CO₂-induced ion and fluid transport in human retinal pigment epithelium

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In the intact eye, the transition from light to dark alters pH, [Ca²⁺], and [K] in the subretinal space (SRS) separating the photoreceptor outer segments and the apical membrane of the retinal pigment epithelium (RPE). In addition to these changes, oxygen consumption in the retina increases with a concomitant release of CO₂ and H₂O into the SRS. The RPE maintains SRS pH and volume homeostasis by transporting these metabolic byproducts to the choroidal blood supply. In vitro, we mimicked the transition from light to dark by increasing apical bath CO₂ from 5 to 15%; this maneuver decreased cell pH from 7.37 ± 0.05 to 7.14 ± 0.06 (n = 13). Our analysis of native and cultured fetal human RPE shows that the apical membrane is significantly more permeable (>10-fold; n = 7) to CO₂ than the basolateral membrane, perhaps due to its larger exposed surface area. The limited CO₂ diffusion at the basolateral membrane promotes carbonic anhydrase–mediated HCO₃⁻ transport by a basolateral membrane Na+/HCO₃⁻ exchanger and increased net steady-state fluid absorption from 2.8 ± 1.6 to 6.7 ± 2.3 µl × cm⁻² × hr⁻¹ (n = 5; P < 0.05). The present experiments show how the RPE can accommodate the increased retinal production of CO₂ and H₂O in the dark, thus preventing acidosis in the SRS. This homeostatic process would preserve the close anatomical relationship between photoreceptor outer segments and RPE in the dark and light, thus protecting the health of the photoreceptors.

The RPE expresses several different HCO₃⁻ transport proteins at the apical and basolateral membranes as illustrated in Fig. 1 (Hughes et al., 1989; Kenyon et al., 1997). As in other epithelia, the activities of these transporters in human RPE can be facilitated by carbonic anhydrases (CAs) expressed in the cytosol (CA II), on the apical membrane (CA IV, CA IX, CA XII, and CA XIV), or on the basolateral membrane (CA IX) (Casey, 2006; Purkerson and Schwartz, 2007; Zhi, C.G., F.E. Wang, T. Banzon, S. Jalickee, R. Fariss, A. Maminishkis, and S.S. Miller, 2007. Membrane-Bound Carbonic Anhydrases in Human Fetal Retinal Pigment Epithelial Cells (hfRPE)). The removal of CO₂ from the SRS can be achieved by diffusion across the RPE or by conversion to HCO₃⁻ using the catalytic activity of CAs for transmembrane transport via HCO₃⁻ transporters, or both. CO₂ diffusion into the RPE drives CA-mediated formation of HCO₃⁻ and protons that acidify the cell. Transporter-mediated HCO₃⁻ entry mitigates this acidification and helps maintain pH homeostasis in the RPE (Kenyon et al., 1997) and in the SRS (Borgula et al., 1989).
The close physiological relationship between HCO$_3^-$ and fluid transport was demonstrated in frog RPE, where steady-state fluid absorption was reduced by ≈70% after the removal of HCO$_3^-$ from both bathing solutions (Hughes et al., 1984). In cultured human fetal RPE (hfRPE), dorzolamide (DZA; nonspecific CA inhibitor) decreases steady-state fluid absorption (Zhi, C.G., F.E. Wang, T. Banzon, S. Jalickee, R. Fariss, A. Maminishkis, and S.S. Miller. 2007. Membrane-Bound Carbonic Anhydrases in Human Fetal Retinal Pigment Epithelial Cells (hfRPE), suggesting the involvement of CA-mediated HCO$_3^-$ transport in fluid transport. These in vitro results are in contrast to in vivo animal studies that suggest that acetazolamide (nonspecific CA inhibitor) enhances retinal adhesion and SRS fluid clearance (Kita and Marmor, 1992; Wolfensberger et al., 2000). In addition, clinical studies showed that some patients with macular edema respond to acetazolamide treatment by increasing SRS fluid clearance (Cox et al., 1988; Fishman et al., 1989), but the underlying physiological mechanisms remain to be determined.

