Chloride Secretion by Bovine Ciliary Epithelium: a Model of Aqueous Humor Formation

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PURPOSE. To study the physiological mechanisms of the Cl transport across the bovine ciliary body-epithelium (CBE).

METHODS. Fresh isolated bovine CBE was mounted in an Ussing-type chamber. The effects of ion substitution and transport inhibitors on electrical measurements and Cl transport were investigated.

RESULTS. The potential difference (PD) across the preparation was 0.55 ± 0.04 mV and was consistently negative at the aqueous side. The short-circuit current (SCC) and tissue resistance (Rt) were found to be 8.0 ± 0.7 μA/cm² and 72 ± 3 Ω/cm², respectively. Both the PD and the SCC of the bovine CBE were found to depend primarily on the concentration of the Cl bath and to a lesser extent on the Na or HCO₃ concentration. At 30 mM Cl, the polarity of the PD and the direction of the SCC were reversed. Reducing the extracellular Na or Cl concentration abolished the net Cl transport into the eye under the short-circuited condition. Bilateral bumetanide (0.1 mM), but not 4,4′-diisothiocyanatostilbene-2-2′-disulfonic acid (DIDS; 0.1 mM), greatly inhibited the Cl transport. Bumetanide, when applied to either side, inhibited the Cl transport. The effect, however, was three times greater on the stromal side than on the aqueous surface. Bilateral heptanol (3.5 mM) and 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB; 0.1 mM) on the aqueous side also inhibited the Cl transport by 80% and 92%, respectively.

CONCLUSIONS. The results suggest that the major Cl influx pathway at the basolateral border in pigmented epithelial cells is through the Na-K-2Cl cotransporter, but not the Cl/HCO₃ and Na/H double exchangers. Intercellular gap junctions between the two cell layers and the NPPB-sensitive Cl channels at the basolateral surface in nonpigmented epithelial cells also play a crucial role in regulating the Cl movement across the functional syncytium. (Invest Ophthalmol Vis Sci. 2000;41:1853–1860)

The aqueous humor of the eye is secreted by the ciliary epithelium. Active cellular electrolyte secretion across this epithelium plays an important role in the aqueous secretion.¹ The ciliary epithelium comprises two epithelial layers: the outer pigmented epithelium (PE), which faces the ciliary stroma, and the inner nonpigmented epithelium (NPE), which faces the posterior chamber. These two epithelial layers are juxtaposed at their apical surfaces through gap junctions² and form a functional syncytium.³

Earlier studies demonstrated active Cl secretion across the ciliary body of cat,⁴ toad,⁵ and rabbit,⁶ although a subsequent study questioned the presence of any active Cl secretion.⁷ Our recent study demonstrated the active Cl transport in bovine CBE, which may be important in aqueous humor formation.⁸

To allow for transepithelial Cl transport to occur across the ciliary epithelium, at least three transport steps are involved: 1) loading of Cl from ciliary stroma into the PE cells through the stromal basolateral surface, 2) passive diffusion of Cl through the gap junctions into the NPE cells, and ultimately 3) efflux of Cl from NPE cells to the posterior chamber.⁹,¹⁰ A number of cotransporters, ion exchangers, ion channels, and Na-K-adenosine triphosphates (ATPases) have been identified in the ciliary epithelium, which may facilitate the vectorial Cl transport across the functional syncytium.¹¹–¹⁶ Currently, two major mechanisms for the uptake of Na and Cl at the basolateral surface in PE cells have been hypothesized (Fig. 1). One of the mechanisms is through a bumetanide-sensitive Na-K-2Cl symporter, and the other mechanism is through a parallel Cl/HCO₃ and Na/H double exchange. Because most of these transporters and ion channels have been characterized only in cultured ciliary epithelial cells, their roles in transepithelial ion transport and their contributions to aqueous secretion are yet to be determined.

