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cGMP Modulates Transport across the Ciliary Epithelium

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Abstract. cGMP reduced the short-circuit current (I_{SC}) when applied to the aqueous surface of isolated rabbit and cat ciliary epithelia. cGMP either stimulated (in the rabbit) or had no effect (in the cat) on I_{SC} when applied to the stromal surface. Addition of the cGMP-mediated hormone atrial natriuretic peptide (ANP) to the stromal (but not the aqueous) surface, or the nitrovasodilator sodium nitroprusside to the stromal surface, inhibited I_{SC} across rabbit ciliary epithelium.

The response to stromal cGMP was partly mediated by K^+ channels at the stromal surface of the rabbit pigmented epithelial (PE) cells, since the effect was inhibited by stromal Ba^{2+}, and was unaffected by Cl^- replacement, by bumetanide, or by DIDS. In contrast, the response to aqueous cGMP was not likely mediated by changing either K^+ or Cl^- channels, based on transepithelial measurements of rabbit ciliary epithelium and complementary whole-cell patch clamping of cultured human nonpigmented ciliary epithelial (NPE) cells. The possibility of interacting effects between cGMP and cAMP in targeting the Na^+,K^+-exchange pump was also considered. Strophanthidin blocked the responses to either aqueous or stromal cGAMP. Applying 10 μM forskolin to generate endogenous cAMP enhanced the subsequent response to aqueous cGMP by ≈80%.

We conclude that cGMP has at least two actions on the ciliary epithelium. The major effect may be to reverse cAMP-mediated inhibition of the NPE Na^+ pumps at the aqueous surface of both rabbit and cat ciliary epithelia. The second effect is likely mediated by increasing K^+-channel and pump activity of the rabbit PE cells at the stromal surface.

Key words: ANP — K^+ channels — Na^+ pump — cAMP — Whole-cell patch clamping — Cultured NPE cells

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Introduction

On a systemic level, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and NO on the one hand [3, 13, 28, 40, 58], and angiotensin II (AII) [12] on the other, exert opposing effects on vasomotion; the natriuretic peptides and AII also oppose each other in regulating natriuresis. This integrative balance is partly mediated through changes in the intracellular concentration of cGMP (cyclic guanosine-3′,5′-monophosphate), which is increased by natriuretic peptides [30, 31] and NO [58] and decreased by AII [12]. Published data indicate that the ciliary epithelium can respond, and/or has receptors, to all of these transmitters [8, 32, 35, 38, 41, 45, 48].

Considerably less is known about cGMP than about cAMP, the first cyclic nucleotide identified as a second messenger. However, as considered in the Discussion, several published data suggest that these two cyclic nucleotides exert opposite effects on aqueous humor secretion. These observations raise the intriguing possibility that cGMP and cAMP form a pair of opposing second messengers whose interaction regulates secretion of aqueous humor.

In the current manuscript, we explore the role of cGMP in modulating ion transport across the isolated ciliary epithelium. This epithelium is responsible for secreting the aqueous humor and consists of two cell layers, the pigmented epithelial (PE) cells facing the stroma and the nonpigmented epithelial (NPE) cells abutting the aqueous humor. Gap junctions provide intercommunicating pathways among the NPE and PE cells and between the two cell layers, but tight junctions surround only the NPE cells [52]. In the current study, transepi-

thelial electrophysiologic measurements have been complemented by whole-cell patch clamping of cultured nonpigmented ciliary epithelial (NPE) cells. The results are interpreted within the framework of a heuristic model of aqueous humor secretion. Finally, the manuscript presents evidence that the cGMP-mediated transmitters ANP and NO both modulate ion transport across the rabbit ciliary epithelium.

Materials and Methods

Transepithelial Measurements

Adult male Dutch belted rabbits weighing 1.8–2.4 kg were purchased from Penn Dutch Laboratory Animals (Denver, PA) and Ace Animals (Boyertown, PA). The rabbits were anesthetized with 25–40 mg/kg sodium pentobarbital (Abbott Laboratories, North Chicago, IL) and sacrificed by injecting air into the marginal ear vein. Cats were euthanized by administering 80–100 mg/kg sodium pentobarbital. Treatment and sacrifice of all animals was in accordance with the ARVO Resolution on the Use of Animals in Research. After enucleation, the iris-ciliary body (I-CB) was isolated by the method of Krupin et al. [33].

The pupil and central area between pupil and ciliary processes were occluded with a Lucite disc, and the I-CB was mounted between the two halves of a Lucite chamber, using a modification of previously described techniques [33]. In particular, a primary seal of the tissue-contact surfaces was created by application of vacuum grease, facilitating a leak-proof preparation with greatly reduced compression of the chamber assembly and attendant edge damage. Tissues were bathed with a Ringer's solution bubbled with 95%/O_2/5% CO_2, and containing (in mM): 110 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 30 $NaHCO_3$, 1.2 KH_2PO_4, 15.0 HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], and 10.0 glucose, at a pH of 7.4 and osmolality of 310 mOsm. Where appropriate, tissues were also bathed with a chloride-free Ringer's solution containing (in mM): 114.7 sodium methylsulfonate, 3.5 $KHCO_3$, 1.2 $MgSO_4$, 1.9 $CaSO_4$, 25.3 $NaHCO_3$, 1.2 KH_2PO_4, 15.0 HEPES, and 10.0 glucose. The transepithelial potential was clamped at 0 mV, and the short-circuit current monitored on a chart recorder.

Whole Cell Patch Clamping of NPE Cells

The NPE cell line studied (ODM/SV40) was derived from the human nonpigmented ciliary epithelium [42]. As previously described [18], cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and 50 μg/ml gentamycin, at 37°C in 5% CO_2. Cells were passaged each 6–7 days, and after becoming confluent, were studied within 6–10 days after passage. Cells were harvested by trypsinization, resuspended, and permitted to settle and attach to glass coverslips, which were then mounted in a perfusion chamber [69].

Micropipettes were double-pulled from Corning No. 7052 glass, coated with Sylgard, and fire polished. The filling solution consisted of (in mM): 20.0 NaCl, 110.0 K^+ aspartate, 1.0 $MgCl_2$, 10.0 HEPES, 5.0 K_4BAPTA, and 5.0 MgATP, at a pH of 7.2. Sufficient $CaCl_2$ was included to generate a free Ca^{2+} concentration of 200 nM. With this solution, the micropipettes displayed resistances of 3–7 MΩ. The seals formed commonly had resistances of ≈10–20 GΩ. After rupturing the membrane patch, the open-circuit potential was noted and the current-voltage relationship determined by analyzing the current responses to a series of voltage pulses.

Data were acquired using List L/M-EPC7 electronics and associated headstage. Filtering was conducted with an eight-pole low-pass Bessel filter (100–500 Hz). The sampling rate was adjusted to be 2.5–3.0 times faster than the corner frequency of the filter to avoid aliasing the signals during the test pulses. The access resistance was always far less than the membrane resistance, so that series resistance compensation was not used.

Chemicals

All chemicals were reagent grade. Sodium nitroprusside, atriopeptin (atrial natriuretic peptide), cGMP in the forms of 8-Br-cGMP (8-bromoguanosine 3:′5′-cyclic monophosphate) and dibutyryl-cGMP (n^2,2′-O-dibutyrylguanosine 3′:5′-cyclic monphosphate), and IBMX (3-isobutyl-1-methylxanthine) were all obtained from The Sigma Chemical, St. Louis, MO. The diastereomer (R_p) of adenosine 3′-5′-(cyclic)phosphorothioate (cAMPS) and staurosporin were purchased from Biomol (Plymouth Meeting, PA). NPPB [5-nitro-2-(3-phenylpropylamino)-benzoate] was a generous gift from Prof. Rainer Greger.

