The Role of NaKCl Cotransport in Blood-to-Aqueous Chloride Fluxes across Rabbit Ciliary Epithelium

Richard B. Crook, Kana Takahashi, Alden Mead, Jonathan J. Dunn, and Marvin L. Sears

PURPOSE. To evaluate the role of NaKCl cotransport in short-circuit current (Isc) and chloride fluxes across rabbit ciliary epithelium mounted in a Ussing-type chamber.

METHODS. Bilayered intact ciliary epithelium free of stroma was obtained after perfusion and dissection of rabbit eyes and mounted in an Ussing-type chamber. The effects of bumetanide and other drugs on Isc and transepithelial 36Cl fluxes in bicarbonate-containing Ringer’s were determined. Immunoblot analysis was performed by standard techniques.

RESULTS. Bumetanide (100 μM) applied to the blood (pigmented epithelium [PE]) side of the ciliary bilayer caused a dose-dependent decrease in Isc from 18.2 ± 2.2 to 10.4 ± 1.4 μA/cm² (43%). Bumetanide applied to the aqueous (nonpigmented epithelium [NPE]) side of the tissue inhibited Isc by only 12%. Immunoblots of dissected NPE and PE tissue probed with an antibody to mammalian NaKCl cotransporter detected approximately 10 times more NaKCl cotransporter protein in PE than in NPE. 36Cl flux studies revealed a PE-to-NPE chloride flux of 180.3 ± 37.2 μEq/cm² per hour and an NPE-to-PE flux of 72.3 ± 22.9 μEq/cm² per hour, indicating a net PE-to-NPE flux of 108.0 ± 31.3 μEq/cm² per hour across rabbit ciliary epithelium. Bumetanide inhibited the PE-to-NPE chloride flux by 52% but did not inhibit the NPE-to-PE flux. Isoproterenol (10 μM) added to the PE side of the bilayer increased Isc by a dose-dependent 53%. Prior addition of bumetanide to the PE side blocked the increase due to isoproterenol by 37%. Isoproterenol (10 μM) stimulated the PE-to-NPE chloride flux by 75% but had no stimulatory effect on the NPE-to-PE chloride flux. 4,4’Diisothiocyanatostilbene-2,2’disulfonic acid (DIDS) inhibited Isc when added to either side of the bilayer but was more potent at low concentrations (<100 μM) when added to the NPE side and more potent at higher concentrations (>100 μM) when added to the PE side. Prior addition of 1 mM DIDS to the NPE side decreased isoproterenol stimulation of Isc by 56%.

CONCLUSIONS. NaKCl cotransporters located primarily on the blood side of rabbit ciliary epithelium contribute to aqueous-negative Isc and to blood-to-aqueous chloride transport across the tissue in bicarbonate-containing medium. DIDS-inhibitable mechanisms, possibly including HCO₃⁻Cl exchange and Cl channels, also play a role. Isoproterenol stimulation of Isc involves coordinate upregulation of PE-side NaKCl cotransport and an NPE-side DIDS-inhibitable mechanism(s). (Invest Ophthalmol Vis Sci. 2000;41:2574–2583)

The formation of aqueous humor in the eye involves blood-to-aqueous active ion transport by the ciliary body epithelium. However, the identity of the ions, the transport of which drives fluid flow, has been controversial. Sodium, chloride, bicarbonate, and potassium have all been suggested to be transported transepithelially and thus drive fluid transport by ciliary epithelium. Ion transport mechanisms that transport these ions have been characterized in ciliary epithelium (reviewed in References 7 and 8), but evidence of a direct role in transepithelial ion fluxes has in most cases been absent.

Early studies using iris–ciliary body from amphibia suggested that net chloride transport is supported by this tissue, but subsequent studies in rabbits failed to confirm this finding. Recent evidence obtained with bovine ciliary epithelium has demonstrated that bumetanide, a loop diuretic inhibitor of NaKCl cotransport, reduces net blood-to-aqueous chloride flux across the tissue by more than 90%. This finding supports previous evidence for a loop diuretic–sensitive component of short-circuit current (Isc) in shark, rabbit, and dog ciliary body preparations. NaKCl cotransport is a chloride entry mechanism found in epithelial and nonepithelial tissues that plays a key role in salt and fluid flow across several pumping epithelia including kidney, intestine, and retinal pigment epithelium. NaKCl cotransport has been detected in cultured nonpigmented ciliary epithelial (NPE) cells and pigmented ciliary epithelial (PE) cells and has been found to be responsible for regulatory volume increase in bovine PE. However, in rabbit, the species in which ion transport across...
ciliary epithelium has been the most extensively studied, direct evidence for NaKCl cotransport involvement in transepithelial anion fluxes across rabbit ciliary epithelium has been lacking.