In vivo studies of retinal metabolism indicate an increase in SRS CO$_2$ level after the transition from light to dark (Wangsa-Wirawan and Linsenmeier, 2003). The present in vitro study investigates how an increase in apical CO$_2$ level can alter the activities of apical and basolateral membrane ion transporters to drive solute-linked fluid transport from the SRS to the choroid. We show that the RPE basolateral membrane has a significantly lower CO$_2$ permeability than the apical membrane. Therefore, an increase in apical CO$_2$ would cause an accumulation of intracellular CO$_2$ that is subsequently converted into HCO$_3^-$ by the catalytic activity of CA II; this in turn stimulates HCO$_3^-$ efflux at the basolateral membrane. We also show that the increase in apical CO$_2$ affects several ion transporters at the apical and basolateral membranes that would lead to a net increase in Na, Cl, and HCO$_3^-$ absorption and active solute-linked fluid transport across the RPE. The transition from light to dark is accompanied by an increase in photoreceptor metabolism. In vivo, the RPE can respond to the increased metabolic load by increasing the clearance of CO$_2$/HCO$_3^-$ from the SRS. This would help protect and maintain the health and integrity of the retina–RPE complex by preventing acidosis in the SRS and an abnormal separation of retina and RPE.

**MATERIALS AND METHODS**

**hfRPE and bovine RPE preparation, and hfRPE culture**

The methods used to extract intact native hfRPE monolayers and grow hfRPE cultures have been described previously (Maminishkis et al., 2006). Bovine RPE choroid was excised from fresh bovine eyes as described previously (Kenyon et al., 1997). The research presented in this study was performed in accordance with the tenets of the Declaration of Helsinki and the National Institutes of Health (NIH) institutional review board.

**Intracellular pH (pH$_i$) measurements**

Cultured hfRPE monolayer grown on a porous polyester membrane transwell filter was incubated at room temperature and 5% CO$_2$ for 30 min in Ringer solution containing 8 µM BCECF-AM (Invitrogen) pH-sensitive dye, 0.1% DMSO, and 0.01% pluronic acid. After incubation with BCECF-AM, the tissue was incubated in control (5% CO$_2$) Ringer for another 30 min before mounting on a mesh (250 µM) in a modified Ussing chamber (exposed surface area of 7.1 mm$^2$). The Ussing chamber was mounted on the stage of an axiovert-200 microscope (Carl Zeiss, Inc.) equipped with a 20x plan-neofluar objective. The hfRPE was continuously perfused with Ringer solution (equilibrated with 5% CO$_2$ at 36.5°C) at a flow rate of 2 ml × min$^{-1}$. Excitation photons (440/480 nm) were generated by a xenon light source, and the specific wavelengths were selected with a monochromator (Polychrome IV; Photonics). The emission fluorescence signals were captured with a photomultiplier tube (Thorn EMI). pH$_i$ calibrations were performed by perfusing high-K calibration solutions (at pH 6.8, 7.2, and 7.6) containing 20 µM nigericin into both solution baths. The average calibration parameters were used to linearly correlate fluorescence intensity to pH$_i$ for all pH-imaging experiments. We estimated the rate of dye loss from the RPE by calculating the percentage rate of loss of intracellular BCECF ($k_{440}$) at intervals of 0.5 min with the following equation (Bevensee et al., 1995):

$$k_{440} = \frac{I_{440}^{pre} - I_{440}^{post}}{\Delta t \times I_{440}^{pre}} \times 100,$$

where ($I_{440}^{pre} - I_{440}^{post}$) is the difference in BCECF fluorescence intensity between Δt = 0.5-min intervals. From 30 experiments, the average $k_{440}$ was −2.21 ± 0.50% × min$^{-1}$.

**Intracellular Na measurements**

Cultured hfRPE monolayers were incubated in control Ringer containing 22 µM SBFI-AM (Invitrogen), 0.18% DMSO, and 0.02% pluronic acid for 1 h at room temperature and 5% CO$_2$. After incubating with SBFI-AM, the hfRPE was mounted onto the modified Ussing chamber. Fluorescent signals corresponding to intracellular Na concentration ([Na$_i$]) were obtained by alternat- ing the excitation wavelength between 340 and 380 nm. A three-point calibration was performed at the end of the experiment (Harootunian et al., 1989). In brief, high K$^+$ (85-mM) calibration solutions containing 0, 10, or 30 mM [Na] and 10 µm gramicidin were perfused simultaneously to both the apical and basal baths. The fluorescence ratio for each [Na]$_i$ was used to obtain a calibration curve for the experiment.

**Transepithelial potential (TEP) and total tissue resistance (R$_T$) measurements**

TEP was measured with a pair of calomel electrodes in series with Ringer solution agar (4% wt/vol) bridges placed in the apical and basal baths of the Ussing chamber. The electrophysiology of the RPE was described previously in detail (Hughes et al., 1998). All TEP recordings are moving averages of 3 s. The R$_T$ was calculated from Ohm’s law,

$$R_T = \frac{\Delta TEP \times Area}{Current},$$

where ΔTEP is the TEP deflection when a 3-µA Current was passed across the hfRPE monolayer (once every minute) with Ag/AgCl electrodes, and Area is the cross-sectional surface area of the RPE. All hfRPE culture preparations had an R$_T$ ≥ 200 Ω × cm$^2$.