Statistics

Unless otherwise stated, values are presented as means ± SE. The probability of the null hypothesis was calculated with Student's *t*-test.

Results

Effects of cGMP on Baseline Short-circuit Current across Isolated Iris-Ciliary Body

The baseline short-circuit current (I_{SC}) was 7.2 ± 0.4 μA and the difference in electrical potential was –0.58 ± 0.03 mV across 71 issues from 56 rabbits. Addition of a membrane-soluble form of cGMP (dibutyryl-cGMP) to the aqueous surface produced a dose-dependent inhibition of I_{SC} (Table (*A*), Fig. 1*D–G*). Consistent reductions were noted at 1 and 10 mM, but not at 100 μM. Unexpectedly, cGMP at the stromal surface produced the opposite effect, stimulating I_{SC} in a dose-dependent fashion (Table (B), Fig. 1*A–C*). Both the inhibitory and stimulatory actions of cGMP were reversible and repeatable with the same tissue. The sidedness of the cGMP responses contrasts with the report that the cyclic nucleotide cAMP stimulates I_{SC} when added to either surface of rabbit I-CB [15].

The addition of 0.1–10 mM cGMP increased the osmolality of the bath by 0.6–6%. We wished to clarify whether the addition of cGMP exerted its effects solely by altering tonicity. For this purpose, we modified the baseline Ringer's solution by substituting 30 mM sodium methylsulfonate for an equimolar concentration of NaCl. Under these conditions, increasing amounts of cGMP could be added to either surface (isotonically replacing the sodium methylsulfonate) without altering either the concentrations of the permeant ions or the osmolality

Table. Responses of I_{SC} (μA) across rabbit ciliary epithelium after applying cGMP in the absence and presence of transport inhibitors.[a]

A. Aqueous cGMP Conditions	0.1 mM	1.0 mM	10.0 mM
1. Baseline	−0.167 ± 0.076 (6)	−0.802 ± 0.063 (46)[b]	−2.113 ± 0.299 (8)[b]
2. Ba^{2+} (5 mM)	−0.267 ± 0.033 (3)[e]	−1.233 ± 0.105 (6)[b]	−3.167 ± 0.285 (3)[d]
3. 0 Cl^-	−0.150 ± 0.150 (2)[f]	−0.600 ± 0.000 (2)[f]	−1.250 ± 0.250 (2)[f]
4. NPPB (100 μM)		−0.550 ± 0.050 (2)[f]	

B. Stromal cGMP Conditions	0.1 mM	1.0 mM	10.0 mM
1. Baseline	0.100 ± 0.058 (4)	0.438 ± 0.082 (14)[b]	1.383 ± 0.237 (4)[c]
2. Ba^{2+} (5 mM)	0.000 ± 0.000 (6)	0.067 ± 0.072 (6)	0.650 ± 0.284 (4)
3. 0 Cl^-	−0.067 ± 0.067 (3)	0.333 ± 0.333 (3)	0.733 ± 0.406 (3)
4. Bumetanide[g]	0.000 ± 0.000 (3)	0.300 ± 0.129 (4)	2.325 ± 0.206 (4)[c]
5. DIDS (0.5 mM)	0.300 ± 0.000 (2)[f]	0.300 ± 0.100 (2)[f]	1.500 ± 0.100 (2)[f]

[a] Numbers of experiments are entered in parentheses. [b] $P < 0.001$. [c] $P < 0.005$. [d] $P < 0.01$. [e] $P < 0.02$. [f] Uncertainty calculated as half the difference between two experimental results. [g] Bumetanide was applied to the stromal surface at concentrations of 0.1 mM ($N = 3$) and 1.0 mM ($N = 1$).

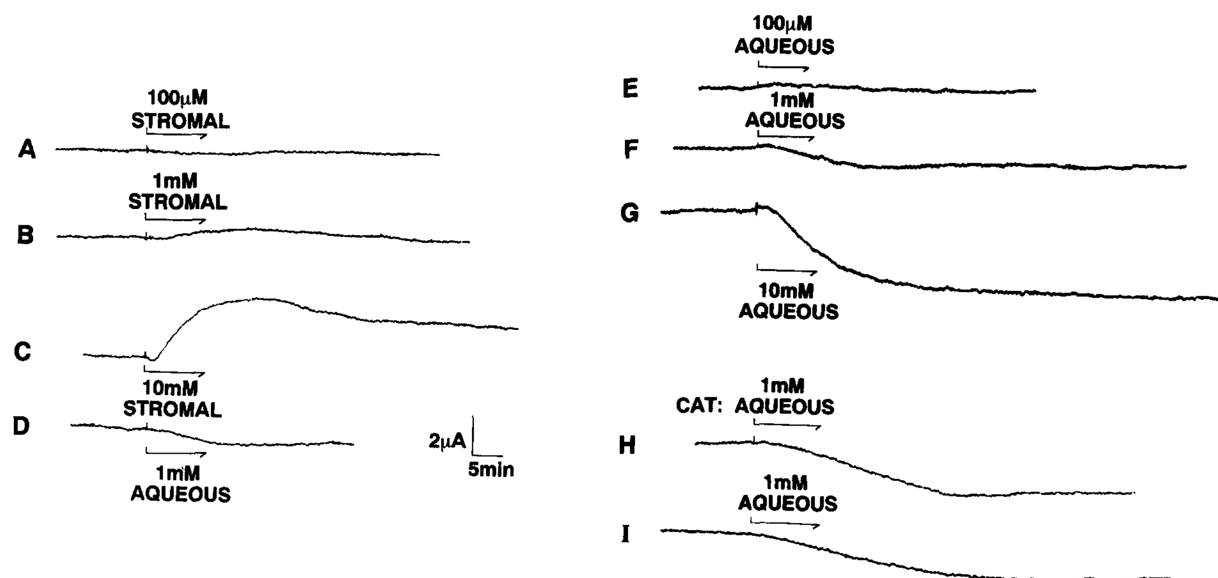

Fig. 1. Continuous recordings of short-circuit current (I_{SC}) across two isolated I-CB preparations from the rabbit (*A–D* and *E–G*) and two from the cat (*H* and *I*) during addition of dibutyryl cGMP to the side indicated. Stromal cGMP stimulated I_{SC} across the rabbit ciliary epithelium, and aqueous cGMP reduced the I_{SC} of preparations from both species in dose-dependent fashions.

(301–307 mOsm) of the medium. As observed with the anisosmotic additions, stromal cGMP stimulated (Fig. 2*A–C*), and aqueous cGMP reduced (Fig. 2*D–F*) the short-circuit current in dose-dependent fashions. These results indicate that cGMP exerts physiologic effects on transport by the ciliary epithelium, independent of any osmotic effects.