We previously demonstrated a net Cl flux in bovine CBE,⁸ which was shown to be an active physiological process, because it could be blocked by loop diuretics. The inhibitory effect of bumetanide on Cl transport was indicative of the presence of Na-K-2Cl cotransporter somewhere in the syncytium. In the present study, we tried to map the transmembrane events—that is, how the Cl is transported through the two cell layers. We studied the effects of several transport inhibitors.
METHODS

Dissection and Electrical Measurements

The method was described in our last report and is briefly summarized here. Freshly enucleated bovine eyes were collected from a local abattoir and stored in a refrigerator. The dissection was usually performed within an hour of death. On removal of the cornea, a sector of sclera was peeled from the choroid. The incision ran tangentially to the globe, beginning at the anterior chamber angle. Subsequently, a sector of intact iris ciliary body was carefully isolated from the lens zonules and the vitreous. The intact bovine CBE was mounted in a modified Ussing–Zerahn type chamber, and only the ciliary body was exposed to the chamber cavity. The area of the chamber cavity was increased over that used in our last experiment from 0.25 cm² to 0.30 cm². In addition, modification was made to minimize the sideways movement between the two half chambers during clamping. Ringer solution (pH 7.4) was continuously perfused through each side of the chamber separately at the rate of 10 ml/h. The control HEPES-buffered Ringer solution contained (in mM) 113.0 NaCl, 4.6 KCl, 21.0 NaHCO₃, 0.6 MgSO₄, 7.5 d-glucose, 1.0 glutathione (reduced form), 1.0 Na₂HPO₄, 10.0 HEPES, and 1.4 CaCl₂. The solution was bubbled with 95% O₂ and 5% CO₂ for 15 minutes before the experiments. For the HCO₃-free solution, it was bubbled with air. A precalibrated DC heating pad maintained the temperature of the chamber at approximately 35°C to 37°C. Electrical parameters including spontaneous potential difference (PD), short-circuit current (SCC), and total electrical resistance (R) across the preparations were constantly monitored by the Dual Voltage Clamp-1000 (World Precision Instruments, Sarasota, FL). The electrical resistance was determined by passing a known current through the preparation and observing the change in the magnitude of the PD. The tissue resistance (Rt) was then calculated in terms of the difference between the total and the blank resistance.

Figure 2 illustrates the PD-sensing device used in our experiments. Essentially, it consisted of two potential-sensing tubes that were connected to each other by a by-pass arm through three-way stopcocks. At the beginning of the experiment, both the potential-sensing tubes and the by-pass arm were filled with normal Ringer (NRR) solution. The potential sensing tubes were then fitted into the PD-sensing arms of the chamber. By switching the three-way stopcocks to position A, any junction potentials between the 0.9% NaCl and Ringer solutions or associated with the Ag/AgCl electrodes could be nullified. The stopcocks were then switched back to position B to allow the measurement of the electrical parameters across the preparation in the chamber. At that point, the fluid junction was exactly the same as in position A (0.9% NaCl and Ringer solutions). The offset potential was checked frequently with the by-pass arm and stopcocks throughout the experiment.

In the ion substitution experiments, the two potential sensing tubes and the by-pass arm were removed temporarily.
They were flushed and refilled with the substituted solution, before they were connected to the chamber again. The offset potentials were adjusted to zero again through the bypass arm, as previously described, before the electrical measurements were taken. Therefore, the changes in the electrical parameters were entirely due to responses of the preparations to the changes in the solution components.

**Measurement of Radiolabeled Cl Flux**

In the initial experiments, after a brief stabilization period, Ringer solution with a particular drug was perfused to the preparation until its full effect on the SCC was demonstrated. Later, a radiolabeled (hot) solution with that drug was perfused and allowed to equilibrate before the flux measurements, as described earlier. In the subsequent experiments, we loaded the drug together with the hot solution and skipped the drug-only treatment. The results were not different from those before, and the electrical parameters remained stable throughout the experiments.

The unidirectional Cl fluxes were determined under the short-circuited conditions. To minimize the variability between individual eyes, only those data from paired measurements (i.e., using the same eye) were compared. This was achieved by mounting two preparations from each eye: One preparation was for the measurement of influx (Jfas: stroma-to-aqueous), and the other preparation was mounted in another identical chamber for backflux measurement (Jsa: aqueous-to-stroma). The net flux was the difference between Jfas and Jsa. In other words, a single net flux data point was obtained from each eye. After a 60-minute equilibration period, perfusates from both half chambers were collected separately with two scintillation vials. The samples were taken from each side at 12-minute intervals until a stable flux was obtained. The radioactivity of all vials. The samples was measured with a liquid scintillation counter (Wallac 1414 Winspectral DSA; Wallac, Helsinki, Finland) after the samples was measured with a liquid scintillation counter (Wallac 1424 Wallac, Helsinki, Finland) after the samples was measured with a liquid scintillation counter (Wallac 1424 Wallac, Helsinki, Finland)