**Estimating intrinsic buffering capacity**

The intrinsic buffering capacity (β, mM/pH units) of the hfRPE cells was determined by using a previously described method.
(Weintraub and Machen, 1989) and was fitted to a third-order polynomial: \( \beta = -93.4 \, pH^3 + 2150.4 \, pH^2 + 16483.6 \, pH + 42065.6 \) for \( pH < 7.35 \), and for \( 7.35 \leq pH \leq 7.7 \), \( \beta = 9.06 \). The total buffering capacity (\( \beta_{total} \)) was then calculated with the equation, \( \beta_{total} = \beta + \beta_{\text{HCO}_3} = \beta + 2.5[H\text{CO}_3^-] \). \( [\text{HCO}_3^-] \) was estimated from the Henderson-Hasselbalch equation with the assumption that intracellular CO\(_2\) level is 5%. \( H^+ \) flux was determined by multiplying \( \beta_{total} \) by an estimate of the initial \( dpH/dt \) determined from the \( pH \) response.

**Steady-state fluid transport measurements**

hfRPE monolayers cultured on porous membrane were mounted in a modified Ussing chamber, and the rate of transepithelial water flow (steady-state fluid absorption rate [\( J_V \)]) was measured using a refined capacitance probe technique as described previously (Hughes et al., 1984; Maminishkis et al., 2006). The TEP and \( R_t \) of the hfRPE monolayer were measured by injecting bipolar currents via Ag/AgCl pellet electrodes that were connected to the solution baths with agar layer. The steady-state fluid absorption rate \( J_V \) was calculated with the Henderson-Hasselbalch equation with the assumption that intracellular CO\(_2\) level is 5%. 

**RESULTS**

Apical or basal CO\(_2\)-induced pH responses in hfRPE

Fig. 2 A shows that increasing CO\(_2\) from 5 to 13% in the apical or basal baths acidified the hfRPE by ≈0.25 and ≈0.04, respectively. Data from 13 experiments show that 13% apical CO\(_2\) decreased \( pH \) by 0.25 ± 0.03, from 7.37 ± 0.05 to 7.14 ± 0.06; in contrast, the 13% basal bath CO\(_2\)-induced acidification (\( \Delta pH = 0.03 \pm 0.01 \)) was almost eightfold smaller. Similarly in Fig. 2 B, decreasing CO\(_2\) from 5 to 1% in the apical or basal baths alkalinized the hfRPE by ≈0.35 and ≈0.03, respectively. In four experiments, 1% apical or basal bath CO\(_2\) alkalinized the cell by 0.41 ± 0.05 and 0.03 ± 0.03, respectively. The CO\(_2\)-induced changes in TEP and \( R_t \) were relatively small and not significant statistically. In freshly isolated native hfRPE preparations, 13% apical CO\(_2\) also caused significantly larger acidification (\( \Delta pH = 0.29 \pm 0.04 \)) than 13% basal CO\(_2\) (\( \Delta pH = 0.03 \pm 0.02; n = 4 \)). This difference in the apical/basolateral CO\(_2\)-induced \( pH \) response is even more pronounced in bovine RPE choroid preparations. No \( pH \) response to 13% basal CO\(_2\) was
observed, but a significant acidification was produced by 13% apical CO\(_2\) (\(\Delta pHi = 0.39 \pm 0.09\); \(n = 6\)).

Tight junctions physically separate the RPE apical and basolateral membranes. Disruption of this barrier, by removing bath Ca\(^{2+}\) and Mg\(^{2+}\), could provide a paracellular pathway for movement of CO\(_2\) from the basal bath to the apical membrane. To test this notion, we increased basal bath CO\(_2\) (from 5 to 13%) 15 min after the removal of Ca\(^{2+}\) and Mg\(^{2+}\) from both bathing solutions. Upon Ca\(^{2+}\) and Mg\(^{2+}\) removal, \(R_t\) rapidly decreased at a rate of 17.3 \(\pm\) 7.3 \(\Omega\) \(\times\) cm\(^2\) \(\times\) min\(^{-1}\) (\(n = 5\)). However, after 15 min in Ca\(^{2+}\)- and Mg\(^{2+}\)-free Ringer, the 13% basal bath CO\(_2\) induced acidification was not significantly different than control (0.02 \(\pm\) 0.01 vs. 0.02 \(\pm\) 0.01; \(n = 5\); \(P > 0.05\)), suggesting that basal bath CO\(_2\) does not enter the apical membrane by diffusing across the tight junctions. When Ca\(^{2+}\) and Mg\(^{2+}\) were restored to the solution baths, \(R_t\) slowly recovered to a rate of 11.9 \(\pm\) 4.0 \(\Omega\) \(\times\) cm\(^2\) \(\times\) min\(^{-1}\), but the recovery rate decreases significantly after \(\approx\)15–20 min, and \(R_t\) recovered by only \(\approx\)40%.