Until now, we have studied I-CB preparations dissected only from the rabbit [14, 34, 62]. Since in some ways, the I-CB of the cat is closer to that of the primate [9, 10, 70], we have also utilized the I-CB from this second species in the present work. In four experiments conducted with the tissues from two cats, 1 mM aqueous cGMP had the same inhibitory effect on the cat as on the rabbit preparation, reducing I_{SC} by 1.9 ± 0.5 μA (Fig. 1*H–I*). Once again, the response was side-specific, albeit not identical with the rabbit response. Addition of 1 mM cGMP to the stromal surface had no significant effect in two experiments. The basis for the difference in responses of the cat and rabbit preparations to stromal cGMP is unclear. The difference does not reflect uptake and binding by pigment granules since pigmented rabbits

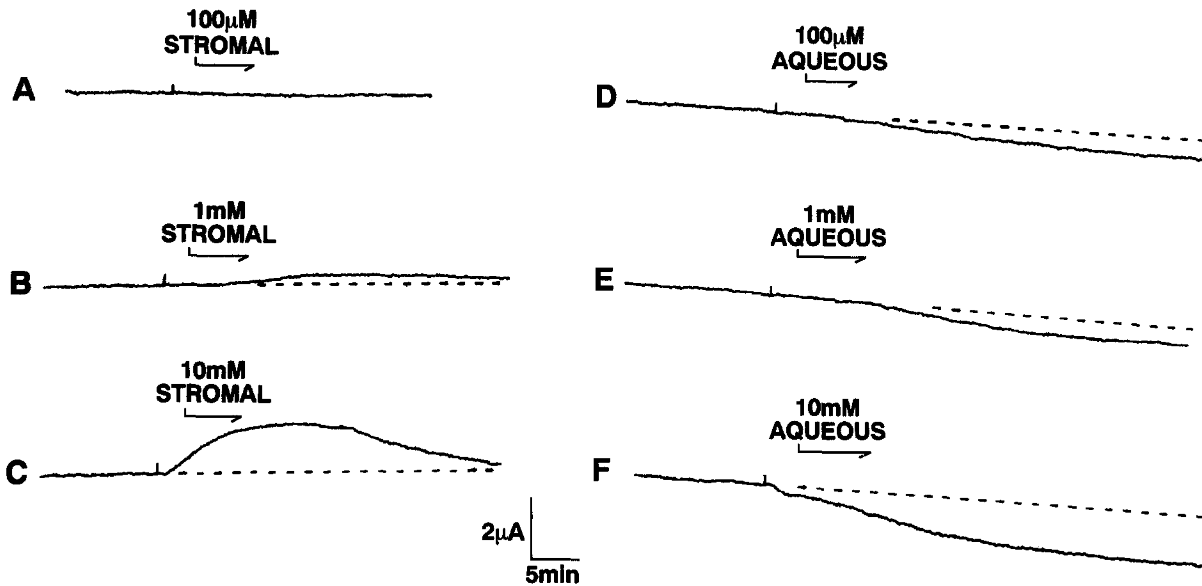

Fig. 2. The isosmotic application of cGMP to the rabbit iris-ciliary body produced the same effects on I_{SC} as those displayed by Fig. 1*A–G*. The interrupted lines in this and other figures are extensions of the baseline time courses drawn by eye for purposes of clarification.

were used. The different responses could have reflected differences in accessibility of the cGMP to the basolateral membranes of the PE cells. This possibility was examined by subsequently adding the cardiotonic steroid strophanthidin to the stromal solution. Application of this pump inhibitor did abolish the I_{SC} across the cat I-CB. We conclude that the differential responses of the two preparations to stromal cGMP likely reflects differences in the transport characteristics of the PE basolateral membranes from the two species.

Effects of Agents Stimulating Intracellular cGMP Production

Although exogenous cGMP clearly altered transport across the ciliary epithelium, it was unclear whether the intracellular cGMP concentrations attained necessarily corresponded to physiologic levels. For this reason, we also administered a hormone and a nitrovasodilator whose actions are thought to be mediated by cGMP.

As noted in the Introduction, the natriuretic peptides are known to increase intracellular cGMP [30, 31]. In each of three rabbit preparations, 1 nM stromal ANP reduced I_{SC} by 0.2–0.7 μA, with further inhibitions elicited as the concentration was increased to 100 nM in two preparations (Fig. 3). At a lower concentration (0.1 nM), a clear inhibition was noted in only one of three tissues. Application of aqueous ANP (0.1–100 nM) had no detectable effect in an additional experiment.

Nitrovasodilators have also been reported to stimulate cGMP accumulation in many tissues by generating NO [21]. One such agent (nitroprusside) is known to stimulate cGMP production in ciliary processes, with a maximal response at 0.5–10 μM [45]. In preparations from three rabbits, 0.2 μM stromal nitroprusside inhibited the I_{SC} by 0.9 ± 0.3 μA.

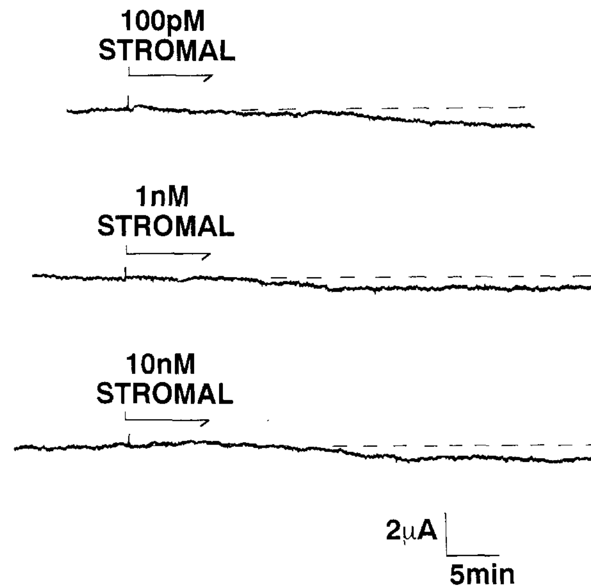

Fig. 3. Short-circuit current (I_{SC}) across isolated rabbit I-CB during addition of increasing concentrations of atrial natriuretic peptide (ANP) to the stromal reservoir. ANP had no effect from the aqueous surface.

Since the effects of both ANP and nitroprusside were (like aqueous cGMP) inhibitory, we have tentatively concluded that ANP- and nitroprusside-stimulated cGMP exerts its dominant effect on the epithelial cells abutting the aqueous surface, i.e., on the NPE cells. The

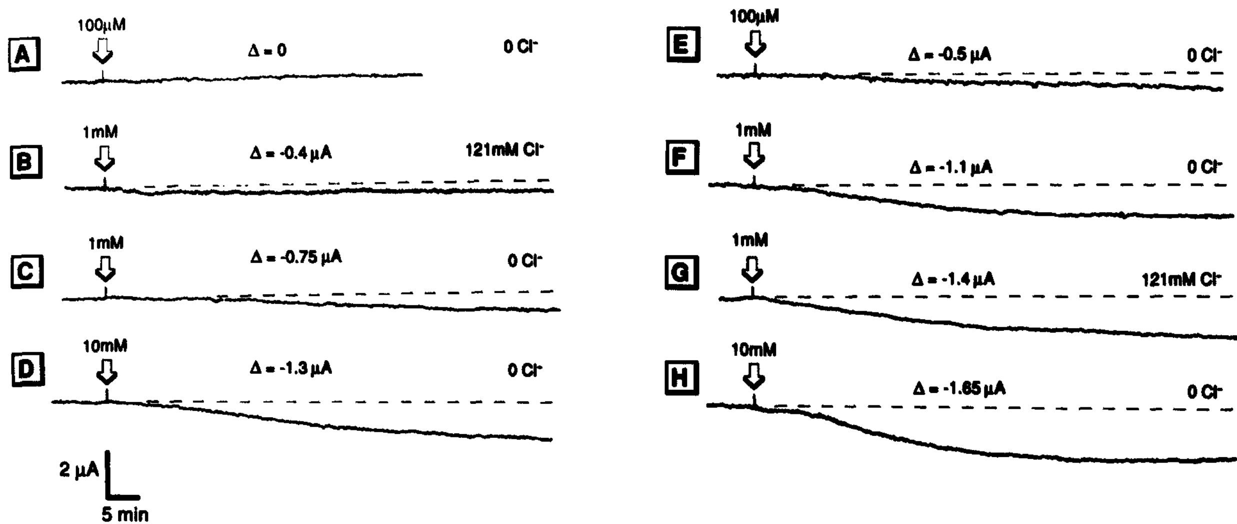

Fig. 4. Effect of external Cl^- on response to aqueous cGMP. Panels *A–D* and *E–H* were obtained from two different rabbit preparations. The traces are presented in order of increasingly large inhibitory responses. With the I-CB of panels *A–D,* the inhibitory effect of 1 mM aqueous cGMP was slightly smaller in the absence of external Cl^- (trace *B*), than in its presence (trace *C*). The opposite was true for the preparation of panels *E–H (cf.* trace *F* with trace *G*).

sidedness of the ANP likely reflects its binding largely at the basolateral membranes of the PE cells [8], with the second messenger cGMP then diffusing through gap junctions to target the NPE cells.