<table>
<thead>
<tr>
<th>Conditions</th>
<th>n</th>
<th>Baseline</th>
<th>Altered Solution</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 mM Cl</td>
<td>5</td>
<td>-0.45 ± 0.08</td>
<td>-0.05 ± 0.07†</td>
<td>-0.51 ± 0.16</td>
</tr>
<tr>
<td>30 mM Cl</td>
<td>4</td>
<td>-0.46 ± 0.16</td>
<td>+0.44 ± 0.20†</td>
<td>-0.54 ± 0.13</td>
</tr>
<tr>
<td>24 mM Na (choline)</td>
<td>5</td>
<td>-0.62 ± 0.15</td>
<td>-0.15 ± 0.12</td>
<td>-0.41 ± 0.10</td>
</tr>
<tr>
<td>24 mM Na (NMDG)</td>
<td>5</td>
<td>-0.91 ± 0.19</td>
<td>-0.70 ± 0.23</td>
<td>-1.03 ± 0.38</td>
</tr>
<tr>
<td>BCF (HCO3-free solution)</td>
<td>6</td>
<td>-0.48 ± 0.06</td>
<td>-0.30 ± 0.07†</td>
<td>-0.36 ± 0.08†</td>
</tr>
</tbody>
</table>

Results are in mean millivolts ± SEM. In reduced Cl and HCO3-free conditions, equimolar amounts of gluconate was exchanged for the Cl and HCO3 in the Ringer solution. In reduced Na conditions, the Na concentration was replaced by an equimolar amount of choline or NMDG. BCF, HCO3-free solution.

*P < 0.05.
†P < 0.01.

**Pharmacologic Agents in Ringer Solution**

In the Na substitution experiments, the Na concentration in the Ringer solution was reduced to 24 mM (the control Na concentration being 136 mM) by exchanging it for an equimolar amount of choline or N-methyl-D-glucamine (NMDG). In reduced Cl and HCO3-free media, the Cl and HCO3 were similarly replaced by equimolar amounts of gluconate or cyclamate.

The radiolabeled isotope of [36Cl] was purchased from Amersham Radiochemicals. Other pharmacologic agents used were: 3-[aminosulfonyl]-5-[butylamino]-4-phenoxybenzoic acid (bumetanide), 4,4′-disothiocyanatostilbene-2,2′-disulfonic acid (DIDS), 5-[N,N-dimethyl]-amiloride hydrochloride (DMA), and dimethyl sulfoxide (DMSO; all from Sigma, St. Louis, MO); 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB; BIOMOL Research, Plymouth Meeting, PA); heptanol (Fluka Chemie, Buchs, Switzerland). All these chemicals were dissolved in DMSO before they were added to the Ringer solution. The final concentration of this solvent in the Ringer solution was adjusted to 0.1%. Preliminary experiments showed that DMSO has no effect on the electrical parameters and Cl flux (data not shown).

**RESULTS**

**Electrical Parameters**

Stable electrical parameters were achieved within 60 minutes of equilibration. Twenty-eight preparations (paired preparations from 14 eyes) bathed with NRR solution were used to establish the control for the electrical parameters. Radiolabeled Cl was subsequently added to these preparations for the measurement of basal Cl secretion. The PD across the preparations was 0.55 ± 0.04 mV, consistently negative at the aqueous side. The SCC and the R0 were found to be 8.0 ± 0.7 μA/cm2 and 72 ± 3 Ω/cm2 respectively. These electrical parameters remained stable for 4 to 5 hours. Both the PD and the SCC were found to be higher than those in our previous report. This may have been due to an improvement in the mounting technique. The effects of ion substitutions on the electrical parameters are summarized in Tables 1 and 2. The polarity and magnitude of the PD and the SCC were found to depend primarily on the concentration of Cl in the solution. At 60 mM Cl, when the Cl was replaced by gluconate, the PD was immediately abolished,

\[ SCC = J \cdot z \cdot F \]

where \( z \) represents the valence of Cl and \( F \) represents Faraday’s constant.
TABLE 2. Effects of Cl, Na, and HCO₃ Substitutions in Ringer Solution on SCC Measurements across Isolated Bovine CBE