The ciliary epithelium forms the innermost segment of the ciliary body and is composed of two contiguous epithelial layers: a PE layer overlain on the luminal side by an NPE layer. Tight junctions link cells in the NPE layer but not the PE layer. NPE and PE cell layers communicate through gap junctions and appear to function as a syncytium. Thus, both epithelial layers may play roles in aqueous inflow, although the NPE layer alone has been reported to support aqueous flow.

The recent development of a rabbit ciliary epithelium preparation free of iris and with greatly reduced stromal contamination represents a potentially improved model for the study of vectorial ion transport by this tissue. As with other rabbit preparations, this preparation is strongly dependent on bicarbonate for maintenance of Isc. Isc is increased after application of agonists known to increase aqueous humor formation, suggesting that this preparation may be useful for the study of hormonal control of fluid flow across ciliary epithelium. In the present study, we evaluated the role of NaKCl cotransport in ion fluxes across rabbit ciliary epithelium. We report that NaKCl cotransporters located primarily on the blood (PE) side of ciliary epithelium significantly contribute to Isc and blood-to-aqueous chloride fluxes across this tissue.

**MATERIALS AND METHODS**

**Chemicals**

(±)Isoproterenol, bumetanide, ouabain, 4,4’diisothiocyanatostilbene-2,2’disulfonic acid (DIDS), and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO); sodium decyl sulfate (SDS) and 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS) from Behring–Calbiochem (La Jolla, CA); TPCK, Pefabloc (AEBSF), aprotinin, chymostatin, pepstatin, and leupeptin from Boehringer–Mannheim (Indianapolis, IN); acrylamide, bisacrylamide, and colchicine from Fisher Scientific (Pittsburgh, PA).

**Rabbit Ciliary Epithelium Preparation**

Rabbit ciliary epithelial bilayers devoid of stroma were prepared as previously described. In brief, rabbits were anesthetized intramuscularly with 0.5 ml of a 1:1 mixture of ketamine chloride (50 mg) and xylazine hydrochloride (50 mg) followed by an infusion of 3000 U sodium heparin. After 5 minutes, the animals were killed and their eyes enucleated. Excess tissue was removed, and the two long posterior ciliary arteries were identified and cannulated with polyethylene tubing. The arteries were perfused with a peristaltic pump at a rate of 0.5 ml/min with calcium magnesium-free Dulbecco’s modified medium (DMM) for 2 minutes and with DMH with 0.1% collagenase for 10 minutes. Solutions were oxygenated and maintained at 37°C. Final perfusion was with DMH plus 10% fetal calf serum at 30°C for 10 minutes. The iris ciliary body was removed microsurgically, and the floating epithelial bilayer was dissected.

**NaKCl Cotransport in Ciliary Body Epithelium**

The tenets of the ARVO Statement for the Use of Animals in Ophthalmic Research were followed and institutional animal experimentation committee approval was granted for these studies.

**Electrophysiological Measurements**

These measurements were performed as previously described, with the following modifications. The tissue was mounted in an Ussing-type chamber with four electrode ports. The volume of each chamber was 3.5 ml with electrode ports for both voltage and current. The electrodes (World Precision Instruments, New Haven, CT) were of the calomel type with 3.5% agar-3 M KCl bridges and connected to a voltage/current clamp apparatus (model DVC-1000; World Precision Instruments). The signals were transmitted to an analog digital amplifier (MacLab; World Precision Instruments) and after amplification were charted, recorded, and stored in a computer (Macintosh SE/30; Apple Computer, Cupertino, CA; with MacLab Chart V 3.0 software; World Precision Instruments).

