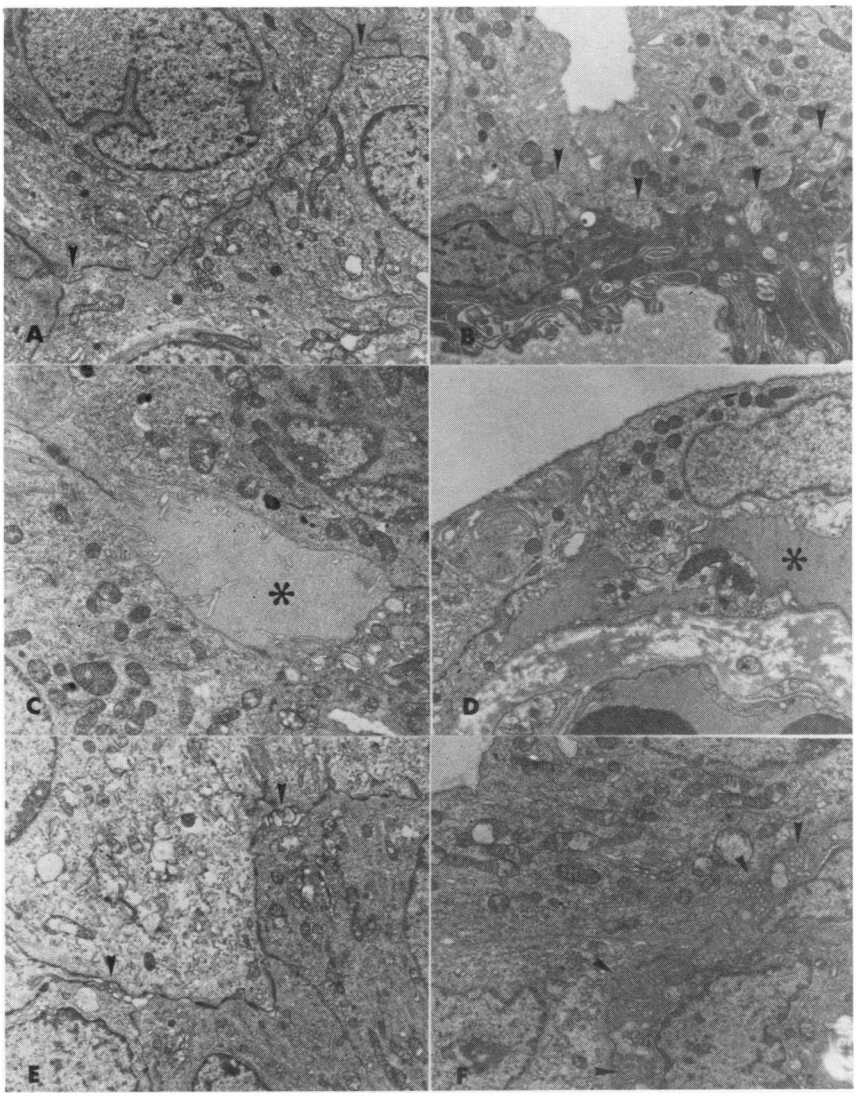

FIG. 11. Ultrathin sections through anterior ciliary processes **(A,C,E)** and iridial processes **(B,D,F)** one hour after instillation of talc suspension in rabbit cul de sac **(A,B)**, one hour after 1 percent topical forskolin suspension **(C,D)**, and four hours after intravenous acetazolamide 25 mg/kg **(E,F)**. *Arrows* indicate normal ciliary channels **(A,B)**, unchanged ciliary channels after intravenous acetazolamide **(E,F)**, but enlarged ciliary channels after topical forskolin. Villiform, once interdigitated, cytoplasmic processes can be seen in separated state **(*)** within interapical fluid. × 5,500 Note that zonular occludens, desmosomes, and gap junctions are not disturbed (*Courtesy K. Kondo*).

DISCUSSION

The finding that IOP is significantly reduced by topical application of the beta blocker, timolol, has caused a major revolution and scramble in ocular pharmacology. New investigations have been pursued that have led to the conclusions that *stimulation* of the ciliary epithelial adenylate cyclase complex can reduce IOP by reducing net aqueous inflow [28,29,32,42]. Timolol indeed may lower IOP by a

mechanism other than beta blockade [28,43,44]. Furthermore, the recognition that increased adenylate cyclase activity within the secretory ciliary epithelia of the eye can reduce IOP has led to new therapeutic possibilities [31,45].