<table>
<thead>
<tr>
<th>Conditions</th>
<th>n</th>
<th>Baseline</th>
<th>Altered Solution</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 mM Cl (bilateral)</td>
<td>5</td>
<td>-5.3 ± 0.7</td>
<td>-0.2 ± 1.0†</td>
<td>-5.8 ± 1.9</td>
</tr>
<tr>
<td>30 mM Cl (bilateral)</td>
<td>5</td>
<td>-5.0 ± 1.4</td>
<td>+4.4 ± 2.3†</td>
<td>-6.4 ± 0.7</td>
</tr>
<tr>
<td>24 mM Na (choline)</td>
<td>5</td>
<td>-5.8 ± 1.1</td>
<td>-1.4 ± 1.2‡</td>
<td>-3.9 ± 0.5</td>
</tr>
<tr>
<td>24 mM Na (NMDG)</td>
<td>5</td>
<td>-8.0 ± 1.2</td>
<td>-2.6 ± 0.7ª</td>
<td>-8.7 ± 2.9</td>
</tr>
<tr>
<td>BCF (HCO₃-free solution)</td>
<td>6</td>
<td>-6.1 ± 0.7</td>
<td>-4.5 ± 1.1</td>
<td>-5.0 ± 1.2</td>
</tr>
</tbody>
</table>

Results are in mean microamps per square centimeter ± SEM.

ªP < 0.05.
†P < 0.01.

and the SCC was reduced by approximately 95%. Reducing Cl to 30 mM even reversed the polarity of the PD. In a similar experiment shown in Table 3, cyclamate replaced Cl in the solution. The sequential reduction of Cl in the solution on both sides also produced a stepwise decline in the PD and SCC. This finding is comparable to results shown in Tables 1 and 2. In experiments with either reduced Na concentration or HCO₃-free media, inhibition of the PD and the SCC was also observed. However, the inhibitory effect was less drastic than that of when Cl was reduced, and a negative PD was preserved. The effects of ion substitution on the electrical parameters were reversible, except in HCO₃-free media in which an incomplete recovery was seen.

The effects of different transport inhibitors on electrical measurements are shown in Table 4. Significant depolarization and inhibition of SCC were observed with bilateral 0.1 mM of either DIDS or bumetanide. Bilateral 0.1 mM DMA, however, had no significant effect on the electrical parameters. NPPB (0.1 mM) abolished the PD, and the SCC when added to the aqueous side but had no effect when added to the stromal side. Heptanol (3.5 mM) on both sides substantially decreased the PD and the SCC by 80%.

Unidirectional Measurements of Cl Flux

Unidirectional Cl flux was measured in paired tissues under short-circuit conditions. Re-examination of Cl secretion with the modified chamber demonstrated a substantial net Cl influx under baseline conditions (Table 5), which is similar to the results in our previous report.8 No significant difference in the electrical resistance of the paired tissues was found. The net Cl flux of 1.03 µEq/cm² per hour was used as a reference for comparison with the other experimental conditions. With the modified chamber, we noted that the magnitude of the unidirectional Cl fluxes were reduced in both directions by 25% to 29%, but had no effect on the net Cl transfer. This implies that the modified chamber was more sensitive in detecting any net ion transport. The net Cl influx corresponded to a current of approximately 27.6 µA/cm², which was approximately 3.5 times larger than that of the measured SCC.

The effects on Cl transport of reducing the concentrations of Cl and Na in the solution are summarized in Table 6. At 60 mM Cl, the net Cl flux was inhibited by 50%, and at 30 mM the net Cl flux was abolished. The Cl transport was also dependent on the concentration of Na in the solution, because the net Cl flux was almost abolished at 24 mM Na. The Cl transport across the bovine CBE was also sensitive to various transport inhibitors, and the results are shown in Table 6. Bumetanide, when applied bilaterally or on the stromal side only, effectively inhibited net Cl flux by 86% and 54%, respectively. In addition, bumetanide slightly inhibited Cl flux (approximately 18%), when applied to the aqueous surface (data not shown). In contrast, bilateral DIDS (0.1 mM) did not inhibit the unidirectional Cl flux in either direction, although a substantial inhibition of the SCC was observed. Furthermore, NPPB (0.1 mM) on the aqueous side or heptanol (3.5 mM) on both sides significantly inhibited the net Cl influx.

DISCUSSION

Cl-Dependent PD and SCC

In rabbit ciliary epithelium, the electronegativity of the transepithelial PD was shown to be dependent on the HCO₃ and Cl concentrations in the bath.17,18 Our results indicate that in

TABLE 3. Effects of Sequential Reduction of Cl Concentration in Ringer Solution on Both PD and SCC Measurements

<table>
<thead>
<tr>
<th>Parameters</th>
<th>NRR</th>
<th>60 mM Cl</th>
<th>30 mM Cl</th>
<th>NRR</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD (mV)</td>
<td>-0.85 ± 0.18</td>
<td>-0.18 ± 0.20ª</td>
<td>+0.21 ± 0.12†</td>
<td>-1.00 ± 0.22</td>
</tr>
<tr>
<td>SCC (µA/cm²)</td>
<td>-6.1 ± 1.6</td>
<td>-1.1 ± 1.2*</td>
<td>+1.2 ± 0.6†</td>
<td>-8.3 ± 2.3</td>
</tr>
</tbody>
</table>

Instead of using gluconate, cyclamate was exchanged for Cl in the bath. Results are means ± SEM (n = 6).

