Apical and basolateral $\text{CO}_2\text{-HCO}_3^-$ permeability in cultured bovine corneal endothelial cells

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Bonanno, J oseph A., Yi Guan, Sergey J elamskii, and X iao J un Kang. Apical and basolateral $\text{CO}_2\text{-HCO}_3^-$ permeability in cultured bovine corneal endothelial cells. Am. J. Physiol. 277 (Cell Physiol. 46): C545–C553, 1999.—Corneal endothelial function is dependent on $\text{HCO}_3^-$ transport. However, the relative $\text{HCO}_3^-$ permeabilities of the apical and basolateral membranes are unknown. Using changes in intracellular pH secondary to removing $\text{CO}_2\text{-HCO}_3^-$ (at constant pH) or removing $\text{HCO}_3^-$ alone (at constant $\text{CO}_2$) from apical or basolateral compartments, we determined the relative apical and basolateral $\text{HCO}_3^-$ permeabilities and their dependencies on Na+ and Cl−. Removal of $\text{CO}_2\text{-HCO}_3^-$ from the apical side caused a steady-state alkalization (+0.08 pH units), and removal from the basolateral side caused an acidification (−0.05 pH units). Removal of $\text{HCO}_3^-$ at constant $\text{CO}_2$ indicated that the basolateral $\text{HCO}_3^-$ fluxes were about three to four times the apical fluxes. Reducing perfusate Na+ concentration to 10 mM had no effect on apical flux but slowed basolateral $\text{HCO}_3^-$ flux by one-half. In the absence of Cl−, there was an apparent increase in apical $\text{HCO}_3^-$ flux under constant-pH conditions; however, no net change could be measured under constant-CO2 conditions. Basolateral flux was slowed ~30% in the absence of Cl−, but the net flux was unchanged. The steady-state alkalization after removal of $\text{CO}_2\text{-HCO}_3^-$ apically suggests that $\text{CO}_2$ diffusion may contribute to apical $\text{HCO}_3^-$ flux through the action of a membrane-associated carbonic anhydrase. Indeed, apical $\text{CO}_2$ fluxes were inhibited by the extracellular carbonic anhydrase inhibitor benzamidine and partially restored by exogenous carbonic anhydrase. The presence of membrane-bound carbonic anhydrase (CAIV) was confirmed by immunoblotting. We conclude that the Na+-dependent basolateral $\text{HCO}_3^-$ permeability is consistent with Na+-$\text{nHCO}_3^-$ cotransport. Changes in $\text{CO}_2\text{-HCO}_3^-$ flux in the absence of Cl− are most likely due to Na+-$\text{nHCO}_3^-$ cotransport-induced membrane potential changes that cannot be dissipated. Apical $\text{HCO}_3^-$ permeability is relatively low, but may be augmented by $\text{CO}_2$ diffusion in conjunction with a CAIV.

bicarbonate permeability; epithelial transport; carbonic anhydrase
apical membrane could be supplemented by CO₂ diffusion and conversion to HCO₃⁻ by CAIV.

MATERIALS AND METHODS

Cell culture. Bovine corneal endothelial cells (BCEC) were cultured to confluence on glass coverslips or 13-mm AnoDisc (Whatman; Fisher Scientific) filters as previously described (3). Briefly, primary cultures from fresh cow eyes were established in T-25 flasks with 3 ml of DMEM, 10% bovine calf serum, and an antibiotic-antimycotic (100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone); gassed with 5% CO₂-95% air at 37°C, and fed every 2–3 days. These were subcultured to three T-25 flasks and grown to confluence in 5–7 days. The resulting second-passage cultures were then subcultured onto coverslips or filters, reaching confluence within 5–7 days. Cells were transferred to 1% serum-DMEM for at least 24 h before experiments.

Solutions and chemicals. The composition of the HCO₃⁻-rich Ringer solution used throughout this study was (in mM) 150 Na⁺, 4 K⁺, 0.6 Mg²⁺, 1.4 Ca²⁺, 118 Cl⁻, 1 HPO₄²⁻, 10 HEPES, 28.5 HCO₃⁻, 2 glucose, and 5 glucose. Ringer solutions were equilibrated with 5% CO₂ and pH was adjusted to 7.50 at 37°C. HCO₃⁻-free Ringer (pH 7.5) was prepared by equimolar substitution of NaHCO₃ with sodium gluconate. Low-HCO₃⁻ Ringer (2.85 mM; pH 6.5) was prepared by replacing 25.65 mM NaHCO₃ with sodium gluconate. Cl⁻-free Ringer was prepared by equimolar replacement of NaCl with sodium gluconate. Low-Na⁺ Ringer (10 mM) was prepared by replacement of 140 mM NaCl with 140 mM N-methyl-D-glucamine chloride. Osmolarity was adjusted to 300 ± 5 mosM with sucrose.