Mechanism of Action of Stromal cGMP

cGMP clearly inhibits short circuit current when applied to the aqueous phase of rabbit or cat I-CB. In contrast, stromal cGMP either has little effect (in cat) or actually stimulates short circuit current (in rabbit). This sidedness strongly suggests that cGMP is exerting at least two different actions on ciliary epithelial transport. In principle, the results obtained by transepithelial analysis could reflect changes in: ion channel conductances, transmembrane ionic gradients, turnover rates of rheogenic symports or antiports, or currents through the Na,K-exchange pumps of the NPE and PE cells. cGMP has been reported to exert effects on multiple channels: inhibiting the epithelial 28-pS, poorly-selective Na^+ channel [37]; stimulating K^+ channels in a number of cells [1, 20, 27, 38, 53, 63, 65]; and stimulating the cation-nonselective phototransduction channel of the outer segments of rods and cones [71].

In the presence of 5 mM stromal Ba^{2+}, 1–10 mM stromal cGMP exerted no statistically significant effect (Table (*B*)). In contrast, the responses were not substantially changed by: replacing Cl^- with methylsulfonate, blocking the $Na^+/K^+/2Cl^-$ symport [24, 67] with 0.1–1.0 mM bumetanide, or by inhibiting $Na^+/2HCO_3^-$ symport activity [68] with 0.5 mM DIDS (Table (*B*)). We conclude that the stimulatory effect of stromal cGMP is partly mediated by activation of K^+ channels.

Mechanism of Action of Aqueous cGMP

Insofar as application of cGMP to the aqueous humor produced an effect opposite to that observed at the stromal surface, we have presumed that the aqueous cGMP is acting primarily on the nonpigmented ciliary epithelial cells abutting that surface. The mechanism underlying the action of cGMP at the aqueous surface has been less clear than at the stromal surface. Unlike the results noted with stromal Ba^{2+}, 5 mM aqueous Ba^{2+} did not inhibit the response to aqueous cGMP (Table (A)). Likewise, substituting methylsulfonate for Cl^- had little effect on the response to aqueous cGMP in duplicate experiments (Table (A)). The time courses are displayed in Fig. 4*A–D* and *E–H.* Increasing concentrations of cGMP (0.1, 1.0 and 10.0 mM) produced increasing inhibitions of I_{SC}, even in the absence of external Cl^- (traces *A, C,* and *D,* and *E, F,* and *H,* respectively). In the subsequent presence of external Cl^-, the response to 1 mM cGMP was slightly smaller in one experiment (trace *B*) and slightly larger in the other (trace *G*). In an additional experiment 0.1 mM aqueous bumetanide did not abolish the response to 1 mM aqueous cGMP.

We have also tested the action of NPPB, an effective blocker of the Cl^- channels of nonpigmented ciliary epithelial cells [19, 69] (Table (*A*)). In two experiments, 1 mM aqueous cGMP was added before introducing NPPB, in the presence of 100 μM aqueous NPPB, and after washing out the NPPB. Similar inhibitory effects were measured during the three periods of observation: –0.6, –0.6 and –0.6 μA with one preparation, and –0.7, –0.5, and –0.9 μA with the second.

These results led us to tentatively conclude that

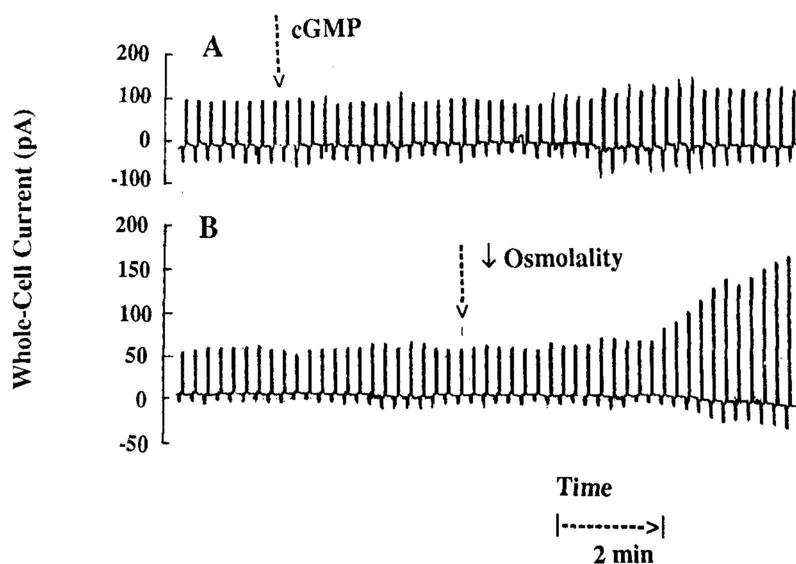

Fig. 5. Continuous recordings of whole-cell current during addition of 1 mM dibutyryl-cGMP (*A*), and during later reduction of osmolality (*B*) of solution perfusing the same cell. V_m was held at −43 mV, and sequentially pulsed to −84 mV and +49 mV at 15-sec intervals. The values of holding and test potentials were chosen to approximate the Nernst potentials for Cl^-, K^+ and Na^+, respectively. The small increase in whole-cell conductance noted after adding cGMP was not consistently observed in other experiments. In contrast, the same cell displayed the characteristic increase in conductance triggered by a 10% reduction in osmolality (established by omitting sucrose from the perfusate).

aqueous cGMP affects neither the K^+ nor the Cl^- channels of the NPE basolateral membrane.

Whole Cell Patch Clamping of Cultured NPE Cells

The question of the potential role of ion channels in mediating the effect of cGMP on the NPE cells was also addressed by ruptured-membrane whole-cell patch-clamping of ODM/SV40 cells derived from human NPE cells [42]. Many (and possibly all) of the transport properties of NPE cells in intact ciliary epithelia are retained by this cell line [14].

Our initial protocol was to keep the membrane at a holding potential of −80 mV. A prepulse of +60 mV was then applied for ≈900 msec, depolarizing V_m and activating a set of outwardly-rectifying K^+ channels [69]. V_m was subsequently clamped at a different value (ranging from −100 to +60 mV) during successive sequences of voltage pulses. At the conclusion of each sequence, V_m was restored to the holding potential. From these measurements, we were able to characterize the current-voltage relationship of both stationary and nonstationary channels contributing to the whole-cell currents. In a series of 7 experiments, we found that cGMP exerted small complex effects on the whole-cell currents (*data not shown*). Both small increases and decreases in conductance were observed, occasionally at different times in the same cell.