ªP < 0.05.
†P < 0.01.
TABLE 5. Re-Examination of Basal Cl Secretion in Paired Tissues with Normal Ringer Solution Under Short-Circuited Conditions

<table>
<thead>
<tr>
<th>Condition</th>
<th>Jsa (µEq/cm² per h)</th>
<th>Jas (µEq/cm² per h)</th>
<th>Net Flux (µEq/cm² per h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unidirectional Cl flux</td>
<td>4.74±0.26</td>
<td>3.71±0.17</td>
<td>1.03±0.28</td>
</tr>
<tr>
<td>Tissue resistance R</td>
<td></td>
<td>72±5</td>
<td>71±4</td>
</tr>
</tbody>
</table>

Values are means ± SEM, (n = 14). Jsa, stroma-to-aqueous flux; Jas, aqueous-to-stroma flux.

* P < 0.005.

bovine CBE, both the PD and the SCC are more susceptible to changes in the Cl concentration than the HCO₃⁻ concentrations, irrespective of the ions substituted (glucose or cyclamate). A positive PD was observed at a low Cl concentration, but this was not the case with HCO₃⁻-free media. Reducing the extracellular Cl concentration diminished the rate of Cl entry into the PE and therefore both the electrical measurements and the Cl transport were reduced. This evidence strongly indicates that extracellular Cl is important to the transepithelial Cl transport. At 30 mM Cl, which abolished the transepithelial Cl transport, the positive PD could be due to the resultant activities of NaK-ATPases at both cell layers, because they were working in an opposite direction (Fig. 1). Differential expression of NaK-ATPase isoforms, which also display a different affinity for Na, have been reported among bovine PE and NPE cells. Furthermore, NaK-ATPase was found to be more abundant in bovine NPE cells than in PE cells. In view of this, the potential difference generated by the NaK-ATPase in the respective cell layers is different, which may be attributed to the observed positive PD under low-CI conditions. Indeed, we cannot rule out the possibility of a net translation of cation(s) into, or a net removal of anion(s) from, the posterior chamber under CI reduction. Additional investigations are needed to test this hypothesis.

Our result was different from those in the studies by Kishida et al. and Krupin et al. who reported a positive PD in rabbit ciliary body under HCO₃⁻-free conditions. The reason for the difference is not fully understood, but variations in the ion transport between different species have been proposed. It is interesting to note that those species (e.g., rabbits) that have an excess of HCO₃⁻ in aqueous humor compared with plasma have a corresponding deficit of Cl in aqueous. The reverse is true for humans. In cattle, the Cl concentration in aqueous is also found to be higher than that of plasma, as in humans, although no information is available for HCO₃⁻ concentration. The high concentration of HCO₃⁻ in the rabbit’s aqueous is thought to be for buffering the acids produced by the anaerobic respiration of the relatively large crystalline lens. From this point of view, it is likely that the mechanisms of transepithelial Cl and HCO₃⁻ transport in the ciliary epithelium are different between species, as reflected in the difference in ionic dependence.

Mechanisms of Transepithelial Cl Transport

Chu and Candia have found that the calculated conductance of Na and Cl is larger than the measured conductance, suggesting the presence of electrically coupled Na and Cl transport. Because the SCC is equal to the algebraic sum of all ion transport processes, the current generated from the transepithelial Cl transport can therefore be larger than the measured SCC if complex ionic transport activities are involved. Our results indicate that the measured SCC was only approximately 30% of the net Cl flux, suggesting that much of the Cl movement across the ciliary epithelium took place in an electroneutral manner. We tried to look at the Na and HCO₃⁻ (unpublished observation) fluxes but failed to show a significant net transfer to account for the apparent discrepancy between the SCC and the Cl fluxes. The exact ion-coupling mechanism remains unclear. However, as the unidirectional Na fluxes are several times larger than the Cl fluxes, even in the absence of net Na transport, we cannot rule out the possibility of coupled NaCl transport. In the present study, we therefore tried to reduce the Na concentration in the solution to see whether it would affect the electrical parameters and unidirectional Cl transport. The Na concentration in the solution was reduced to 24 mM, which is approximately the intracellular Na concentration in most epithelial cells, including rabbit ciliary epithelial cells. This maneuver was expected to dissipate the driving force for Cl uptake, if any, into the PE cells.