The 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) was obtained from Molecular Probes (Eugene, OR). The CAI benzolamide (mol wt 320), benzolamide, and nigericin (10 mM in ethanol) were stored at 20°C.

Measurement of pHᵢ. pHᵢ was measured with the pH-sensitive fluorescent dye BCECF (24). The cells were loaded by incubation in Ringer containing 1–5 μM BCECF-AM at room temperature for 30–60 min. Dye-loaded cells were then kept in Ringer for at least 30 min before use. Fluorescence excitation was provided by a 75-W xenon arc as part of a PTI ratio fluorescence system (Photon Technology, Monmouth Junction, NJ). The excitation wavelengths (495 and 440 nm) were obtained by passing the light through a DeltaRam monochromator. The excitation light was directed to the objective by a dichroic mirror centered at 505 nm. The fluorescence emission collected by the objective was passed through a barrier filter (540 ± 20 nm) and led to a photomultiplier for photon counting. Neutral-density filters (optical density 1–2) were included in the excitation path to minimize photobleaching. Synchronization of excitation with emission measurement and data collection was controlled by Felix software (Photon Technology). Fluorescence ratios were obtained at 1 s⁻¹. The ratio of fluorescence emission to excitation at 495 nm to that at 440 nm (i.e., F₄₉₅/F₄₄₀) was calibrated against pH, by the high-K⁺-nigericin technique (3, 28). A calibration curve, which follows a pH titration equation, has been constructed for BCEC (3).

Immunoblotting. Fresh BCEC were scraped from dissected corneas, placed into ice-cold PBS containing a protease inhibitor cocktail (Complete; Boehringer Mannheim), and centrifuged at low speed for a brief period. Cell pellets were resuspended in 2% SDS sample buffer containing protease inhibitors. Cultured cells were dissolved directly in sample buffer. Both preparations were sonicated (Branson 250) briefly on ice and then centrifuged at 6,000 g for 5–10 min. An aliquot of the supernatant was taken for protein assay by the Bradford method (Bio-Rad). A fresh BCEC was scraped from dissected corneas, placed into ice-cold PBS containing a protease inhibitor cocktail (Complete; Boehringer Mannheim), and centrifuged at low speed for a brief period. Cell pellets were resuspended in 2% SDS sample buffer containing protease inhibitors. Cultured cells were dissolved directly in sample buffer. Both preparations were sonicated (Branson 250) briefly on ice and then centrifuged at 6,000 g for 5–10 min. An aliquot of the supernatant was taken for protein assay by the Bradford method (Bio-Rad). β-Mercaptoethanol (5%) and bromphenol blue were added to the remainder of the supernatant, and the mixture was heated at 80°C for 4 min. The samples were applied to a 12% polyacrylamide gel with a 4.5% stacking gel (60 μg protein/lane). After electrophoresis at 20 mA, proteins were transferred to a polyvinylidene difluoride membrane overnight at 4°C. Membranes were incubated in PBS containing 5% nonfat dry milk for 1 h at room temperature and washed in PBS containing 0.05% Tween two to three times for 5 min. The blots were then incubated with anti-human CAIV primary antibody, kindly provided by W. Sliwka and A. Waheed (St. Louis Univ. School of Medicine). Next, the blots were washed four times with PBS-Tween, incubated with secondary antibody coupled to horseradish peroxidase (Sigma), and finally developed by enhanced chemiluminescence (DuPont). Films were scanned to produce digital images that were then assembled and labeled using Microsoft PowerPoint software.

RESULTS

Two approaches were used to examine apical and basolateral HCO₃⁻ permeabilities of cultured corneal endothelial cells. In the first approach, pHᵢ was measured while cells were perfused in CO₂-HCO₃⁻-rich Ringer, followed by a brief exposure to CO₂-HCO₃⁻-free Ringer at the same pH. This is the constant-pH proto-


Fig. 1. Control intracellular pH (pHi) changes due to HCO₃⁻ removal. A: constant-pH protocol. CO₂-HCO₃⁻-rich Ringer, pH 7.5, was replaced by HEPES-buffered CO₂-HCO₃⁻-free Ringer, pH 7.5, first on apical side only then on basolateral side. B: constant-CO₂ protocol. Perfusion HCO₃⁻ concentration ([HCO₃⁻]) was reduced from 28.5 mM at pH 7.5 to 2.85 mM at pH 6.5, and both apical and basolateral solutions were gassed with 5% CO₂. Apical and basolateral sides were exposed to low-[HCO₃⁻] Ringer during periods indicated (boxes).