A second voltage protocol was also applied in an effort to better detect the time course of any cGMP-mediated conductance effects. Rather than determining the full current-voltage relationship at discrete time points, the whole-cell currents were continuously monitored at only 3 voltages: (i) E_K, the Nernst potential for K^+ (approximately −84 mV), (ii) E_{Cl}, the Nernst potential for Cl^- (approximately −43 mV), and (iii) either E_{Na}, the Nernst potential for Na^+ (approximately +49 mV), or 0 mV. This continuous-recording technique was used in four technically satisfactory experiments. Despite the improved resolution, we still found no consistent, reproducible change in whole-cell current following perfusion with cGMP at a concentration (1 mM) which elicited substantial transepithelial effects. For example, the whole-cell currents of Fig. 5 changed very little after exposure to cGMP, but the same cell was able to respond strikingly to a different, subsequent stimulus (10% hypotonicity). In two of these experiments, 50 μM IBMX was included in the perfusate to minimize hydrolysis of the cGMP by endogenous phosphodiesterase.

We have concluded that cGMP does not significantly affect those baseline ion channels providing the major contribution to whole-cell conductance in cultured NPE cells. This finding is consistent with our inability to detect changes in K^+ or Cl^- conductance following addition of cGMP to the aqueous medium bathing intact rabbit ciliary epithelium.

Potential Role of Na^+, K^+-exchange Pump in Mediating Action of Aqueous cGMP

In view of our inability to detect evidence of ion channel-mediated actions of cGMP on NPE cells from either transepithelial or whole-cell patch experiments, we considered the possibility that aqueous cGMP might inhibit I_{SC} by stimulating positive current through the pump into the aqueous. As considered in the Discussion, this possibility could not be rigorously addressed within the framework of the present study. However, the data of Fig. 6 are consistent with this possibility. Following the application of 50 μM aqueous strophanthidin to inhibit the pump, 1 mM aqueous cGMP had no effect on the short-circuit current (Fig. 6*B*), in contrast to the typical

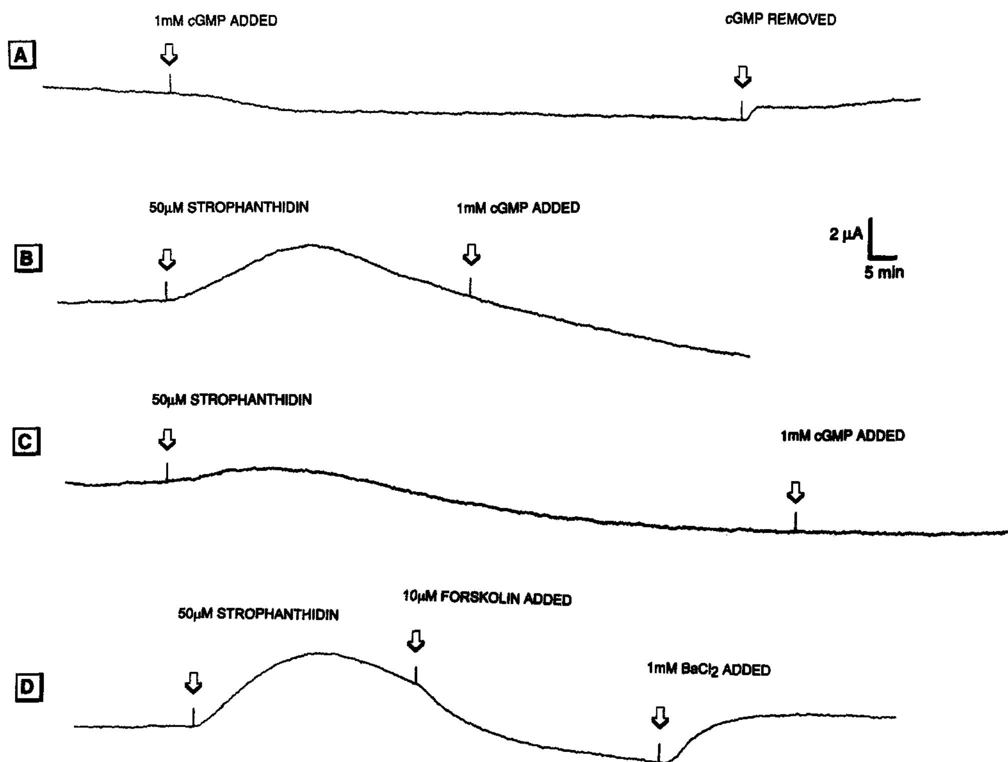

Fig. 6. Effect of aqueous strophanthidin on response to aqueous cGMP. (*A*) Prior to introducing strophanthidin, 1 mM cGMP produced a typical inhibition of I_{SC}. (*B*) The trace is a continuation of the record of panel *A*, displaying the transient stimulation and subsequent inhibition by 50 µM strophanthidin. Addition of 1 mM aqueous cGMP during the declining phase had no effect on the time course of the I_{SC}. (*C*) In another experiment, 50 µM strophanthidin was again added to the aqueous bath, but the 1 mM aqueous cGMP was withheld until the I_{SC} reached a steady state. Once again, the cGMP produced no detectable effect. (*D*) In another separate experiment, pretreatment with 50 µM strophanthidin reversed the subsequent response to 10 µM aqueous forskolin. Instead of the characteristic stimulation, the forskolin now inhibited the I_{SC}, an effect which could be reversed by the later addition of 1 mM Ba^{2+}.

inhibition noted earlier with the same preparation (Fig. 6*A*). The cGMP of Fig. 6*B* was added during the declining phase of current. When the addition of aqueous cGMP was delayed until a steady state was reached, again no effect was detected (Fig. 6C). The strophanthidin did not prevent the tissue from responding to other agents. For example, the subsequent addition of 10 µM forskolin reduced I_{SC} in two other experiments, an effect reversed by aqueous Ba^{2+} (Fig. 6*D*). Additionally, in 3 experiments the current-elevating effect of stromal cGMP was reversibly eliminated following stromal strophanthidin (*data not shown*).

Interaction of cGMP and cAMP

Recently, cAMP-dependent kinase (PKA) has been reported to phosphorylate and inhibit the Na^+,K^+-activated pump of ciliary epithelial cells [22, 23]. The data of Figs. 4–6 were consistent with the possibility that aqueous cGMP might also act on the pump of the NPE cells. Since both agents might be targeting the pump, we examined the effect of cAMP on the response to aqueous cGMP.

In a series of seven experiments, 10 µM forskolin was applied to the aqueous surface to stimulate the catalytic subunit of adenylyl cyclase, generating endogenous cAMP. As previously demonstrated [16], forskolin produced an increase in short-circuit current (ΔI_{SC} = 0.8 ± 0.1 µA, $P < 0.001$). In the present study, that stimulation was transient without significant alteration of the steady-state level (ΔI_{SC} = −0.3 ± 0.2 µA). In the presence of forskolin, 1 mM aqueous cGMP reduced I_{SC} by 0.93 ± 0.09 µA (Fig. 7*B*). The magnitude of this inhibition was greater than that produced by cGMP either before forskolin was added (Fig. 7*A*) or after forskolin was washed out from the same tissue (Fig. 7*C*). The effects of forskolin on the response to cGMP by seven preparations are summarized in the bar graph of Fig. 7*D*. Wherever possible, we have tried to compare the effects of forskolin + cGMP (experimental period) to the mean of the results in the control periods obtained with cGMP alone, before and after the experimental period. In two experiments, the cGMP effect was not measured after the experimental period, so that the comparison was made only with the initial control application of cGMP. If anything, this latter comparison will underestimate the magnitude of the forskolin-induced enhancement, since the