Tissues in the chambers were bathed in balanced salt solution (122 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 3 mM Na2PO4, 25 mM NaHCO3, 5 mM dextrose, and 0.3 mM glutathione [pH 7.4]; PLUS; Alcon, Fort Worth, TX), which was bubbled with 5% CO2 and 95% O2. The temperature was maintained between 30°C and 34°C by heating the mantle.

After the tissue was mounted, at least 30 minutes was allowed to permit the baseline Isc to achieve a steady state. Tissue preparations were discarded if steady state could not be reached or if the absolute value was less than 15 μA/cm2. Basal values for electrical properties of rabbit ciliary epithelium were: Isc = 18.5 ± 2.0 μA/cm2; transepithelial potential (TEP) = 677 ± 27 μV (NPE side negative), and resistance = 40 ± 3 Ω/cm2. Resistance was intermittently checked, and compensation for solution resistance was achieved as described. All measurements of current were obtained at V = 0.

**36Cl Fluxes**

After establishment of a stable Isc, 5 to 10 μCi 36Cl was added in 100 μl Ringer’s to one side of the bilayer preparation, and 100 μl Ringer’s without isotope was added to the other. After 5 minutes (to allow mixing), 100-μl aliquots were removed from both chambers at designated intervals (usually 5 or 10 minutes). These were added to 1 ml of scintillant and counted in the 32P channel in a scintillation counter (Packard; Meriden, CT). Fluxes were determined in both directions with each bilayer preparation, with the flux in the second direction determined after washing of both chambers to remove label. Experiments were performed varying the initial direction, with no detectable effect on the results.

Unidirectional chloride fluxes were calculated using the equation

\[ J = C \cdot [\bar{i}] / L \cdot I / A \]

where \( J \) is unidirectional flux (in microequivalents per square centimeter per hour), \( I \) is radioactivity in the loading chamber at \( t = n \) (in Curies), \( C \) is radioactivity in the collecting chamber at \( t = n \) (in Curies), \([\bar{i}]\) is chloride in the loading chamber at \( t = 0 \) (in equivalents), \( t \) is collection duration (in hours), and \( A \) is exposed area of tissue preparation (in square centimeters).
Dissection of PE and NPE Layers

Dissection was performed as described with the following modifications. After treatment of the enucleated eye with collagenase and removal of the ciliary bilayer as described, the bilayer was placed in a calcium-free medium (F10/minimum essential medium [MEM] with 33 mM mannitol and 3.4 mM EGTA, [pH 7.5]), and incubated at 37°C for 20 to 30 minutes. The NPE and PE layers could then be gently teased apart using microsurgical instruments. The cuboidal PE could be visually distinguished from columnar NPE, as confirmed by dissections of NPE and PE from pigmented rabbits. Contamination of each layer by the other was estimated to be less than 15% based on visual examination.

Protein was determined by the method of Peterson and DNA by the method of Leyva and Kelley.

Immunoblot Analysis

Immunoblot analysis was performed as described with the following modifications. Dissected tissue was washed two times with PBS and then placed in 1 ml homogenization buffer (250 μM TPCK, 209 μM AEBSF, 1.45 μM pepstatin, 3.3 μM chymotrypsin, 6.25 U/ml chymotrypsin, 8.63 μM leupeptin, 5 mM EDTA, 140 mM NaCl, and 20 mM HEPES [pH 7.4]). Samples were homogenized on ice with 20 strokes of a motorized pestle (Fisher Scientific) set at three. The homogenates were centrifuged at 1000g for 5 minutes and the resultant supernatants for 5000g for 15 minutes. These supernatants were centrifuged at 37,000g for 30 minutes, and the pellets were resuspended in 1% SDS and a 50-μl aliquot saved for protein assay. The remainder was heated for 10 minutes at 95°C and an aliquot (15-25 μl) was electrophoresed in 2% SDS, 50 mM dithiothreitol, 50 mM Tris-HCl [pH 6.8], 6% glycerol and 0.01% Serva Blue G on a 7.5% SDS acrylamide gel. After electrophoresis at 30 mV to nitrocellulose overnight, the protein adhering to the blot was visualized with 1% ponceau S, coated with blocking buffer (5% nonfat dry milk in PBS-0.1% Tween-20 [pH 7.4]) and exposed overnight at 4°C to a monoclonal antibody (T435, 1:10,000 in blocking buffer). After the blot was washed five times in blocking buffer, it was incubated for 2 hours at room temperature with 1:2500 rabbit anti-mouse horseradish peroxidase-labeled IgG (Amersham), followed by four washes in blocking buffer, one wash in PBS-0.1% Tween-20, and a final wash in distilled water. Antigen was detected by enhanced chemiluminescence (Amersham).