In this approach, pHi will be affected by both CO₂ and HCO₃⁻ fluxes. In the second approach, the test Ringer had reduced HCO₃⁻ concentration ([HCO₃⁻]) and pH, but the same CO₂ concentration ([CO₂]). This is the constant-CO₂ protocol. In this approach, pHi will be affected by HCO₃⁻ fluxes and the reduced Ringer pH. Both approaches, however, focus on the fact that net HCO₃⁻ efflux should lead to a drop in pHi. Figure 1A shows the effect on pHi after removal of CO₂-HCO₃⁻ sequentially from the apical side and then the basolateral side by the constant-pH protocol. On both the apical and basolateral sides there was an initial rapid alkalinization due to rapid efflux of CO₂. On the apical side, the alkalinization was larger (0.17 vs. 0.05 pH units) and was followed by a small acidification (−0.035 pH units). This acidification reflects HCO₃⁻ efflux across the apical membrane. A new steady-state pH_i was reached within 2–3 min and was always above the baseline pH_i. When CO₂-HCO₃⁻-rich Ringer was reintroduced, there was a rapid acidification of the same magnitude as that of the initial alkalinization. This was followed by a recovery to the baseline pH_i. On the basolateral side, Fig. 1A shows that after the initial alkalinization from CO₂ efflux, there was a sharp acidification below the baseline (−0.15 pH units) and then a recovery to a new steady state ~0.05 pH units below the baseline. These data are summarized in Table 1. The smaller initial alkalinization and deeper and more rapid acidification on the basolateral side indicate that HCO₃⁻ efflux is greater on the basolateral side than on the apical side. The concurrent CO₂ and HCO₃⁻ effuxes, however, make it difficult to quantitate these differences.

To remove the effects of CO₂ fluxes seen in Fig. 1A, we next used the constant-CO₂ protocol. Figure 1B shows a small drop in pH_i (−0.06 pH units) when HCO₃⁻ was removed from the apical side. However, on the basolateral side, HCO₃⁻ removal caused an initial rapid drop (−0.21 pH units) followed by a small recovery. Table 2 summarizes these responses. In the presence of CO₂-HCO₃⁻, total buffering capacity (β) of corneal endothelial cells is ~55 mM/pH unit (3). By using the largest pH decrease from Table 2, the net equivalent effluxes (ΔpHi × β) from the apical side and basolateral side were calculated to be 2.8 and 11.6 mM, respectively. However, these flux values do not take into account the effect of reduced bath pH on the pHi change. To test this, we measured the drop in pH_i due to changing the bath Ringer pH from 7.5 to 6.5 in CO₂-HCO₃⁻-free Ringer. The pH_i dropped by 0.10 and 0.35 when the apical and basolateral pH values, respectively, were lowered. Taking into account the intrinsic (non-HCO₃⁻) buffering capacity of these cells (10 mM/pH unit) (3), the net H⁺ influx values were 1.0 and 3.5 mM for the apical and basolateral sides, respectively. Thus ~64 and 70% of the initial pH_i drop after removal of HCO₃⁻ at constant CO₂ from the apical and basolateral sides, respectively, are due to HCO₃⁻ efflux. The corrected net HCO₃⁻ effluxes were then 1.8 and 8.1 mM for apical and basolateral sides, respectively, indicating that the HCO₃⁻ permeability of the basolateral side is more than four times that of the apical side. During the time it takes for the initial pHi drop to occur, however, other compensating pH_i regulatory mechanisms may be activated. Therefore, we also calculated the initial HCO₃⁻ flux on the basis of the initial dpHi/dt, where t is time, again subtracting the initial dpHi/dt due to changing the perfusate pH from 7.5 to 6.5. These results are