It has been documented that the NaK-ATPase at the basolateral border in PE cells creates a substantial transmembrane Na gradient and favors the intracellular accumulation of Cl in ciliary epithelium. Provided that the transepithelial Na and Cl transport was independent but in the same direction, reducing the Na concentration would inhibit Na transport solely and should result in a hyperpolarization due to the predominance of Cl transport. However, we found that reducing the Na concentration in the solution depolarized the PD and de-

TABLE 6. Unidirectional Cl Fluxes Measured Under Various Experimental Conditions

<table>
<thead>
<tr>
<th>Condition</th>
<th>Stroma-to-Aqueous</th>
<th>Aqueous-to-Stroma</th>
<th>Net Flux (µEq/cm² per h)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 mM CI</td>
<td>2.78±0.29</td>
<td>86±8</td>
<td>2.27±0.31</td>
<td>89±11</td>
</tr>
<tr>
<td>30 mM Cl</td>
<td>1.28±0.07</td>
<td>97±4</td>
<td>1.27±0.07</td>
<td>89±6</td>
</tr>
<tr>
<td>24 mM Na</td>
<td>4.28±0.32</td>
<td>94±4</td>
<td>4.25±0.30</td>
<td>94±7</td>
</tr>
<tr>
<td>BMT (bilateral)</td>
<td>4.51±0.24</td>
<td>69±3</td>
<td>4.57±0.18</td>
<td>71±5</td>
</tr>
<tr>
<td>DIDS (bilateral)</td>
<td>4.66±0.18</td>
<td>64±3</td>
<td>4.19±0.30</td>
<td>71±5</td>
</tr>
<tr>
<td>Heptanol (bilateral)</td>
<td>4.84±0.41</td>
<td>70±4</td>
<td>3.64±0.30</td>
<td>72±7</td>
</tr>
<tr>
<td>NPPB (aqueous)</td>
<td>5.08±0.32</td>
<td>58±4</td>
<td>4.87±0.48</td>
<td>59±3</td>
</tr>
</tbody>
</table>

The net Cl influx of 1.05 µEq/cm² per hour was used as a reference for comparison to the experimental conditions listed. Values are means ± SEM, and the number in parentheses represents the number of experiments.
creased the SCC. These results imply that Cl may couple with Na in transepithelial transport. Total inhibition of the net Cl influx under reduced Na concentration confirmed the importance of extracellular Na in the Cl transport process. The coupled movement of Na and Cl in the PE cells was achieved by using the Na gradient maintained by the NaK-ATPase.

The coupled movement of Na and Cl may be achieved in many different ways, such as through Na-K-2Cl symport or by parallel Na/H and Cl/HCO₃ antiport. These two mechanisms are not mutually exclusive. It is possible that both mechanisms work simultaneously, although one or the other mechanism may be more pronounced in a given species. A recent study of rabbit ciliary epithelium in which an Ussing chamber was used has shown that the SCC was inhibited by both bumetanide and DIDS, suggesting that both the Na-K-2Cl symport and the Cl/ HCO₃ antiport contribute to the net ion fluxes across the ciliary epithelium.

Na-K-2Cl Cotransport System

Wiederholt and Zadunaisky and Weiderholt et al. demonstrated the inhibitory effect of loop diuretics on both the SCC and the intracellular Cl activity in shark ciliary epithelium. Edelman et al. subsequently analyzed the volume of isolated PE and NPE cells which were subjected to anisosmotic perturbations and transport inhibition. They have suggested that bumetanide-sensitive Na-K-2Cl cotransport constitutes the principal pathway for ion entry into the PE cells. In the present study, bumetanide caused a significant inhibitory effect on both electrical measurements and Cl secretion. The transepithelial Cl transport was inhibited by 85% to 90% when bumetanide was added to both sides. More than 60% of this inhibition (54% of 86%) can be explained by the effect exerted on the stromal surface. We also noted an 18% inhibition of Cl flux when bumetanide was applied to the aqueous side (data not shown). The reason for this is not clear. Evidence for the presence of Na-K-2Cl cotransport has been reported in human NPE cells. Crook and Riese have proposed that Na-K-2Cl cotransporter may be present at the apical borders of NPE cells and may provide an additional pathway for Cl entry into the NPE cells. A more recent study on rabbit ciliary epithelium has even suggested that Na-K-2Cl cotransporter may be present at the basolateral membrane in NPE cells, enhancing the Cl transport into the aqueous humor. The exact mechanism remains to be determined. Our findings indicated that a bumetanide-sensitive Na-K-2Cl cotransport is present primarily at the basolateral membrane in PE cells and contributed to the transepithelial Cl movement in bovine ciliary epithelium.