**Apical membrane electrogenic Na/2HCO\(_3\) cotransporter in hRPE**

We tested apical Na/2HCO\(_3\) cotransport activity by adding apical DIDS and comparing the resultant pH\(_i\) and TEP responses in control Ringer (26.2 mM HCO\(_3\)) versus low HCO\(_3\) Ringer (2.62 mM HCO\(_3\)) in the apical bath (Fig. 3 A). Data from six experiments showed that in control Ringer, apical DIDS acidified the cell by 0.05 \(\pm\) 0.02 and decreased TEP by 1.59 \(\pm\) 0.63 mV, whereas adding apical DIDS in low apical bath [HCO\(_3\)] alkalinized the cell by 0.04 \(\pm\) 0.01 and transiently increased TEP by 0.30 \(\pm\) 0.15 mV; these responses are reversible. The apical DIDS-induced pHi and TEP responses are consistent with the inhibition of an electrogenic HCO\(_3\)-dependent mechanism, whose activity can be reversed by a 10-fold reduction in apical bath [HCO\(_3\)].

To evaluate the potency of 0.5 mM of apical DIDS in the inhibition of the apical membrane Na/2HCO\(_3\) cotransporter, we decreased apical bath [HCO\(_3\)] 10-fold and compared the resultant pH\(_i\) and TEP responses in the presence or absence of apical DIDS (Fig. 3 B). In three experiments, DIDS reduced the apical bath \(\Delta[HCO_3^-]\)-induced TEP response by sevenfold, from 2.1 \(\pm\) 0.2 to 0.3 \(\pm\) 0.2 mV (\(P < 0.01\)). The effect of DIDS on the TEP response was partially reversible after a 5-min washout (\(\Delta\text{TEP} = 1.28 \pm 0.22\) mV). This result suggests that apical DIDS almost completely blocked the apical membrane Na/2HCO\(_3\) cotransporter activity. Surprisingly, the apical bath \(\Delta[HCO_3^-]\)-induced acidification (\(\Delta pHi = 0.10 \pm 0.02\)) was
not significantly affected by DIDS (ΔpHi = 0.09 ± 0.01; n = 3; P > 0.05), suggesting the presence of an electro-neutral and DIDS-insensitive HCO3 transporter at the apical membrane (see Discussion).

Apical bath CO2 may be converted into HCO3 by transmembrane CAs on the apical membrane surface, thus stimulating apical Na/2HCO3 cotransport activity. Therefore, we tested the effect of altering apical bath CO2 on apical Na/2HCO3 cotransport activity by comparing apical DIDS (0.5 mM)-induced pHi and TEP responses in control Ringer (5% CO2) to that in 1 or 13% CO2-equilibrated Ringer (Fig. 4 A). In four experiments, apical DIDS-induced pHi and TEP responses in control Ringer (ΔpHi = 0.05 ± 0.02; ΔTEP = 1.52 ± 0.33 mV) were the same as that in 13% CO2-equilibrated Ringer (ΔpHi = 0.05 ± 0.02; ΔTEP = 1.57 ± 0.67 mV; P > 0.05). Similarly, the apical DIDS-induced pHi and TEP responses in control Ringer (ΔpHi = 0.05 ± 0.02; ΔTEP = 1.66 ± 0.59 mV) were the same as that in 1% CO2-equilibrated Ringer (ΔpHi = 0.06 ± 0.02; ΔTEP = 1.31 ± 0.78 mV; n = 5; P > 0.05). To further test the pHi sensitivity of the apical membrane Na/2HCO3 cotransporter, we perfused 13% CO2-equilibrated Ringer into the apical bath in the presence or absence of 0.5 mM of apical DIDS (Fig. 4 B). If increasing apical bath CO2 increases apical Na/2HCO3 cotransport activity, 13% apical CO2 should cause a larger acidification in the presence of apical DIDS compared with control. However, in the presence of apical DIDS, the 13% CO2-induced acidification (ΔpHi = 0.22 ± 0.03) was the same as control (ΔpHi = 0.22 ± 0.02; n = 4; P > 0.05). Collectively, these results lead to the conclusion that apical Na/2HCO3 cotransport activity is not affected by apical CO2-induced alterations in pHi.