Immunoprecipitation was performed as previously described. Images on x-ray films were scanned into a computer (IBM, Armonk, NY), and the 170-kDa band quantitated (Sigma-gel software; Jandel Scientific, San Rafael, CA), within the linear range of film exposure. Cotransporter protein is expressed as pixels per protein band.

Data Presentation

Data are means ± SE. Significance was determined using Student’s t-test, with P < 0.05 considered to be significant.

RESULTS

The role of NaKCl cotransport in Isc across the rabbit ciliary bilayer was examined by adding 100 μM bumetanide to the PE side of the ciliary bilayer mounted in an Ussing-type chamber (Fig. 1A). Isc decreased by 43% in a dose-dependent manner.
Effect of Bumetanide on Transepithelial Chloride Fluxes

Because the ions contributing to the aqueous-negative current across rabbit ciliary epithelium are not well defined, we asked whether chloride might play a role. Accordingly, $^{36}$Cl$^{-}$ fluxes across the mounted ciliary bilayer were determined (Fig. 5). Fluxes in both directions across the bilayer were linear. The PE-to-NPE flux was consistently two to three times greater than the NPE-to-PE flux (Fig. 5). Data from 10 of 12 experiments showed a PE-to-NPE flux of 180.3 ± 37.2 μEq/cm$^2$ per hour and an NPE-to-PE flux of 72.3 ± 22.9 μEq/cm$^2$ per hour (Table 1). The net PE-to-NPE (blood-to-aqueous) chloride flux was 108.0 ± 31.3 μEq/cm$^2$ per hour ($P < 0.05$). (Note that this may be an overestimate, because the exposed surface area can be significantly larger than that of the chamber aperture itself.) The ratio of PE-to-NPE and NPE-to-PE fluxes was 2.49. In 2 of 12 experiments, flux values in both directions were less than 3% of those shown in Table 1. These data, which were obtained in early experiments, were excluded because the tissues may have been damaged.

We next evaluated the role of NaKCl cotransport in chloride fluxes across the ciliary bilayer. Bumetanide administered to the PE side of the bilayer inhibited the PE-to-NPE chloride flux by 52% (Fig. 6). By contrast, the NPE-to-PE chloride flux was not inhibited by bumetanide, regardless of the side of the bilayer to which the drug was applied. In fact, a slight, but insignificant stimulation of the NPE-to-PE chloride flux by bumetanide was observed ($P > 0.05$). These results suggest that NaKCl cotransport contributes significantly to an aqueous-directed chloride flux across this tissue.

Role of NaKCl Cotransport in Isoproterenol Stimulation of Isc

β-Adrenergic agonists increase the rate of aqueous humor formation in humans$^{36}$ and increase Isc across some rabbit ciliary epithelium.
epithelium preparations. Figure 7A shows that isoproterenol added to the PE side of the bilayer evoked a dose-dependent increase in Isc. At 100 μM isoproterenol, stimulation of Isc was 53% over basal, with a half maximal stimulatory concentration of 2.7 μM. Because prior studies have shown that isoproterenol stimulates NaKCl cotransport in cultured ciliary epithelial cells, we asked whether isoproterenol stimulation of Isc might be due in part to increased NaKCl cotransport activity. Figure 7B shows that 100 μM bumetanide added to the PE side of the bilayer reduced isoproterenol stimulation of Isc by 37%. This suggests that NaKCl cotransport contributes to isoproterenol elevation of Isc.

Effects of DIDS on Isc
Bicarbonate transporting mechanisms have been described in both NPE and PE layers of rabbit ciliary epithelium. DIDS,
a sulfonated distibene that is an inhibitor of HCO₃⁻Cl exchange and some Cl channels. It has been shown in several studies to reduce Isc across mounted ciliary epithelium.