The use of adrenergic *agonists* to reduce IOP dates at least as far back as 1900 when Darier [46] tried subconjunctival injections of adrenalin. Hamburger [47] applied the drug topically in the treatment of glaucoma patients. It was largely a result of the work of Goldmann [48] that it was accepted that topical epinephrine lowered IOP by reducing aqueous humor formation. Weekers et al. [49] were first to show that isoproterenol lowered IOP. Garner et al. [50] reported that not all the pressure reduction by epinephrine could be accounted for by a decrease in inflow. Ballintine [51] and Becker et al. [52], using tonography, measured an increase in outflow facility after topical epinephrine. Studies of denervation supersensitivity and degeneration release in the rabbit, where pseudofacility is unimportant, supported the idea that outflow of aqueous was increased by epinephrine and that the increase was mediated by an alpha receptor [1,4,53–56].

These and other clinical observations, especially by Grant [57,58], prompted the description of the reduction in ocular pressure after topical application of the mixed adrenergic agonist, epinephrine, by phases [59,60]: first, early, an alpha adrenoreceptor effect; second, an intermediate phase lasting several hours which is a beta adrenoreceptor effect; and finally, a long-term effect (universally) of progressive increases in outflow [61]. This long-term effect may be related to increased mucopolysaccharide metabolism in outflow channels [59,62] or perhaps to gradual loss of agonist from its former pigment binding site [63]. This hypothesis did not completely address cellular mechanisms but did tend to explain many clinical observations of the effects on IOP after the use of adrenergic agents.

To avoid the pharmacodynamic complexities of *in vivo* studies, i.e., the small change induced as a sum of the action of many possible receptor sites or of toxic effects on ocular cells, laboratory investigations to characterize the interaction between drug and beta receptor in the ciliary epithelium, the tissue responsible for the secretion of aqueous humor, have been done. In a well-controlled, *in vitro* cell-free system, the drug concentration at the receptor can be determined. It has been possible to quantify the drug-receptor relationship [28]. Beta adrenergic receptors were studied in crude particulate preparations of the ciliary processes of rabbit eyes by a direct ligand-binding assay using ^{125}I-hydroxybenzyl pindolol [64], and by examining the kinetic and regulator properties of adenylate cyclase linked to the beta adrenergic receptors [28].

High-affinity binding sites for ^{125}I-hydroxybenzyl pindolol were found in the same particulate membrane fractions of homogenized ciliary processes as adenylate cyclase activity. Stimulation of adenylate cyclase activity by catecholamines [65] was completely blocked by several beta adrenergic agonists, but not by phenoxybenzamine, an alpha blocker [5,6]. The K_d is comparable to beta adrenergic receptors of other tissues. Neufeld and Page [66] found a higher K_d, possibly a reflection of a technique which included nonspecific binding sites. K_{act} for stimulation of enzyme activity was of the order expected for a beta adrenergic receptor-linked adenylate cyclase, and K_Is for inhibition of l-epinephrine stimulation similar to binding constants for these antagonists to beta adrenergic receptors in other systems. Results similar to those found by Gregory et al. [28] have been obtained in membrane preparations from sheep eyes [67,68] and in monkey [69], rabbit [64,66], and human eyes [30,70,71]. The order of potency of agonist activation indicates that ciliary processes contain a predominance of beta$_2$ adrenergic receptors [30]. Finally, binding

constants determined by the direct ligand-binding technique and by the assay for adenylate cyclase agree. The agreement certainly suggests that the two techniques measure the interaction between beta adrenergic ligands and the beta receptor of the ciliary processes [28].