CA II catalyzes the interconversion of CO2 and HCO3 in the cytosol, and CA inhibition by DZA may affect apical Na/2HCO3 cotransport activity. We tested this notion by decreasing apical bath [HCO3] (10-fold) and compared the resultant pHi and TEP responses in the presence of 250 μM of apical DZA to that in control (Fig. 5). In five experiments, DZA decreased apical bath Δ[HCO3]-induced TEP response by 60% (from 2.25 ± 0.81 to 0.89 ± 0.29 mV; P < 0.01) and increased the pHi response from 0.11 ± 0.01 to 0.19 ± 0.01 (P < 0.01). The effect of DZA on the pHi and TEP responses was partially
state pH in 5 and 13% apical bath CO₂ differed significantly, which required us to use the total buffering capacity of the hRPE to calculate equivalent H⁺ fluxes. In the presence of 13% CO₂-equilibrated Ringer in the apical bath, the basal bath Δ[Cl⁻]-induced acidification was larger in the presence of DZA because CA II inhibition reduces intracellular CO₂/HCO₃ buffering capacity, which compromises the ability of the RPE to buffer the acidification caused by HCO₃ efflux from the apical membrane.

Basolateral membrane Cl⁻/HCO₃ exchanger activity in hRPE
To assess basolateral membrane Cl⁻/HCO₃ exchanger activity, basal bath [Cl⁻] was reduced from 126 to 1 mM, which alkalinized the cell by ≈0.22 (Fig. 6). In three experiments, this alkalinization (∆pHᵢ = 0.18 ± 0.05) was abolished by 0.5 mM of basal DIDS (∆pHᵢ = 0.02 ± 0.01; n = 3; P < 0.05), but this effect was not reversible. Next, we tested the pHᵢ dependence of the Cl⁻/HCO₃ exchanger by comparing the basal bath Δ[Cl⁻]-induced pHᵢ response in 5 versus 13% apical bath CO₂ (Fig. 7 A). The steady-state pHᵢ in 5 and 13% apical bath CO₂ differed significantly, which required us to use the total buffering capacity of the hRPE to calculate equivalent H⁺ fluxes. In the presence of 13% CO₂-equilibrated Ringer in the apical bath, the basal bath Δ[Cl⁻]-induced change in pHᵢ flux was 2.3 ± 1.0 mM × min⁻¹, approximately fourfold smaller than the H⁺ flux in 5% CO₂ (9.0 ± 4.5 mM × min⁻¹; n = 7; P < 0.01); this effect was fully reversible. Fig. 7 B summarizes a parallel experiment in which basal bath [Cl⁻] was reduced in the presence of 1% CO₂-equilibrated Ringer in the apical bath. In this case, the basal bath Δ[Cl⁻]-induced acidification was larger in the presence of DZA because CA II inhibition reduces intracellular CO₂/HCO₃ buffering capacity, which compromises the ability of the RPE to buffer the acidification caused by HCO₃ efflux from the apical membrane.
solution baths (Fig. 9). In three experiments, Na removal reduced the basal bath $\Delta$[HCO$_3^-$]-induced pHi response by more than twofold ($\Delta$pHi = 0.08 ± 0.02) compared with control ($\Delta$pHi = 0.21 ± 0.04; P < 0.05). In addition, the basal bath $\Delta$[HCO$_3^-$]-induced TEP response ($\Delta$TEP = 1.01 ± 0.21 mV) was essentially abolished in the absence of Na ($\Delta$TEP = 0.04 ± 0.07 mV; P < 0.05), and this effect was reversible. This indicates that although reducing basal bath [HCO$_3^-$] causes HCO$_3^-$ efflux via both Cl/HCO$_3^-$ exchanger and Na/nHCO$_3^-$ cotransporter, the TEP response corresponds specifically to Na/nHCO$_3^-$ cotransporter activity due to its electrogenicity and Na dependence. This allows one to distinguish the activity of the Na/nHCO$_3^-$ cotransporter from that of the Cl/HCO$_3^-$ exchanger.