Table 1. Effects on pH_i during CO₂-HCO₃⁻ removal at constant pH

<table>
<thead>
<tr>
<th>Condition</th>
<th>Initial ΔpH</th>
<th>pH Decrease</th>
<th>Steady-state ΔpH</th>
<th>Initial ΔpH</th>
<th>pH Decrease</th>
<th>Steady-state ΔpH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (15)</td>
<td>+0.15 ± 0.02</td>
<td>−0.07 ± 0.04</td>
<td>+0.08 ± 0.05</td>
<td>+0.05 ± 0.01</td>
<td>−0.23 ± 0.02</td>
<td>−0.05 ± 0.01</td>
</tr>
<tr>
<td>Cl⁻ free (6)</td>
<td>+0.15 ± 0.03</td>
<td>−0.15 ± 0.01*</td>
<td>0 ± 0.01*</td>
<td>+0.08 ± 0.02</td>
<td>−0.15 ± 0.02*</td>
<td>−0.07 ± 0.02</td>
</tr>
</tbody>
</table>

Values are means ± SD. pH decrease is intracellular pH (pHi) change subsequent to initial change. Steady-state ΔpH is final pH change from baseline pH_i. Numbers of experiments are in parentheses. *Significantly different from control (P < 0.05).
shown in Table 3 and indicate that, on the basis of initial fluxes, the ratio of basolateral HCO₃⁻ permeability to apical HCO₃⁻ permeability is ∼3.

Na⁺ dependency. The high HCO₃⁻ permeability of the basolateral side is most likely due to the Na⁺-HCO₃⁻ cotransporter. Therefore, reduced Na⁺ concentration ([Na⁺]) should have a greater effect on reducing HCO₃⁻ flux on the basolateral side. Figure 2 shows a set of apical and basolateral responses at normal [Na⁺] (control) obtained by using the constant-pH protocol. The [Na⁺] was then reduced to 10 mM on both sides. This caused the pHᵢ to drop −0.2 units from 7.4 to 7.2, a drop due primarily to reversal of Na⁺-HCO₃⁻ cotransport because Na⁺/H⁺ exchange is not active above pHᵢ 7.15 (3). When apical CO₂-HCO₃⁻ was removed, cells became alkalinized as usual and came to a new steady state at a pHᵢ similar to that of the control. In Fig. 2, it appears that the apical HCO₃⁻ efflux rate was faster than the control rate. This was observed in two of four trials. However, when basolateral CO₂-HCO₃⁻ was removed, the HCO₃⁻ efflux rate was slowed by 51 ± 10% (P < 0.05; n = 4). We conclude that basolateral HCO₃⁻ efflux is diminished in low [Na⁺], but not apical flux, consistent with a Na⁺-HCO₃⁻ cotransporter located basolaterally.

Cl⁻ dependency. Figure 3 shows the apical and basolateral responses under the constant-pH protocol in the absence of Cl⁻. First, it should be noted that the resting pHᵢ (7.45) is significantly higher than that in control Ringer (7.33). This has been shown previously and is due to NaHCO₃ influx via the transporter (Na⁺-HCO₃⁻ influx) secondary to membrane potential depolarization when Cl⁻ is removed (6). When apical CO₂-HCO₃⁻ was removed in the absence of Cl⁻, after the initial alkalinization (+0.15 pH units) there was a significant decrease in pHᵢ (~0.15 pH units), i.e., an increase in HCO₃⁻ efflux, giving no net change in the steady-state pHᵢ. Furthermore, on the basolateral side, the initial alkalinization was slightly larger than that of the control (Fig. 1A) and the ensuing decrease in pHᵢ was significantly diminished. However, the steady-state change in pHᵢ was not significantly different from that for the control. These data are summarized in Table 1. Thus it appears that apical HCO₃⁻ permeability is unmasked by the absence of Cl⁻. This may be due to release of a competitive efflux pathway or due to reduction in basolateral Na⁺-HCO₃⁻ cotransport activity, which is the most likely cause for the reduced basolateral efflux rate (see DISCUSSION).