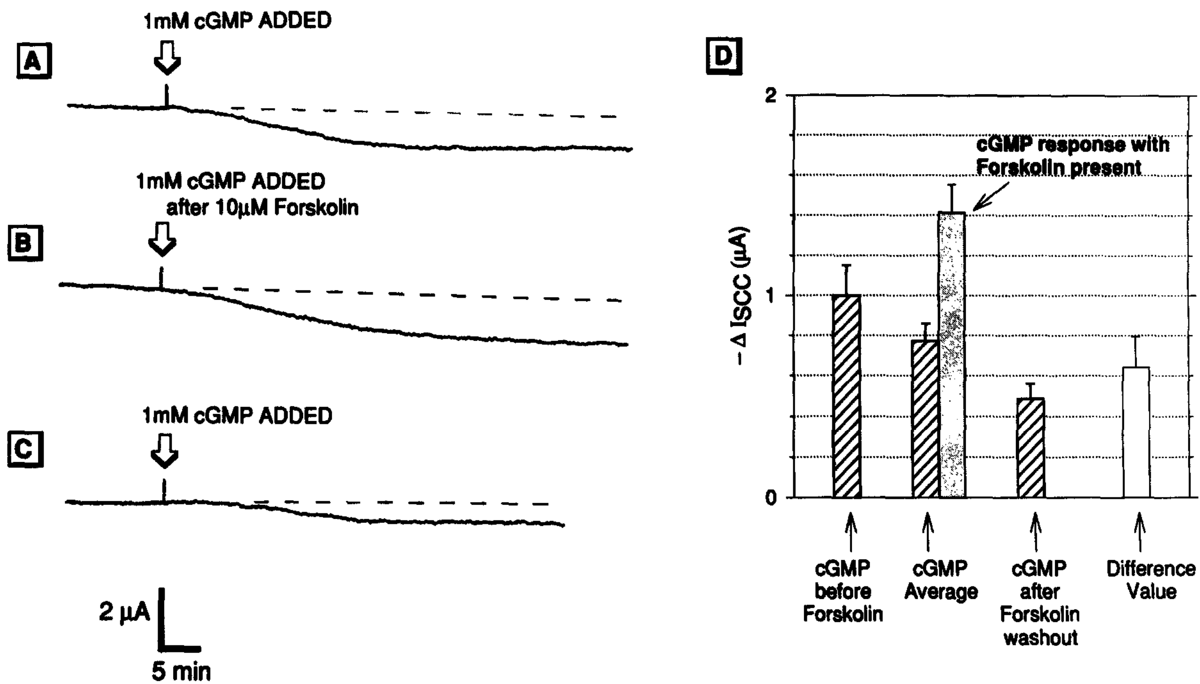

Fig. 7. Interaction of forskolin and aqueous cGMP. cGMP (1 mM) was added successively to the aqueous bath in traces *A–C*, separated by intervening bilateral washouts. In the absence of forskolin, cGMP inhibited I_{SC} by 1.1 µA at the beginning of the experiment (*A*) and by 0.5 µA at its conclusion (*C*). The intermediate addition (B) was conducted after prior addition of 10 µM forskolin, and produced an inhibition of 1.3 µA, considerably larger than the average control value of 0.8 µA (taken as the mean of *A* and *C*). The means ± SE from the results of seven experiments are presented in the bar graph of panel *D*. Before, during, and after adding forskolin, cGMP reduced I_{SC} by 0.67 ± 0.10, 0.93 ± 0.09, and 0.32 ± 0.05 µA, respectively. To facilitate comparison, the initial response has been normalized to 1.0. The bar labeled "cGMP Average" has been calculated from the mean control values in the five experiments where cGMP was added both before and after the period of forskolin exposure, and from the initial cGMP response in two experiments where the response after forskolin washout was not measured. The difference between forskolin-enhanced response to cGMP and the "cGMP Average" ("Difference Value") was significant at the 0.01 probability level.

magnitude of the response to cGMP tended to run down with repetitive applications of the cyclic nucleotide. With this approach, we calculate that pre-exposure to forskolin increased the magnitude of the response to aqueous cGMP by 0.4 ± 0.1 µA ($P < 0.01$), an enhancement of ≈80%.

The interaction of forskolin with PKA inhibitors was examined in four additional experiments. As illustrated in Fig. 8*A*, at a 100 µM concentration the inhibitory diastereomer of cAMPS, R_p [11], itself inhibited I_{SC} when applied to the aqueous surface, but was unable to prevent (Fig. 8*A*) or reverse (Fig. 8*B*) the effect of 10 µM forskolin in two experiments. We have interpreted this to mean that the R_p dose used externally was adequate to partially reverse the effects of low baseline levels of cAMP, but insufficiently high to inhibit the response to the large increase in cAMP after forskolin. This interpretation is consistent with the known effects of R_p on the activation curves of purified PKA [11], and on calculations based on measured cAMP levels (L. Liu, E. Eta, P. Bhattacherjee and C. Paterson, *submitted*) and the cell volume of cultured nonpigmented ciliary epithelial cells [17]. Even in the presence of 100 µM R_p, 30 µM cAMP fully activates PKA [Fig. 2, ref. 11]. Forskolin likely increases the intracellular cAMP level to >1 mM, 1–2 orders of magnitude higher than the fully activating concentration.

To pursue this further, we turned to staurosporin, which is usually taken to be an inhibitor of protein kinase C, but is also highly effective in blocking PKA at an IC_{50} value only threefold higher [61]. The application of this less specific, but far more powerful, PKA inhibitor to two preparations replicated the effects of cGMP in reversing the forskolin-induced stimulation of I_{SC} (Fig. 8).

Discussion

cAMP and cGMP as Balancing and Opposing Second Messengers

Recent publications suggest that the cyclic nucleotides cAMP and cGMP exert opposite effects on aqueous humor secretion. Topical administration of forskolin to directly activate adenylyl cyclase and increase cAMP pro-