Basolateral Na/nHCO$_3^-$ cotransport: dependence on apical Na-linked transporters

We expected Na-linked transporters at the apical membrane (Fig. 1) to provide substrate that would help drive

Figure 5. CA II dependence of apical membrane Na/2HCO$_3^-$ cotransporter. Low HCO$_3^-$ (2.62 mM) Ringer was perfused into the apical bath to record the initial control response. This maneuver was repeated in the presence of 250 µM of apical DZA. After DZA washout, low apical bath [HCO$_3^-$]-induced control response was obtained. Solid bars above the graphs represent solution changes from control Ringer as described in the legend to Fig. 2.

Figure 6. DIDS sensitivity of basolateral membrane Cl/HCO$_3^-$ exchanger. Low (1 mM) CI Ringer was perfused into the apical bath to record the initial control response. This maneuver was repeated in the presence of 0.5 mM of apical DIDS. After DIDS washout, the low basal bath [Cl]-induced control response was obtained. Solid bars above the graphs represent solution changes from control Ringer as described in the legend to Fig. 2.
the outward transport of Na/nHCO₃ at the basolateral membrane. To test this notion, we first inhibited the apical membrane Na/H exchanger with 1 mM amiloride and observed its effect on the pHı and TEP responses caused by a 10-fold reduction in basal bath [HCO₃⁻]. In five experiments, apical amiloride did not affect basal bath ∆[HCO₃⁻]-induced TEP response (ΔTEP = 1.28 ± 0.58 vs. 1.24 ± 0.50 mV; P > 0.05), indicating that the apical membrane Na/H exchanger does not provide substrate for basolateral Na/nHCO₃ cotransport activity. On the other hand, the basal bath ∆[HCO₃⁻]-induced acidification and H⁺ flux was larger in the presence of apical amiloride (ΔpHı = 0.28 ± 0.05; H⁺ flux = 8.3 ± 1.6 mM × min⁻¹) compared with control (ΔpHı = 0.22 ± 0.03; H⁺ flux = 7.1 ± 1.3 mM × min⁻¹; n = 5; P < 0.05). This observation indicates that the Na/H exchanger normally acts to buffer cell acidification produced by HCO₃⁻ efflux from the basolateral membrane.

Next, we inhibited the Na/K/2Cl cotransporter with 200 µM of apical bumetanide, which did not affect the basal bath ∆[HCO₃⁻]-induced pHı response (ΔpHı = 0.20 ± 0.05 vs. 0.21 ± 0.03 ; n = 4; P > 0.05) and TEP response (ΔTEP = 0.89 ± 0.28 vs. 0.80 ± 0.18 mV; n = 6; P > 0.05). This lack of effect suggests that Na entry via the Na/K/2Cl cotransporter does not contribute significantly to basolateral Na/nHCO₃ cotransport activity. We also evaluated the effect of Na extrusion by the apical membrane Na/K ATPase on the activity of the basolateral membrane Na/nHCO₃ cotransporter. Adding 200 µM ouabain into the apical bath caused an acute TEP decrease (ΔTEP = 0.55 ± 0.47 mV; n = 5), as expected from inhibition of the Na/K ATPase. However, apical ouabain did not affect the basal bath ∆[HCO₃⁻]-induced pHı response (ΔpHı = 0.18 ± 0.02 vs. 0.20 ± 0.02; n = 3; P > 0.05) and TEP response (ΔTEP = 1.26 ± 0.59 vs. 1.17 ± 0.49 mV; n = 5; P > 0.05), indicating that Na extrusion by the Na/K ATPase does not reduce or limit basolateral Na/nHCO₃ cotransport activity.

Figure 7. pH sensitivity of basolateral membrane Cl/HCO₃ exchanger. Low (1 mM) Cl Ringer was perfused into the apical bath to record the initial control response. This maneuver was then repeated in (A) 13% or (B) 1% apical bath CO₂. After returning to control Ringer, low basal bath [Cl⁻]-induced control response was obtained. Solid bars above the graphs represent solution changes from control Ringer as described in the legend to Fig. 2.
suggesting that inhibiting apical \( \text{HCO}_3^- \) entry via the \( \text{Na}/2\text{HCO}_3^- \) cotransporter reduces the \( \text{HCO}_3^- \) supply that drives basolateral \( \text{Na}/\text{nHCO}_3^- \) cotransport. However, apical DIDS increased the basal bath \( \Delta[\text{HCO}_3^-] \) -induced acidification from 0.19 ± 0.03 to 0.23 ± 0.03 (P < 0.05; \( n = 7 \)), and \( \text{H}^+ \) flux from 5.0 ± 1.1 to 6.0 ± 1.1 mM × min\(^{-1} \) (P < 0.05; \( n = 7 \)). This observation suggests that normally, apical \( \text{HCO}_3^- \) entry via the \( \text{Na}/2\text{HCO}_3^- \) cotransporter is a buffer that counteracts the acidification caused by \( \text{HCO}_3^- \) efflux from the basolateral membrane.