In an attempt to quantify the effect of the absence of Cl⁻ on HCO₃⁻ flux, Cl⁻-free experiments were also done by using the constant-CO₂ protocol. Figure 3B shows again the higher starting baseline pHᵢ in the absence of Cl⁻. When HCO₃⁻ was removed from the apical side, pHᵢ went down ∼0.04 units (0.02 ± 0.02 units (mean ± SD); n = 7) taking only ∼10 s; then, within another 10 s, pHᵢ was up 0.07 units (0.05 ± 0.03 units (mean ± SD)). Next the pHᵢ decreased to a steady state that was 0.05 units below baseline. The initial rate of pHᵢ decrease (i.e., HCO₃⁻ efflux) was calculated from this last pHᵢ decrease. Table 2 shows that the initial rate was not significantly different from the control rate. When HCO₃⁻-rich Ringer was added back, pHᵢ quickly went down 0.05 units (0.04 ± 0.02 units (mean ± SD)) and then rose 0.10 units (0.09 ± 0.02 units (mean ± SD)). On the basolateral side, the initial decrease in pHᵢ and the steady-state change in pHᵢ were about the same as those for the control; however, there was a significant reduction (~30%) in the initial rate of decrease (Table 2). Thus the results from Fig. 3, A and B, indicate that basolateral HCO₃⁻ flux is slowed in the absence of Cl⁻; however, the net flux appears to be unaffected. The effect of Cl⁻ on apical flux is more complex: an apparent increase in apical flux under constant-pH conditions yet no effect on net efflux under constant-pH conditions.

Apical CO₂ flux. The steady-state alkalinization observed when apical CO₂-HCO₃⁻ is removed may indicate that apical CO₂ efflux is a significant source of HCO₃⁻, which could be generated by carbonic anhydrase at the surface of the apical membrane. Initially, we investigated the possibility of an extracellular CAIV by examining the effects of the polymer-linked CAI and benzolamide on CO₂-HCO₃⁻-induced changes in pHᵢ by using

Table 2. Effects on pHᵢ during HCO₃⁻ removal at constant CO₂

<table>
<thead>
<tr>
<th>Condition</th>
<th>Apical</th>
<th>Basolateral</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial ΔpH</td>
<td>dpH/min</td>
</tr>
<tr>
<td>Control (8)</td>
<td>−0.05 ± 0.01</td>
<td>−0.070 ± 0.03</td>
</tr>
<tr>
<td>No Cl⁻ (7)</td>
<td>−0.05 ± 0.01</td>
<td>−0.073 ± 0.02</td>
</tr>
</tbody>
</table>

Values are means ± SD. dpH/min, rate of change of pHᵢ in pH units per minute. Numbers of experiments are in parentheses. *Significantly different from control (P < 0.05).

Table 3. Apical and basolateral initial dpH/min and HCO₃⁻ and H⁺ fluxes measured under constant-CO₂ and HCO₃⁻-free conditions

<table>
<thead>
<tr>
<th>HCO₃⁻ Rich</th>
<th>HCO₃⁻ Free</th>
</tr>
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<tbody>
<tr>
<td>Apical</td>
<td>Basolateral</td>
</tr>
<tr>
<td>dpH/min</td>
<td>Total (HCO₃⁻ + H⁺) initial flux</td>
</tr>
<tr>
<td>−0.070 ± 0.033</td>
<td>4.0 (8)</td>
</tr>
<tr>
<td>−0.075 ± 0.004</td>
<td>0.75 (6)</td>
</tr>
<tr>
<td>−0.096 ± 0.003</td>
<td>0.98 (6)</td>
</tr>
<tr>
<td>Basolateral</td>
<td>Initial HCO₃⁻ flux</td>
</tr>
<tr>
<td>−0.192 ± 0.047</td>
<td>10.6 (8)</td>
</tr>
<tr>
<td>−0.14 ± 0.03</td>
<td>3.25</td>
</tr>
<tr>
<td>−0.15 ± 0.03</td>
<td>9.62</td>
</tr>
</tbody>
</table>

Values are means ± SD. Flux (mM/min) = dpH/min × β, where β is buffering capacity. Numbers of experiments are in parentheses.
cells cultured on coverslips. Figure 4A shows that CO$_2$-induced acidification was slowed by 40% (45 ± 23% (mean ± SD); paired t-test; P < 0.05; n = 4) and that alkalinization when CO$_2$ was removed was also slowed by 40% (24 ± 12% (mean ± SD); paired t-test; P < 0.05; n = 4) in the presence of 10 µM polymer-CAI. Figure 4B shows that 10 µM benzolamide slowed CO$_2$-induced acidification by 45% (47 ± 13% (mean ± SD); n = 5) and slowed alkalinization on CO$_2$ removal by 35% (30 ± 8% (mean ± SD); n = 5). These results are consistent with the possibility that CO$_2$ flux is influenced by an extracellular carbonic anhydrase.