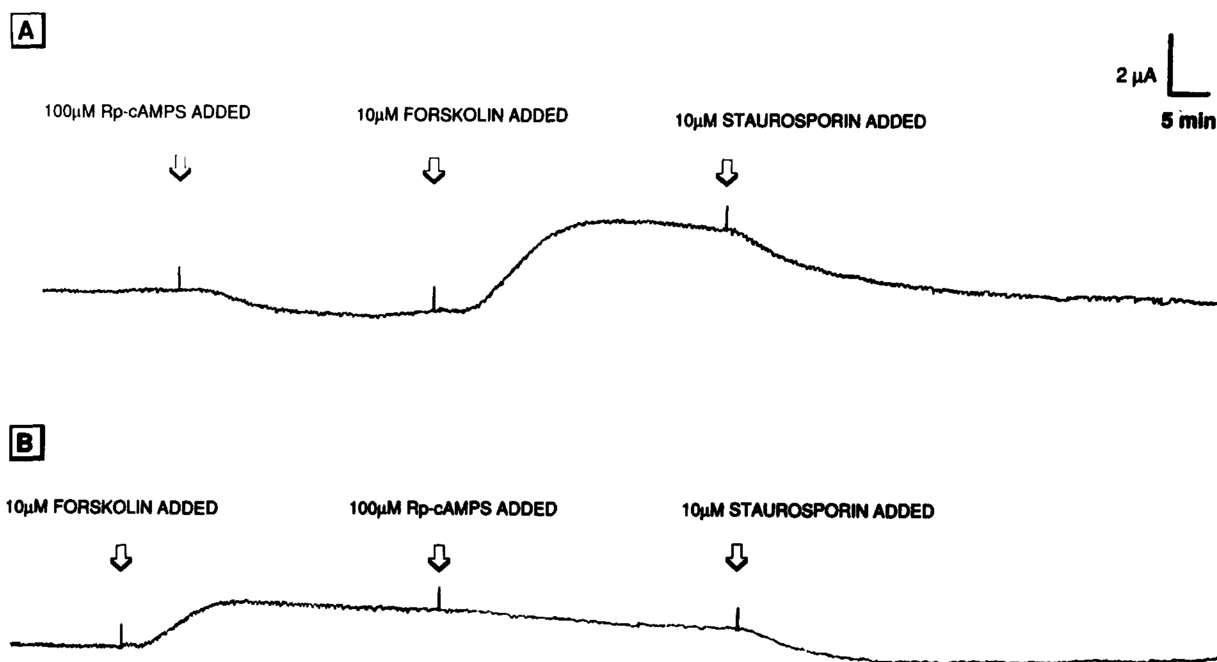

Fig. 8. Interaction of forskolin and PKA inhibitors. At 100 μM, the inhibitory diastereomer of cAMPS, R_p, inhibited I_{SC} when applied to the aqueous surface (*A*), but was unable to prevent (*A*) or reverse (*B*) the effect of forskolin. Application of the nonspecific, but more effective, kinase inhibitor staurosporin [61] largely reversed the action of forskolin at a concentration of 10 μM.

duction reduces both net secretion of aqueous humor and intraocular pressure (IOP) in humans [56]. Similar results are obtained by intravitreal injection of rabbits with Al^{3+} or Be^{2+} and F^- to activate G-protein (G_S), stimulating cAMP production by adenylyl cyclase [46]. Levels of cAMP can also be increased by reducing phosphodiesterase breakdown of the nucleotide. Topical or intravitreal administration of a phosphodiesterase inhibitor, with [47] or without forskolin [43], reduces the IOP of rabbits. Thus, cAMP produces a fall in both secretion and IOP. In contrast, sodium nitroprusside which acts to increase cGMP [21] has been found to increase both aqueous humor secretion and IOP [35]. At low topical concentrations, nitroprusside may not reach the ciliary epithelium, and the major action is to increase outflow [49]. At higher topical concentrations, nitroprusside increases secretion [35], very possibly by increasing intracellular cGMP. The precise effects of ANP on aqueous humor secretion and IOP are not clear. Several investigators have reported that the peptide decreases either IOP alone or both parameters [32, 45, 48, 60]. However, ANP has also been reported to transiently increase aqueous humor secretion in the cynomolgus monkey [55]. The absence of a clear consensus on the functional ocular effects of ANP is not surprising, since the peptide's effects on other organs (particularly the kidney) depend upon the baseline state of the animal [26]. In the case of the IOP, multiple effects of ANP on blood flow, secretory mechanisms and outflow could further confound the interpretation of in vivo results. In the present work, we precluded contributions of these multiple variables by focusing specifically on in vitro tissue and cultured cellular preparations.

Effects of cGMP on I_{SC}

The major finding of the present study is that cGMP does exert dose-dependent and side-dependent effects on the short-circuit current (I_{SC}) across the isolated ciliary epithelium. One effect appears to be an action at the aqueous surface on the nonpigmented ciliary epithelial (NPE) cells, leading to inhibition of I_{SC}. Two observations have led us to conclude that this is the major effect of cGMP. First, preparations from two different species (the rabbit and cat) display the same inhibitory response to aqueous cGMP. In contrast, the responses to stromal cGMP are not identical. Second, stromal application of the hormone ANP, whose actions are mediated by cGMP, also produces an inhibition of I_{SC}. Since most of the ANP receptors are located on the PE cells [8], the hormone likely binds at the PE cell membranes, generating cGMP which then diffuses through gap junctions to the target cells (the NPE cells).

The second effect of cGMP is an increase in I_{SC}, elicited by application to the stromal I-CB surface of the rabbit, but not of the cat. We presume that higher concentrations of intracellular cGMP are required to trigger

this second effect than for the major NPE-targeted effect. Otherwise, ANP would stimulate I_{SC} across the rabbit ciliary epithelium, contrary to observation.

MECHANISMS OF ACTION OF cGMP

cGMP exerts its effects in other tissues by interacting with at least three potential target sites: ion channels [29, 39], cGMP-dependent kinase (PKG) [4, 29], and phosphodiesterase [39, 45]. The effect at the stromal surface is partly mediated by K^+ channels of the PE cells since 5 mM stromal Ba^{2+} abolished the response to 1 mM stromal cGMP (Table (B)). cGMP has been reported to activate K^+ channels in other cells [1, 20, 27, 36, 53, 63, 65].

The major target site of cGMP at the aqueous surface does not appear to be either a K^+ channel or Cl^- channel (Table (A), Fig. 4). Furthermore, cGMP activation of a cation-nonselective channel [71] would be expected to enhance cation movement from aqueous into the NPE cells, thereby increasing I_{SC}, contrary to observation. An alternative conductive target could be the Na^+,K^+-exchange pump of the NPE cells. This possibility is supported by recent reports that another cyclic nucleotide, cAMP, inhibits the pump in renal tubules [2] and NPE cells [22, 23]. cAMP has been reported to act both directly, by phosphorylating a regulatory site on the pump [2, 5, 22, 23], and indirectly, by phosphorylating the threonine-34 residue of DARPP-32 (a modulator of protein phosphatase 1) [2, 5, 64]. The phosphorylated DARPP-32 inhibits dephosphorylation, and thereby reactivation of the pump in renal tubules [2]. DARPP-32 has been found in the NPE cells of the ciliary epithelium [59]. By stimulating phosphodiesterase [45], cGMP could reduce the cAMP concentration, reversing the actions of PKA on the pumps of the NPE cells.

Within the scope of the present study, the role of the pump as a target of aqueous cGMP could not be readily addressed. The most direct approach would be to measure the response to cGMP before and after selectively blocking the pump with cardiotonic steroids. However, after the pump is blocked (increasing I_{SC}), the NPE cells begin to release K^+ into the aqueous medium (reducing I_{SC}) (Fig. 6). The absence of a stable plateau immediately after inhibiting the pump greatly complicates efforts to quantify the response of cGMP administered at that point. On the other hand, if we wait until a steady state is reached after blocking the pump (Fig. 6*C*), the absence of a response to aqueous cGMP could reflect the dissipation of ionic gradients and membrane potentials. Thus, the observations of Fig. 6 indicating that blocking the pump abolishes the subsequent response to aqueous cGMP are consistent with, but do not rigorously prove, the concept that the pump is a target of cGMP. Experiments testing the effectiveness of stromal cGMP during strophanthidin block suggest that (i) cGMP targets pumps on either side of the ciliary epithelium and/or (ii) a pump-derived K^+ gradient is a prerequisite for stromal cGMP to stimulate I_{sc} through PE K^+ channels (inhibitable by Ba^{2+}).