We compared the effects of DIDS on Isc when added to either side of the bilayer (Fig. 9). DIDS inhibited Isc with a half maximal inhibitory concentration (IC₅₀) of 100 μM when added to the

**FIGURE 6.** Effect of bumetanide on unidirectional chloride fluxes. After establishment of a stable rate of 36Cl flux, 100 μM bumetanide was added to one side of the bilayer and vehicle to the other. Samples were then taken and the new slope determined. PE → NPE: Na⁺Cl⁻ added to the PE side and collected on the NPE side; NPE → PE: Na⁺Cl⁻ added to the NPE side and collected from the PE side. No difference was seen in the results as a function of which side received isotope first. Control PE → NPE flux was 0.66 ± 0.24 nanomoles/min · 100 μl. n = 3 to 4. *P < 0.001 comparing treated with untreated controls.

**FIGURE 7.** Isoproterenol stimulation of Isc. (A) Dose response. The indicated concentrations of isoproterenol were added to the PE side, and the change in Isc was recorded at plateau. n = 4 to 5. *P < 0.05, **P < 0.001. (B) Effect of bumetanide on isoproterenol stimulation of Isc. Bumetanide (BUM; 100 μM) or vehicle was added to the PE side. After establishment of a new Isc plateau, 10 μM isoproterenol was added to the PE side and the changes in Isc determined. n = 4. *P < 0.05 comparing BUM + ISO with ISO.

**FIGURE 8.** Effect of isoproterenol on chloride fluxes. After a linear Cl flux in one direction was established, 10 μM isoproterenol was added to the PE side, and the change in Cl flux was determined. Control PE → NPE flux was 0.41 ± 0.21 nanomoles/min · 100 μl. n = 6. *P < 0.001 for stimulated compared with control samples.
PE side but was 10 times more potent when added to the PE side (IC50 = 10 M). At 1 mM DIDS, the highest concentration measured, these potencies reversed as DIDS inhibited Isc by 70% when added to the PE side and by 56% when added to the NPE side.

**Effect of DIDS on Isoproterenol Stimulation of Isc**

The dependence of DIDS’s potency on the side of the bilayer to which it was added raised the possibility that the DIDS-sensitive ion transport mechanisms on each side of the bilayer are not identical. In particular, the data suggest that a mechanism partially inhibited by 10 μM DIDS is present on the NPE side. HCO3−-Cl− exchange is typically inhibited by DIDS with an IC50 of approximately 100 μM.42 In contrast, a chloride channel inhibitable by 10 μM DIDS has been described in rabbit NPE.43 This channel is activated by cyclic adenosine monophosphate (cAMP).43 Because cAMP is elevated by isoproterenol in rabbit ciliary epithelium,43 we reasoned that, if such a mechanism contributes to blood-to-aqueous chloride transport, treatment with DIDS on the NPE side may reduce isoproterenol stimulation of Isc. DIDS added to the NPE side reduced subsequent stimulation of Isc by isoproterenol by 56%, compared with isoproterenol-stimulated controls without DIDS (Fig. 10).

**DISCUSSION**

The effects of loop diuretics on the electrical properties of ciliary epithelium have been investigated for almost two decades. The results of these studies have been mixed, with hyperpolarization,3,12,14,15 depolarization,15 or no effect of the drugs35 reported. Of several studies that compared loop diuretic effects on the blood and aqueous sides of ciliary epithelium, most found little or no effect on the PE side of the bilayer.12-15 The reason for the difference between these results and our finding of a pronounced inhibition of Isc by bumetanide added to the PE side is not clear but may have to do with enzymatic removal of stroma from the bilayer, which may improve access of compounds to the blood side of the tissue.