We next focused on CO$_2$ efflux across the apical membrane using the double-perfusion setup and benzolamide. Figure 5A shows that exposure to 1 µM apical benzolamide caused an immediate 0.05-pH unit drop (0.09 ± 0.04 (mean ± SD); n = 4). Within 5 min, CO$_2$-HCO$_3^-$ was removed from the apical side and the initial alkalinization rate and maximal alkalinization were reduced by 10%. Figure 5B shows a similar experiment with a 10-min exposure to 30 µM benzolamide. The alkalinization rate and maximal alkalinization were reduced by 50%. Note that a brief washout could not reverse the inhibition. Figure 6 summarizes the dose effect of benzolamide on the initial rate and maximal alkalinization of the first test pulse, which occurred within 5 min of exposure to the drug. A longer washout of benzolamide was tried and yielded only limited reversibility. Figure 7A shows that after a 7-min exposure to 30 µM benzolamide, cells were washed for 30 min. Subsequent pulses showed only modest recovery of the initial rate (mean recovery ± SD = 12 ± 5%; n = 4) and amplitude (mean ± SD = 15 ± 13%). Because release of benzolamide during washing appears to be slow, we attempted to reverse its effect by...
exposing cells to exogenous carbonic anhydrase (5 mg/ml). Figure 7B shows significant inhibition during a 7-min exposure to 30 µM benzolamide. Cells were then washed for ~20 min, showing modest recovery of the initial rate (mean recovery ± SD = 6 ± 2.5%; n = 3) and the maximal change in pH (mean ± SD = 5 ± 5%). Cells were then continually exposed to carbonic anhydrase. Subsequent pulses showed that inhibition was partially reversed (59 ± 30% recovery of the initial rate and 85 ± 20% recovery of the amplitude). These results show that extracellular carbonic anhydrase can partially reverse the inhibition of a brief exposure to benzolamide, indicating that benzolamide is acting primarily at the membrane.

Previous studies have indicated that CAIV activity is present in rabbit and mouse corneal endothelia (21, 27). To determine if the CAIV is present in the bovine corneal endothelium, we performed immunoblotting experiments with anti-human CAIV antibodies. The expected range for mammalian CAIV is 39–52 kDa (26). As shown in Fig. 8, the antibody detected a band at 45 kDa in both cultured and freshly isolated bovine corneal endothelia. A minor band at 40 kDa was more prominent in the cultured cells. A band at 27 kDa was observed in both preparations, but was much stronger in the fresh cells.

**DISCUSSION**

Two protocols, constant pH and constant CO₂, were used to examine HCO₃⁻ permeability in corneal endothelial cells. When the constant-pH protocol is used, the relative steady-state pH change after CO₂-HCO₃⁻ removal can give a general impression of the relative apical and basolateral permeabilities. Figure 1A clearly shows greater basolateral HCO₃⁻ efflux. The initial pH₁...
HCO₃⁻ permeability in corneal endothelial cells

constant-CO₂ protocol eliminates the CO₂ fluxes, but membrane potential (4, 11, 14) and that will raise that the brief unilateral exposure to low pH was not needed. The exposure to low bath pH did not increase into the AnoDisc membrane itself, which adds another basal diffusion barrier.

Na⁺ dependency. Previous studies have shown that the corneal endothelial cells possess a Na⁺-dependent, DIDS-sensitive HCO₃⁻ cotransporter that is sensitive to membrane potential (4, 11, 14) and that will raise cytosolic [Na⁺] when exposed to HCO₃⁻ (4). On the basis of the steady-state levels of intracellular and extracellular [HCO₃⁻] and [Na⁺] and the average membrane potential of endothelial cells, we concluded that this cotransporter would have a Na⁺-HCO₃⁻ stoichiometry of ~1:2 and would act as a HCO₃⁻ uptake system (4). Application of the anion transport inhibitor DIDS to the basolateral side produced cell acidification, whereas apical exposure had a more variable effect (6). From these studies, it was concluded that a Na⁺-HCO₃⁻ cotransporter is located on the basolateral side. This conclusion is consistent with our finding, shown in Fig. 2, that basolateral HCO₃⁻ fluxes and not apical fluxes are slowed in low [Na⁺].

Cl⁻ dependency. Cl⁻ has been shown to be essential for fluid transport by the corneal endothelium (31). A recent examination of cultured corneal endothelial cells for Cl⁻/HCO₃⁻ exchange showed little to no anion exchange activity (6), and there is no evidence for other types of Cl⁻-dependent HCO₃⁻ transporters. However, anion channel activity has been demonstrated, and changes, however, are due to CO₂ fluxes, thus making quantitative comparisons of HCO₃⁻ flux difficult. The constant-CO₂ protocol eliminates the CO₂ fluxes, but introduces a low bath pH, for which compensation is needed. The exposure to low bath pH did not increase the rate of BCECF dye leakage from cells, indicating that the brief unilateral exposure to low pH was not detrimental. Using the initial HCO₃⁻ flux yields a basolateral permeability that is about threefold greater than the apical permeability (Table 3). This is probably a low estimate because no compensation was made for the AnoDisc membrane itself, which adds another basal diffusion barrier.