Apical \( \text{CO}_2 \) induced changes in basolateral membrane \( \text{Na}/\text{nHCO}_3^- \) cotransporter activity

Optimal \( \text{Na}/\text{nHCO}_3^- \) cotransporter activity requires a steady supply of \( \text{HCO}_3^- \), and the inhibition of CA II with DZA should reduce \( \text{Na}/\text{nHCO}_3^- \) cotransport across the basolateral membrane. This notion was tested by reducing basal bath \( [\text{HCO}_3^-] \) 10-fold in the presence of 250 \( \mu \text{M} \) DZA in the basal bath (Fig. 11). In a total of nine experiments, DZA reduced the basal bath \( \Delta[\text{HCO}_3^-] \) -induced TEP response by ≈30% (from \( \Delta \text{TEP} = 1.44 \pm 0.80 \) to \( 0.98 \pm 0.50 \text{ mV}; P < 0.01 \)), suggesting that CA II inhibition reduces basolateral \( \text{Na}/\text{nHCO}_3^- \) cotransport activity. In contrast, DZA did not affect the basal bath \( \Delta[\text{HCO}_3^-] \) -induced acidification (\( \Delta \text{pH} = 0.18 \pm 0.03 \)) compared with control (\( \Delta \text{pH} = 0.17 \pm 0.01; n = 4; P > 0.05 \)). This lack of effect probably occurred because the DZA-induced reduction in basolateral membrane \( \text{HCO}_3^- \) efflux is counteracted by a concomitant reduction in intracellular \( \text{CO}_2/\text{HCO}_3^- \) buffering capacity.

A 13% \( \text{CO}_2 \) load applied to the apical membrane should increase basolateral membrane \( \text{Na}/\text{nHCO}_3^- \) cotransporter activity by shifting intracellular \( \text{CO}_2/\text{HCO}_3^- \) equilibrium toward the formation of \( \text{HCO}_3^- \) (facilitated by CA II activity). To test this hypothesis, we made a 10-fold reduction in basal bath \( [\text{HCO}_3^-] \) and compared the resultant \( \text{pH}_i \) and TEP responses in 5 versus 13% apical bath \( \text{CO}_2 \) (Fig. 12 A). With 13% apical bath \( \text{CO}_2 \), the basal bath \( \Delta[\text{HCO}_3^-] \) -induced TEP response (\( \Delta \text{TEP} = 1.35 \pm 0.78 \text{ mV} \)) was ≈20% higher than control (\( \Delta \text{TEP} = 1.11 \pm 0.67 \text{ mV}; n = 9; P < 0.05 \)). However, there was no change in basal bath \( \Delta[\text{HCO}_3^-] \) -induced \( \text{pH}_i \) response in

**Figure 8.** DIDS sensitivity of basolateral membrane \( \text{Na}/\text{nHCO}_3^- \) cotransporter. Low \( \text{HCO}_3^- \) (2.62 mM) Ringer was perfused into the basal bath to record the \( \text{pH}_i \), TEP, and \( \text{R}_\text{f} \) responses, first in the absence and then in the presence of 0.5 mM of basal DIDS. After DIDS washout, low basal bath \( [\text{HCO}_3^-] \) -induced control response was obtained. Solid bars above the graphs represent solution changes from control Ringer as described in the legend to Fig. 2.

**Figure 9.** Na dependence of basolateral membrane \( \text{Na}/\text{nHCO}_3^- \) cotransporter. Low \( \text{HCO}_3^- \) (2.62 mM) Ringer was perfused into the basal bath to record the initial control response, and this maneuver was repeated in the absence of Na in both the apical and basal baths. After returning to control Ringer, low basal bath \( [\text{HCO}_3^-] \) -induced control response was obtained. Solid bars above the graphs represent solution changes from control Ringer as described in the legend to Fig. 2.
the presence of 13% apical bath CO₂ (H⁺ flux = 6.0 ± 1.3 mM × min⁻¹) compared with control (H⁺ flux = 6.1 ± 2.3 mM × min⁻¹; n = 9; P > 0.05). Presumably, 13% apical CO₂ did not significantly alter H⁺ flux caused by basal bath Δ[HCO₃] because the CO₂-induced increase in HCO₃ efflux via the Na/nHCO₃ cotransporter was offset by concomitant inhibition of the pH₃-sensitive Cl/HCO₃ exchanger, thus producing no observable change in net H⁺ flux.