WORKING HYPOTHESIS

For clarity, the possible bases for the transport effects of cGMP (as balancing and opposing cAMP) are presented within the minimalist model of aqueous humor secretion of Fig. 9. More comprehensive models of the transport mechanisms [66, 14] and intracellular signalling [17] are considered elsewhere. cAMP is pictured as exerting at least three effects on the NPE cells, two of which tend to increase I_{SC}. The major effect of cAMP is hypothesized to be inhibition of the Na^+,K^+-exchange pump through cAMP-dependent kinase (PKA) in two different ways: directly phosphorylating (and thereby inhibiting) the pump [2, 5, 22, 23], and indirectly (through PKA activation of DARPP-32) preventing pump dephosphorylation by protein phosphatase 1 [2, 57]. These two actions will inhibit net secretion of aqueous humor. The third action is taken to be an activation of K^+ channels (which stimulates secretion) [17]. A fourth, less well-defined action (not included in the figure) is the activation of L-type Ca^{2+} channels [51] which are present in ciliary epithelial cells [25]. The full implications of these Ca^{2+} channels for aqueous humor secretion are unclear, but intracellular Ca^{2+} is known to play a supportive role in maintaining the activity of the NPE Cl^- channels [17]. We propose that the major role of cGMP in the NPE cells is to activate cAMP phosphodiesterase, lowering the cAMP concentration, and thereby reducing cAMP-dependent kinase activity. Baseline protein phosphatase activity should then dephosphorylate the pump, DARPP-32, and the K^+ and Ca^{2+} channels, reversing the putative actions of cAMP. This concept is supported by the observation that cGMP activates particulate cAMP phosphodiesterase of ciliary process homogenates [45]. In addition, cGMP-dependent kinase may possibly activate protein phosphatase 2A accelerating dephosphorylation of the pump and DARPP-32, as suggested for GH_4C_1 cells [64]. We further propose that the effect of cGMP exerted on the PE cells is to stimulate K^+ channels, an effect noted in renal tubules [50, 54].

The model of Fig. 9 potentially resolves two apparent paradoxes in the literature. First, the observations that cAMP reduces aqueous humor secretion but increases I_{SC} have prompted speculations concerning possible compartmentalization of cAMP pools and receptor-independence of some adrenergic-stimulated effects [15]. These possibilities may prove correct, but a simpler explanation is that the increase of short-circuit current (positive current from aqueous to stroma) reflects a decrease in net pump current in the opposite direction (from NPE cell to aqueous) (Fig. 9). The second apparent paradox which has long puzzled investigators and

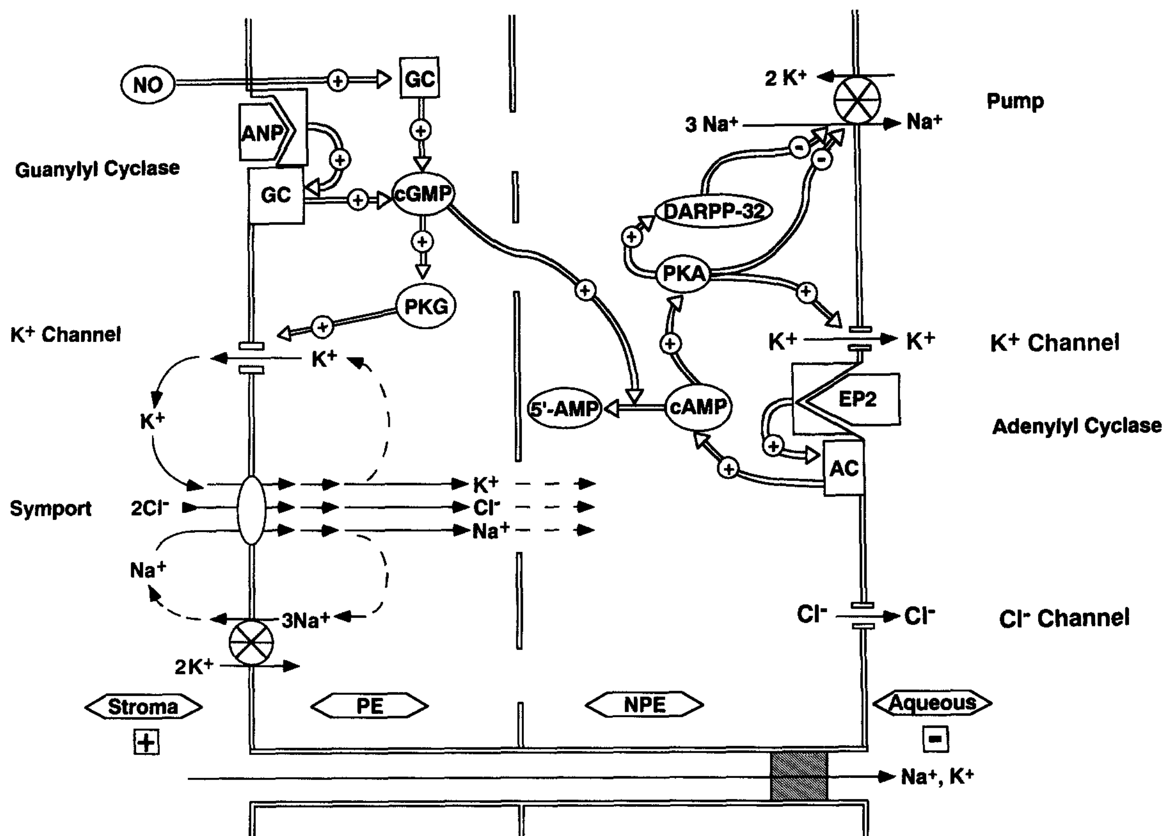

Fig. 9. Working hypothesis of role of cGMP in regulating secretion of aqueous humor. Guanylyl cyclase (GC) in the ciliary processes is stimulated by both ANP and NO to generate cGMP [45]. Occupancy of EP_2 prostaglandin receptors activates adenylyl cyclase (AC) to produce cAMP in the ciliary epithelium [6, 7]. cGMP has been reported to stimulate cAMP phosphodiesterase [45], accelerating hydrolysis of cAMP to the inactive 5′-AMP. In addition, cGMP is presented as activating cGMP-dependent kinase (PKG) and stimulating K^+ channels of the PE cells. This putative action of PKG would account for the stimulatory effect of stromal cGMP we have observed, and is consistent with observations in other cells [1, 20, 27, 36, 53, 63, 65]. Three of the reported actions of cAMP have also been included in the model: (i) cAMP-dependent kinase (PKA) has been thought to inhibit the Na,K-activated pump by phosphorylating a regulatory site [2, 5, 22, 23]; (ii) removal of the phosphate group has been reported to be blocked by PKA-mediated phosphorylation of DARPP-32, which inhibits protein phosphatase 1 activity [2, 57]; and (iii) cAMP increases the K^+ permeability of NPE cells [17]. DARPP-32 has been found in the NPE cells of the ciliary epithelium [59]. cGMP is presented as stimulating phosphodiesterase, thereby reducing the cAMP concentration and reversing the actions of PKA on NPE pumps [and on the K^+ channels and Ca^{2+} channels (*not depicted*)] [45]. cGMP may similarly stimulate the pump within PE cells.

clinicians is that both β-agonists (which increase cAMP levels) and β-antagonists (which reduce cAMP levels) lower aqueous humor secretion [44]. This paradox can be resolved if baseline levels of cAMP are sufficient to activate NPE K^+ and Ca^{2+} channels (stimulating secretion), but too low to inhibit the pump. Increasing the cAMP concentration (with a β-agonist) would then inhibit the NPE pump, reducing secretion. On the other hand, decreasing the cAMP level from baseline (with a β-antagonist) would deactivate the K^+ and Ca^{2+} channels, also reducing secretion.

While accommodating published data and our preliminary results, the model of Fig. 9 is a working hypothesis. The observation that forskolin-mediated activation of the putative pump inhibitor (cAMP-activated kinase) enhances the effect of aqueous cGMP (Fig. 7) is consistent with that hypothesis.

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