Na⁺ dependency. Previous studies have shown that the corneal endothelial cells possess a Na⁺-dependent, DIDS-sensitive HCO₃⁻ cotransporter that is sensitive to membrane potential (4, 11, 14) and that will raise cytosolic [Na⁺] when exposed to HCO₃⁻ (4). On the basis of the steady-state levels of intracellular and extracellular [HCO₃⁻] and [Na⁺] and the average membrane potential of endothelial cells, we concluded that this cotransporter would have a Na⁺-HCO₃⁻ stoichiometry of ~1:2 and would act as a HCO₃⁻ uptake system (4). Application of the anion transport inhibitor DIDS to the basolateral side produced cell acidification, whereas apical exposure had a more variable effect (6). From these studies, it was concluded that a Na⁺-HCO₃⁻ cotransporter is located on the basolateral side. This conclusion is consistent with our finding, shown in Fig. 2, that basolateral HCO₃⁻ fluxes and not apical fluxes are slowed in low [Na⁺].

Cl⁻ dependency. Cl⁻ has been shown to be essential for fluid transport by the corneal endothelium (31). A recent examination of cultured corneal endothelial cells for Cl⁻/HCO₃⁻ exchange showed little to no anion exchange activity (6), and there is no evidence for other types of Cl⁻-dependent HCO₃⁻ transporters. However, anion channel activity has been demonstrated, and altering bath Cl⁻ concentration ([Cl⁻]) can have profound effects on endothelial membrane potential, which in turn will secondarily affect Na⁺-nHCO₃ cotransport flux. Further, a limited amount of HCO₃⁻ permeability through Cl⁻ channels can be demonstrated in the form of cell alkalization in the absence of Na⁺ in response to cAMP activation of anion permeability (2). Figure 3, A and B, shows that basolateral HCO₃ efflux is slowed in the absence of Cl⁻. This may indicate a Cl⁻ dependency for Na⁺-nHCO₃ cotransport; however previous studies have shown no such dependency in cells grown on coverslips or in freshly isolated cells (4). Further, Tables 1 and 2 indicate that the net change in pH₁ was the same in the absence of Cl⁻ as in the control, arguing that the efflux rate is reduced, but not the net flux. The most likely explanation is that the absence of Cl⁻ slows the dissipation of membrane potential changes during Na⁺-nHCO₃ flux. For example, during Na⁺-nHCO₃ influx the membrane potential hyperpolarizes, slowing further Na⁺-nHCO₃ influx. The hyperpolarization could be partially offset by Cl⁻ efflux through anion channels, because Cl⁻ is above its electrochemical equilibrium (5). However, in the absence of Cl⁻, this depolarization cannot be offset by Cl⁻ and HCO₃⁻ flux is therefore slowed. Further studies are needed to investigate this possibility.

The effect of the absence of Cl⁻ on apical HCO₃⁻ flux was more complex. Under the constant-pH protocol, apical HCO₃⁻ net efflux was greater than control efflux (Fig. 3A; Table 1). This is the opposite of what is expected for an apical Cl⁻/HCO₃⁻ exchanger, but might be explained by the presence of apical anion channels that have some HCO₃⁻ permeability (2, 6, 7). If the inherent permeability of an apical channel to Cl⁻ is higher than its permeability to HCO₃⁻, then this together with the higher bath and cytoplasmic [Cl⁻] under control conditions would limit HCO₃⁻ access to the channel. However, when Cl⁻ is absent, this competition is removed and greater HCO₃⁻ flux can occur. Another possibility, as explained above, is that Na⁺-nHCO₃ cotransport activity is slowed, which allows the limited apical HCO₃⁻ efflux to have a greater effect on pH₁. Under the constant-CO₂ protocol, the initial rate of HCO₃⁻ efflux appeared to be the same as the control rate. However, this is difficult to know for certain because the pH₁ first went down quickly (~0.02 pH units), then up quickly (~+0.05 pH units), then down again at a rate comparable to the control rate. When HCO₃⁻ was added back, pH₁ quickly went down, then rose 0.1 units back to the baseline. These transient changes cannot be due to CO₂ fluxes, but possibly they are due to small membrane potential changes. If apical anion channels with HCO₃⁻ permeability are present, then apical HCO₃⁻ removal would cause a small depolarization, increasing Na⁺-nHCO₃ cotransport flux transiently, which could explain the transient increase in pH₁. When apical HCO₃⁻ is added back, a small hyperpolarization could take place, transiently depressing Na⁺-nHCO₃ cotransport and causing the transient decrease in pH₁. Again, these transients are not observed in the controls because the presence of Cl⁻
would limit the effect of changing apical $[\text{HCO}_3^-]$ on the membrane potential. Clearly, these possibilities will require further testing.