Thus, although inconsistencies have been found *in vivo* in studies of inflow and of outflow after administration of adrenergic agonists, it has been established on a molecular level that stimulation of the beta adrenergic receptor leads to activation of membrane-bound adenylate cyclase and to an accelerated rate of production of ciliary intracellular cyclic AMP. *The accelerated production of cyclic AMP stimulated by nonadrenergic agents is consistently associated with decreased IOP as a consequence of decreased net aqueous inflow* [72]. Stimulation of the adenylate cyclase receptor complex by a few molecules of cholera toxin gives physiologic, chemical, and anatomic evidence for the pressure-regulating role of the adenylate cyclase receptor complex in the ciliary epithelial tissue of the eye. Exquisitively low doses of cholera toxin, 2.4×10^{-11}M, delivered either by close arterial injection or by intravitreal injection, lowered IOP dramatically by reducing net aqueous inflow [29,33]. The fall in IOP was neither accounted for by a drop in blood flow (which actually increased) nor by any increases in outflow facility [33].

Observations made with forskolin fit the findings with cholera toxin. Forskolin significantly reduces IOP in rabbits, monkeys, and man [31]. Topical application of a suspension of this drug lowers rabbit IOP in a dose-dependent fashion. In rabbits, a single drop of a 1.0 percent suspension decreases net aqueous inflow by nearly 50 percent while increasing ciliary blood flow 2.5-fold. *In vitro,* forskolin acts as a direct (cell surface reception not required) potent stimulator of adenylate cyclase in both rabbit and human ciliary processes. The effects of forskolin are not blocked by timolol, as would be expected, and the presence of forskolin significantly enhances the stimulation of cyclic AMP production achieved by isoproterenol.

Still other agents that stimulate adenylate cyclase activity reduce eye pressure by reducing net aqueous inflow. These preparations include several commercial compounds of the gonadotropin hormones, especially hCG and FSH [32]. hCG, *in vitro,* stimulates rabbit ciliary adenylate cyclase activity in crude membrane preparations of ciliary epithelia. Thus, the adenylate cyclase receptor complex in the secretory tissue of the eye, the ciliary epithelia, when activated by a variety of pathways, reduces the net rate of aqueous inflow and the level of IOP (Fig. 12).

Diverse stimulators of adenylate cyclase, cholera toxin, gonadotropins, and forskolin cause IOP and aqueous flow to fall dramatically. How does the adenylate cyclase receptor complex in the ciliary epithelia act to reduce aqueous inflow? Cholera toxin has been known to induce watery diarrhea by stimulating an intestinal epithelial adenylate cyclase with consequent efflux of sodium and water from the lumen of the intestine [73]. Cholera toxin increases the production of cerebrospinal fluid by the choroid plexus and increases endolymph production in the inner ear [74–76]. In all these instances stimulation of adenylate cyclase activity with accelerated production of cyclic AMP in the epithelial cell causes the movement of water from the basal to the apical portion of the cell and thence into the lumina. The cell polarity of the ciliary epithelia is reversed because the optic vesicle invaginates during development of the eye. The polarity of the cell can explain differences in directional movement of fluid produced by the epithelia of these different organs. The polarity of the nonpigmented epithelium (apex toward the pigmented epithelium and the blood) suggests that the fluid results from absorption across the basolateral or

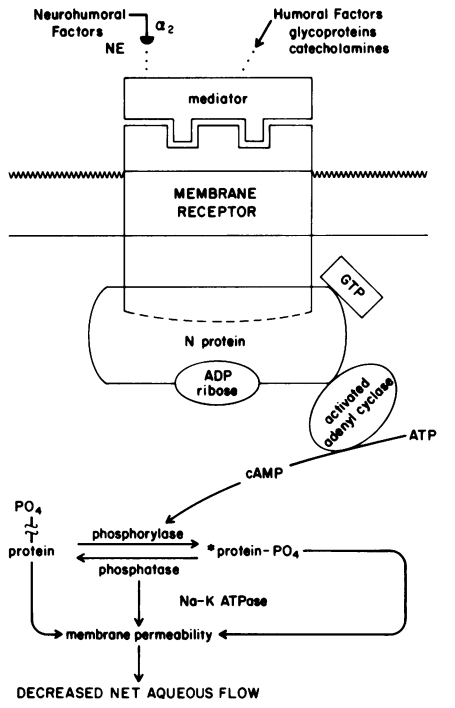

FIG. 12. When mediators act on a beta membrane-bound receptor, the catalytic moiety of the adenylate cyclase complex is activated via the coupling protein, N, that binds GTP. It is possible, in a manner similar to the exogenous ribosylating action of cholera toxin, that endogenous ADP ribosylation of the N protein occurs within the cytoplasm of the cell. In this ribosylated state of the N coupling protein, an associated GTPase is inhibited, an effect that keeps adenylate cyclase activated. (The regulatory role of the guanine nucleotide may include an amplifying effect by GTP and a dampening effect by GDP.) Cyclic AMP is now produced at an accelerated rate and activates or deactivates a phosphorylation system which may directly regulate membrane permeability or may indirectly regulate the rate of formation of aqueous humor by altering the rate at which sodium is presented to an Na-K-ATPase pump.