The inhibitory effect of bumetanide on Isc was side specific. It was 3.5 times more effective when added to the PE side than to the NPE side of the bilayer. This functional asymmetry corresponded to an asymmetric distribution of NaKCl cotransporter protein. PE membranes possessed 9.5 times the 170-kDa cotransporter protein found in the NPE layer. These results suggest that the NaKCl cotransporter is localized primarily to the PE layer, which is consistent with a function as a chloride entry mechanism for blood-to-aqueous chloride transport across the tissue. The reason for the greater difference between PE and NPE cotransporter protein levels (9.5-fold) compared with bumetanide inhibition of Isc (3.5-fold) is not known. However, bumetanide is known to slowly cross cell membranes (Chris Lytle, personal communication, August 1998). If Isc inhibition by NPE-side bumetanide were due to partial access of NPE-side bumetanide to the PE side, then Isc inhibition by NPE-side bumetanide in Figure 3A would be an upper estimate, and the PE-to-NPE ratio of bumetanide inhibition of Isc could be higher than we have reported. The present immunologic findings are similar to those of a recent study using freshly isolated bovine NPE and PE cells, in which 80% of NaKCl cotransporter protein was found in the PE layer.34 In addition, an immunofluorescence study found cotransporter protein concentrated on the basolateral surface of bovine PE cells.
cells, with no detectable signal on the NPE basolateral surface. 46

The rabbit bilayer supported a robust net PE-to-NPE chloride flux, with a blood-to-aqueous rate two to three times the aqueous-to-blood rate. Earlier studies in rabbit found rapid but equivalent NPE-to-PE and PE-to-NPE chloride fluxes. 5,9,10 The reason for the difference between the latter results and ours is not clear but again could be a function of the preparation. Calculated chloride currents greater than the measured Isc have been reported in several studies. 3,10,11,49,50 Similarly, the present data also indicate a calculated chloride current much greater than the measured Isc, which suggests that the bulk of chloride transport probably occurs in an electroneutral fashion in our preparation as well.

NaKCl cotransport appeared to contribute to approximately 50% of blood-to-aqueous (PE-to-NPE) chloride flux across rabbit ciliary epithelium (Fig. 6). No role of NaKCl cotransport in aqueous-to-blood (NPE-to-PE) chloride flux could be detected, suggesting that the effect of NaKCl cotransport was unidirectional under the conditions used in this study. Recently To et al., 11 in studies of ion transport across bovine ciliary epithelium, found that bumetanide applied to both sides of an Ussing chamber almost completely inhibited net chloride flux. Thus NaKCl cotransport may play a larger role in bovine than in rabbit ciliary epithelium chloride fluxes. DIDS has no significant effect on either Isc or chloride transport in bovine ciliary epithelium 11 in contrast to the rabbit, where DIDS strongly inhibited Isc. Therefore, the role of bicarbonate in ion fluxes in ciliary epithelium may also vary with species. Rabbit aqueous humor contains more bicarbonate than human plasma, whereas human aqueous humor contains more chloride than does human plasma. 51

The present data imply that approximately half of Isc and of blood-to-aqueous chloride transport may be due to mechanisms other than NaKCl cotransport. These mechanisms are not identified in the present work. However, it has been well documented that bicarbonate is required for normal current in rabbit ciliary epithelium 29,58,52 and a role for bicarbonate transport mechanisms has been proposed by several groups. Parallel roles for NaKCl cotransport and HCO3-Cl exchange as chloride entry mechanisms have been reported in arterial muscle. 53 In airway submucosal glands, inhibition of NaKCl cotransport causes a compensatory increase in HCO3-Cl exchange. 54 Butler et al. 38 and Wolosin et al. 59 have detected HCO3-Cl exchange in NPE and in PE (where it is Na dependent), which is consistent with our finding that 1 mM DIDS lowered Isc significantly when added to either side of the bilayer. We found that addition of both bumetanide and DIDS to the PE side reduced Isc almost to zero (data not shown).

Effect of Isoproterenol

β-Adrenergic agonists are the best known stimulators of aqueous humor formation in man and primates. 36,55 Glaucoma drugs such as timolol and betaxolol may derive their hypotensive efficacy from blockade of this stimulation. 36 In other species, however, the role of β-adrenergic agents has been less clear. In rabbit, adrenergic agonists have been variously reported to elevate Isc 30,56,57 or to reduce it. 58,59 The reason for this disparity is not known.