Apical CO$_2$ flux. When CO$_2$-HCO$_3^-$ is removed there is an initial alkalinization due to the rapid efflux of CO$_2$. The rate and extent of this initial response are influenced by the concurrent rate of HCO$_3^-$ efflux. For example, a small alkalinization is observed when basolateral CO$_2$-HCO$_3^-$ is removed because the concurrent HCO$_3^-$ efflux is large. The opposite response, a high sustained alkalinization, is observed on the apical side, indicating that CO$_2$ efflux exceeds HCO$_3^-$ efflux. Thus a significant component of apical HCO$_3^-$ flux may be in the form of CO$_2$, which then could be converted rapidly to CO$_2$ by a CAIV. Inhibiting the conversion of CO$_2$ to HCO$_3^-$ at the membrane can reduce the local CO$_2$ diffusion gradient (16, 25) and thus slow CO$_2$ flux. If CO$_2$ efflux is slowed, then the rate and extent of alkalinization will be reduced because HCO$_3^-$ efflux will have a proportionally greater effect on pH. Both the polymer-linked CAI and benzolamide significantly reduced the rate and extent of pH change in endothelial cells cultured on coverslips when CO$_2$-HCO$_3^-$ was removed (Fig. 4), indicating that CO$_2$ fluxes can be influenced by a CAIV. Similarly, when apical CO$_2$ efflux was examined in the presence of benzolamide, the initial rate and extent of alkalinization were significantly reduced (Figs. 5–7). Furthermore, as previously noted for muscle (25), the reversibility of benzolamide inhibition was small even after 30 min of washout. We used exogenous carbonic anhydrase (5 mg/ml) in an attempt to restore the membrane activity (or possibly scavenge bound benzolamide) as was shown for the kidney (29). Exposure to carbonic anhydrase restored 59% of the initial alkalinization rate and 85% of the total alkalinization (Fig. 7B). These results are consistent with benzolamide, acting primarily at the membrane, inhibiting a carbonic anhydrase that enhances CO$_2$ diffusion across the membrane.

Initial immunofluorescence reports indicated that only CAII and not CAIV was associated with the corneal endothelium (9). However, more recently, strong corneal endothelial apical membrane-associated carbonic anhydrase activity has been demonstrated histochemically in the CAII-deficient mouse (21) and in the normal rabbit (27). We used Western blotting to determine if CAIV immunoreactivity was present in the endothelial cells. Positive bands for both cultured and freshly isolated cells (40–45 kDa) were observed in the correct range (39–52 kDa) for mammalian CAIV (26). A strong band was also seen at 27 kDa for the fresh cells. This may be related to the mechanical scraping used to collect the fresh tissue. Together with some unavoidable proteolysis, the scraping may yield more fragmentation. Taken together, the immunoblot results, polymer-CAI data, benzolamide data, and the demonstrated histochemical activity at the apical membrane strongly suggest that CAIV is present on the apical membrane. Similar types of physiological experiments can be used to determine if a basolateral CAIV could be present and could increase the availability of HCO$_3^-$ for Na$^+$ and CO$_2$ uptake.

In summary, the permeability of the corneal endothelium to HCO$_3^-$ is at least three times greater for the basolateral membrane than for the apical membrane. The Na$^+$-HCO$_3^-$ cotransporter, located on the basolateral membrane, provides robust uptake of HCO$_3^-$. Na$^+$-HCO$_3^-$ cotransport is slowed in the absence of Cl$^-$, most likely because of changes in membrane potential that cannot be dissipated. Because of the low apical HCO$_3^-$ permeability, intracellular [HCO$_3^-$] builds up and HCO$_3^-$ is converted to CO$_2$ by CAII. The accumulated CO$_2$ could augment apical HCO$_3^-$ flux given the presence of CAIV on the apical membrane.

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