It is worth noting that when bumetanide was applied to the stromal side, it not only inhibited the influx, but also stimulated the backflux. This phenomenon also applied to some other experimental conditions such as bilateral heptanol. The exact reason is not known. An early study of toad CE has suggested that Cl backflux was entirely a passive and diffusional flux. However, a later report has indicated that in addition to the secretive pathway, there is a reabsorptive pathway through which Cl can be returned to the stromal side. There are many ion transporters present on the surface of the NPE cells, which can regulate the "active" ion reabsorption into the functional syncytium. We speculate that in normal secretion of aqueous, the cotransporter works in such a way that it dominates the directional influx of Cl across the ciliary epithelium by driving the Cl out of the cells into the posterior chamber. However, when the cotransporter in the PE cells is blocked (as with bumetanide), the influx decreases and the intracellular Cl concentration drops accordingly. Owing to the decrease in the directional driving force of Cl, the reabsorption of Cl increases to replenish the intracellular Cl. In that case, the Cl backflux is increased because more Cl is returned into the stroma. A new equilibrium is ultimately reached when the influx and backflux are similar in magnitude.

Cl/HCO₃ and Na/H Antiport System

Evidence for the role of Cl/HCO₃ and Na/H exchangers in ion transport across the ciliary epithelium have been presented in the literature: a reduction of extracellular HCO₃ concentration abolished the net Cl influx in rabbit; Cl uptake was stimulated by an outwardly directed HCO₃ gradient on cultured bovine PE cells; both PE and NPE contained more Cl when incubated with HCO₃-rich media; and recovery of intracellular pH of the rat's ciliary epithelial cells from acid load was dependent on the presence of external Na and was inhibited by amiloride, whereas pH recovery from alkali load was inhibited by either replacement of external Cl or inclusion of DIDS. Such evidence supports a model of Cl/HCO₃ antiport working in parallel with an Na/H exchanger, which together mediate the uptake of Na and Cl into the functional syncytium. Na and Cl were exchanged for H and HCO₃. The intracellular pH and carbonic anhydrase indirectly governed the double-exchange mechanism. Our results show that bilateral DIDS (0.1 mM) significantly decreased the SCC by half, but interestingly it had no effect on the unidirectional Cl flux in either direction. The reason for this phenomenon is not clear. We suggest that the correlation between the electrical parameter measurements and ion fluxes is not simple, possibly because of the complex syncytial structure of the ciliary epithelium. The SCC reflects the summation of all electrogenic activities across this bilayered structure; however, the exact ionic components contributing to the SCC have yet to be elucidated. Until the whole picture is known, the contribution of Cl transport to the SCC will remain unclear. Simultaneous abolition of electrical measurements and Cl flux was seen in some of the experimental conditions, such as with aqueous NPPB and bilateral heptanol. However, with 60 mM Cl, the electrical parameters were greatly inhibited, but a substantial portion of Cl flux remained. Krupin et al. suggested that a change in the SCC does not necessarily permit the identification of any change in solute transfer. Further study may be required to clarify the relationship between the Cl flux and the SCC in the ciliary epithelium.

In theory, DIDS may have inhibited 10% to 15% of Cl flux that was not inhibitable by bumetanide (86% inhibition). However, this amount of inhibition may not be easily identified with an Ussing-type setup. We therefore suggest that Cl/HCO₃ may play a minor role in transepithelial Cl transport. It is possible that DIDS acted on other electrogenic pathways, which may have been involved in housekeeping functions, such as the regulation of intracellular pH. This pathway remains unknown and awaits further experimentation.