In the ciliary process the beta receptor may be a $beta_2$ receptor rather than both $beta_1$ and $beta_2$. Whether these receptors are distributed evenly or differently across the epithelia and vasculature is not known at this time.

Beside beta receptors, there are alpha receptors in this scheme. There may be a class of alpha (α) receptors that are linked to inhibit adenyl cyclase. These have not yet been demonstrated for ocular tissue. There may occur other alpha (α) receptors, $alpha_1$, that are post-synaptic, and not linked to adenylate cyclase. These may influence the ciliary process through calcium as a second messenger to produce either vascular or, less likely, epithelial (secretory) effects. These have been demonstrated in the lacrimal and parotid gland, but not for ciliary process. Finally, there are presynaptic, $alpha_2$ receptors that act in feedback, to inhibit norepinephrine synthesis. The effect of these receptors in the ciliary process is unknown at present but they are known to function elsewhere, as in the retina (Modified from Sears and Mead [32]).

basal surfaces of the nonpigmented epithelium with secretion from the apices of this cell layer into the interapical ciliary channels. Evidence from other solute and water-secreting epithelia such as rabbit ileum and colon, frog stomach and cornea, and dogfish rectal gland, among others, indicates that apical exit is enhanced by increased production of cyclic AMP [77–79]. For example, an increase in the apical permeability to chloride and water occurs after an accelerated production of cyclic AMP in the corneal epithelium. There are no direct measurements of chloride activity in these cells, so further work is required to establish the hypothesis that intracellular cyclic AMP may regulate solute and water movement via the apex of the nonpigmented epithelium.

The path for this fluid movement from the apices of the nonpigmented epithelium

is probably via the intercellular "ciliary channels" into which numerous microvilli project from the apical parts of pigmented epithelial and nonpigmented epithelial cells. This area provides an expandable space into which water and metabolites may be secreted. This channel is prominently seen between the two ciliary epithelial layers of the iris (iridial processes) in the rabbit and in the anterior part of the ciliary processes in both rabbit and man. These channels enlarge after stimulation of adenylate cyclase [41] but junctions between cells are not disrupted. (Interestingly, enlargement of these channels was previously described [80] but the author's attention was drawn to the increase in the smooth endoplasmic reticulum associated with the use of topical isoproterenol.) The interapical fluid then could find its way from the ciliary channels into the stroma of the ciliary processes across the pigmented epithelial cell layer, reducing net inflow of aqueous into the posterior chamber [81]. We have found, by electron microscopy, after intra-arterial or intravitreal cholera toxin or hCG or after topical application of forskolin that fluid appears in the space between the pigmented and nonpigmented ciliary epithelium before the IOP decreases. This effect is manifested by an increase in the size of the "ciliary channels." Application of isoproterenol results in similar, though more modest, microanatomical changes. The source of this fluid is not absolutely certain. It is barely possible that it represents a transudate across the pigmented epithelium. The polarity of the nonpigmented epithelium taken together with two other facts suggests that the fluid results from absorption across the basal lateral or basal surfaces of the nonpigmented epithelium with secretion from the apices of the nonpigmented epithelium. Two supporting facts are: *(1)* evidence from all other solute and water-secreting epithelia with regard to the function of the apices [82], and *(2)* the osmotic gradient for water is toward the ciliary stroma from the posterior chamber under ordinary circumstances where the stroma has a protein concentration of 70 percent that of the plasma [83]. Further increases would not act to reverse this outward direction of fluid movement, a movement that would be enhanced by cyclase-mediated flow of solute and water from the apices of the nonpigmented epithelium.