In the present studies, isoproterenol caused an increase in both Isc and blood-to-aqueous chloride flux across the rabbit bilayer (cf. Figs. 7 and 8). However, isoproterenol did not stimulate NPE-to-PE (aqueous-to-blood) chloride flux. The magnitude of isoproterenol stimulation of the PE-to-NPE chloride flux was similar to the reported increase in the rabbit nocturnal rate of aqueous humor formation over the diurnal rate. 47 It has been suggested that the circadian rhythm of aqueous formation may depend on the concentration of circulating epinephrine in some species. 36

The present data indicate that NaKCl cotransport contributes to the isoproterenol increase in Isc, because pretreatment with bumetanide partially blocked the Isc increase. The simplest interpretation of these data is that isoproterenol elevates Isc in part by stimulating NaKCl cotransport activity in rabbit ciliary epithelium, as it does in human PE cells. 37 Whether cotransport is stimulated through the cAMP pathway as it is in PE cells 57 and whether cotransport stimulation is secondary to other cellular responses to isoproterenol are unknown.

It seems likely that additional mechanisms may be involved in the isoproterenol increase in Isc, because bumetanide did not completely block the increase. In some tissues, the rates of chloride influx and efflux are coordinately regulated by hormones. 60 Our finding that a DIDS-inhibitable mechanism(s) on the NPE side of the bilayer is also stimulated by isoproterenol raises the possibility that coordinate control mechanisms for chloride influx and efflux may also exist in ciliary epithelium. Although the NPE-side transport mechanism(s) has not been identified, a DIDS-inhibitable chloride channel present on the NPE basolateral membrane that is activated by cAMP has been reported in both rabbit and bovine NPE. 45,61 The effect of DIDS on isoproterenol stimulation of Isc when applied on the PE side was not determined, although DIDS lowered the rate of aqueous-directed 36Cl transport stimulated by isoproterenol (data not shown).

A model summarizing the findings of the present study is shown in Figure 11. This is necessarily a partial description, but two points can be made. First, the NaKCl cotransporter resides primarily on the PE side of the bilayer and serves as a chloride entry mechanism. The second mechanism responsible for half
of chloride influx was not identified by our work, but anion exchangers have been proposed to provide blood-side chloride entry in rabbit, so this possibility is also indicated. Second, isoproterenol, which increased both Isc and PE-to-NPE chloride flux, stimulated NaKCl cotransport on the PE side and a DIDS-inhibitable mechanism on the NPE side. Thus, the model depicts coordinate adrenergic upregulation of both chloride entry into the PE layer (through NaKCl cotransport) and chloride exit across the NPE basolateral membrane into the posterior chamber (through a chloride channel or other mechanism). Whether isoproterenol also stimulates chloride entry through a second mechanism such as an anion exchanger is not yet known. Of course, the effects of isoproterenol on ion fluxes may be more complex than is indicated in this provisional model.

A recent study has proposed that NaKCl cotransporters are present on both PE and NPE basolateral surfaces. In the presence of bicarbonate, an NPE basolateral cotransporter is proposed to serve a primary role as an efflux mechanism for blood-to-aqueous chloride transport, whereas the PE cotransporter provides efflux for a smaller flux of chloride moving from aqueous to blood. In the present study, where chloride effluxes were directly measured, NaKCl cotransport was found only to be involved in chloride entry into PE cells preparatory to aqueous-to-blood chloride transport. No evidence for NaKCl cotransport’s contributing to aqueous-to-blood (NPE-to-PE) Cl fluxes was detected, nor was an NPE-side NaKCl cotransporter contributing to chloride efflux into the aqueous detected. The reason for the difference between the results of this study and ours is not clear but could be due to differences in methods of tissue preparation and/or other experimental conditions.

The central role played by NaKCl cotransport in blood-to-aqueous anion transport across rabbit ciliary epithelium raises the possibility that drugs and hormones that alter its activity could be used to modulate the rate of aqueous humor formation. They could be used to lower intraocular pressure in ocular hypertensive patients or to raise intraocular pressure in patients with ocular hypotension after vitrectomy. The present finding that isoproterenol stimulates NaKCl cotransport in the ciliary bilayer appears to be the first direct evidence for hormonal control of an ion transport mechanism directly involved in transepithelial ion fluxes across ciliary epithelium.

Acknowledgments

The authors thank Christian Lytle, University of California, Riverside, for generously providing the T4 antibody against the NaKCl cotransporter and Jon Polansky for encouragement and inspiration.

References