Our results were in contrast to those reported by McLaughlin et al. on rabbit ciliary epithelium. They proposed that the dominant entry pathway of NaCl uptake is through a parallel Cl/HCO₃ and Na/H antiport based on the measurements of ion contents in the PE and NPE cells. Although different species and methodologies were adopted, the exact
junctional communication. Our finding suggests that much of the Cl movement across the ciliary epithelium is through a transcellular pathway rather than a paracellular route. We noted a 7% to 8% decrease in $R_t$ after bilateral heptanol, the concentration of Cl efflux into the posterior chamber has been suggested not to interfere with other cellular functions except for the uncoupling of gap junctions, which allow free passage of ions from the PE cells to the NPE cells. Similar ionic contents between PE and NPE cells have been demonstrated by electron-probe x-ray microanalysis, suggesting the existence of highly effective transcellular communication between the two cell layers. Our results indicate that bilateral 0.05% heptanol (equivalent to approximately 3.5 mM) inhibited both electrical parameters and net Cl efflux by approximately 80%. This was in agreement with a rabbit study in which the SCC was inhibited by 85% to 90% with 3 mM heptanol. This concentration has been shown not to interfere with other cellular functions except for the uncoupling of junctional communication. Our finding suggests that much of the Cl movement across the ciliary epithelium is through a transcellular pathway rather than a paracellular route. We noted a 7% to 8% decrease in $R_t$ after bilateral heptanol, the exact reason for this decrease is not clear. It is possible that heptanol has other physiological effects on the paracellular pathway, such as loosening the tight junction. Further studies are required to verify this hypothesis.

**Cl Efflux**

The efflux of Cl into the posterior chamber has been suggested to play an important role in aqueous secretion. Intracellular accumulation of Cl was found in the NPE cells of shark and rabbit above its electrochemical equilibrium, therefore driving the passive movement of Cl from the NPE cells to the posterior chamber through the Cl channel. The role of Cl channel or related conductive pathways in aqueous secretion have been documented: Cl channel blockers such as NPPB and DIDS were shown to inhibit the regulatory volume decrease of canine and transformed human NPE cells; changes in membrane potential of NPE cells induced by extracellular Cl substitution and beta-adrenergic stimulation could be prevented by DIDS; and currents activated by hypotonic solution in patch clamping can be prevented by pretreatment with NPPB and DIDS. In the present study, the differential SCC response of NPPB on the stromal and the aqueous sides indicates the NPPB-sensitive Cl conductive pathway is present predominantly at the NPE cells. It has been shown that NPPB may have other effects such as blocking the K channel. However, blocking the cation conductance at the aqueous side should induce hyperpolarization rather than depolarization of the PD as observed when NPPB was added to the aqueous surface. Potassium channel blocker BaCl$_2$ (1 mM) at the aqueous side resulted in a substantial hyperpolarization and did not affect the inhibitory effect of NPPB on the PD and the SCC (Table 7). However, it was difficult to affirm the specificity of NPPB on Cl channel in our setup definitively. It indicated that NPPB blocked primarily the anion, possibly the Cl channels, but not the K channel. The inhibition of Cl transport by aqueous NPPB indicated that the Cl channel is the major efflux pathway into the aqueous humor.

**CONCLUSION**

Our study examined the transmembrane events that contribute to Cl transport across the bovine CBE. We propose that the bumetanide-sensitive Na-K-2Cl symport is the major influx pathway for Cl in the PE cells. The Cl/HCO$_3$ and Na/H double exchangers play a minor role in facilitating the Cl uptake into membrane potential of NPE cells induced by extracellular Cl channel or related conductive pathways in aqueous secretion. The role of Cl channel or related conductive pathways in aqueous secretion have been documented: Cl channel blockers such as NPPB and DIDS were shown to inhibit the regulatory volume decrease of canine and transformed human NPE cells; changes in membrane potential of NPE cells induced by extracellular Cl substitution and beta-adrenergic stimulation could be prevented by DIDS; and currents activated by hypotonic solution in patch clamping can be prevented by pretreatment with NPPB and DIDS. In the present study, the differential SCC response of NPPB on the stromal and the aqueous sides indicates the NPPB-sensitive Cl conductive pathway is present predominantly at the NPE cells. It has been shown that NPPB may have other effects such as blocking the K channel. However, blocking the cation conductance at the aqueous side should induce hyperpolarization rather than depolarization of the PD as observed when NPPB was added to the aqueous surface. Potassium channel blocker BaCl$_2$ (1 mM) at the aqueous side resulted in a substantial hyperpolarization and did not affect the inhibitory effect of NPPB on the PD and the SCC (Table 7). However, it was difficult to affirm the specificity of NPPB on Cl channel in our setup definitively. It indicated that NPPB blocked primarily the anion, possibly the Cl channels, but not the K channel. The inhibition of Cl transport by aqueous NPPB indicated that the Cl channel is the major efflux pathway into the aqueous humor.

**References**


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