The net solvent flux into the posterior chamber is very likely dependent on the active transport of sodium. The system is probably similar to the model of a standing gradient for osmotic flow related to a relatively tight, moderately high resistance junction between the nonpigmented epithelia. Studies of rates of entry of nascent CO_2 into the posterior chamber after acetazolamide tend to indicate that solvent and bicarbonate move together and that the one-way entry of sodium and bicarbonate is reduced by equimolar amounts. Evidence to couple the activity of the carbonic anhydrase system to sodium movement is still being searched for, however. Sodium could enter the epithelia by the antiporter H^+/Na system, perhaps similar to the renal tubule. The exchange of H^+ for Na^+ from the stroma resulting in a movement of Na and HCO_3 into the posterior chamber is not unreasonable. Other systems, such as those for chloride transport or cotransport, have not been well studied in the ciliary epithelia and remain to speculate about (see schema Fig. 13).

The sum of the forces for the movement of aqueous humor from the stroma into the posterior chamber and from the posterior chamber into the ciliary channels between the epithelial cell layers will determine a total "net" flow (Fig. 13). In this view the adenylate cyclase receptor complex would be regulatory, adjusting net aqueous inflow. The flow will, in the final analysis, be mediated by enzyme complexes in the cell membranes. The secretion of a substance against its concentration gradient across an animal cell plasma frequently involves the coupling of that movement to

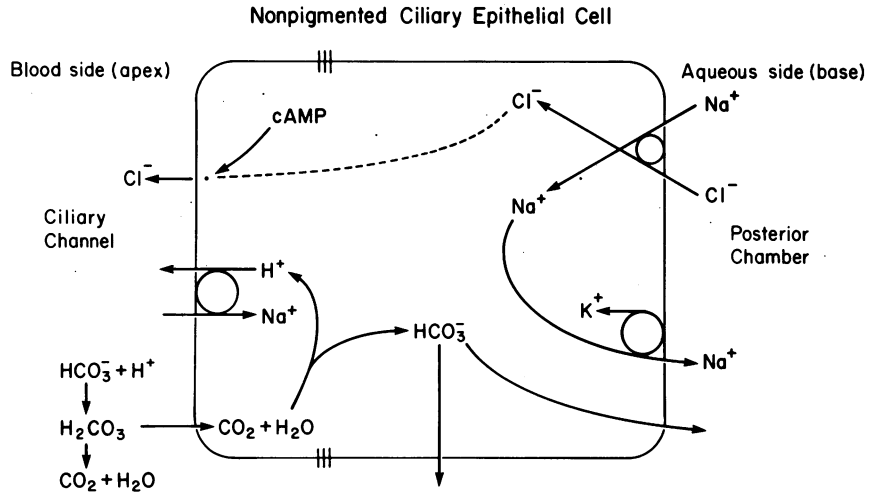

FIG. 13. A model proposed herein for two-way transport in the nonpigmented ciliary epithelia that includes a cAMP-mediated increase in apical permeability to chloride in response to a secretory stimulus and the conventional dogma of a bicarbonate-dependent (perhaps sodium-coupled) mechanism for movement of water into the posterior chamber.

the electrochemical gradient of sodium created by ATPases. Thus, intracellular ions from either of the above "opposing" processes may be further regulated by membrane-bound Na-K ATPase activity. Whether it is this enzyme that is a substrate for phosphorylases activated by cyclic AMP is an issue under study. The demonstration of cyclic AMP-dependent protein kinase activity within the pigment epithelial cell layer [84] may represent a first step toward the clarification of the mechanism by means of which this activity may be influenced. Identification of the phosphorylated proteins and further studies of phosphorylation in the isolated, dissociated, or cultured non-pigmented epithelium will be important. In this layer the energetics and enzyme systems for transport are known to be present [85–87].

A combined biochemical, physiological, pharmacological, and anatomical approach has shed additional light on the molecular mechanisms of IOP regulation. The role of ciliary adenylate cyclase appears to be central and may represent a "final common pathway" in eye pressure regulation. Furthermore, these investigations have drawn attention to an exciting new area of ocular pharmacology. If the reduction of IOP sufficient to arrest and/or delay optic nerve damage is one goal in glaucoma treatment, then the stimulation of ciliary cyclic AMP production by a compound such as forskolin represents fertile ground for future research.

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