In similar experiments, we reduced basal bath [HCO₃] in 1% apical bath CO₂ (Fig. 12 B). This maneuver should reduce free HCO₃ in the cell and subsequently decrease basolateral membrane Na/nHCO₃ cotransport activity. With 1% apical bath CO₂, the basal bath Δ[HCO₃]-induced TEP response (ΔTEP = 0.37 ± 0.32 mV) was more than fivefold smaller than control (ΔTEP = 1.26 ± 0.74 mV; n = 5, P < 0.01). This result indicates that intracellular CO₂ is a significant source of HCO₃ supply for the basolateral membrane Na/nHCO₃ cotransporter. 1% apical CO₂ increased the basal bath Δ[HCO₃]-induced equivalent H⁺ flux from 7.2 ± 1.8 to 10.7 ± 1.9 mM × min⁻¹ (n = 5; P = 0.04). The H⁺ flux in the presence of 1% apical bath CO₂ was larger probably because the resultant alkalinization activated the Cl/HCO₃ exchanger more than the reduction in Na/nHCO₃ cotransport activity.

We showed that 13% apical CO₂ increased the basal bath Δ[HCO₃]-induced TEP response (Fig. 12 A), suggesting that 13% apical CO₂ activates the basolateral membrane Na/nHCO₃ cotransporter, which should decrease [Na]. However, 13% apical CO₂ increased [Na] from 15.7 ± 3.3 to 24.0 ± 5.3 mM (n = 6; P < 0.05). This suggests that one or more Na entry pathways are affected by 13% apical CO₂. To test whether 13% apical CO₂-induced acidification activated the Na/H exchanger, we compared the effect of 1 mM amiloride on the steady-state pHᵢ of the RPE in control Ringer (5% CO₂) to that in 13% CO₂-equilibrated Ringer. In three experiments, adding 1 mM amiloride into the apical bath did not cause any change in steady-state pHᵢ flux. This suggests that one or more Na entry pathways are affected by 13% apical CO₂.
The model in Fig. 1 predicts that 13% apical CO$_2$ would increase net Na, Cl, and HCO$_3$ absorption, producing an increase in $J_V$ across the RPE. Fig. 13 A shows that 13% CO$_2$ increased $J_V$ from $\approx$5 to 9 µl × cm$^{-2}$ × hr$^{-1}$, and Fig. 13 B shows that 1% CO$_2$ decreased $J_V$ from $\approx$7 to 2.5 µl × cm$^{-2}$ × hr$^{-1}$. In four experiments, increasing CO$_2$ from 5 to 13% in both solution baths increased $J_V$ by more than twofold (from $2.8 \pm 1.6$ to $6.7 \pm 2.3$ µl × cm$^{-2}$ × hr$^{-1}$; $n = 5$; $P < 0.05$). In another set of experiments, decreasing CO$_2$ from 5 to 1% in both solution baths decreased steady-state fluid absorption by $\approx$60% (from $8.8 \pm 3.9$ to $3.4 \pm 1.1$ µl × cm$^{-2}$ × hr$^{-1}$; $n = 4$; $P < 0.05$).

**DISCUSSION**

The retinal photoreceptors are among the most metabolically active cells in the body (Winkler et al., 2008). All of the oxygen used by the photoreceptors is supplied by the choroidal blood supply, as indicated by the steep decrease of PO$_2$ from $\approx$70 mm Hg in the choriocapillaris to $\approx$0 mm Hg at the photoreceptor cilium, which...
This CO2 load would be mainly dissipated by the choroidal blood supply because it features a very high flow rate (Alm and Bill, 1987) and a relatively short diffusion path from the inner segments (Oyster, 1999). We perfuse 13% CO2-equilibrated Ringer to the hfRPE apical membrane to mimic our estimate of the in vivo increase in CO2 that occurs in the SRS after the transition from light to dark (from 5 to 10 ± 3%; see Appendix 1). The present results show that in hfRPE, CO2 is more effectively transported across the apical membrane compared with the forms the junction between inner and outer segments (Birol et al., 2007) and is the site of the most dense mitochondrial packing in the retina (Stone et al., 2008). Oxygen consumption at this location increases ≈1.5–3 times after dark adaptation, mainly from increased ATP consumption needed to maintain the photoreceptor dark current (Wangsa-Wirawan and Linsenmeier, 2003). The increase in retinal oxygen consumption leads to a proportionate increase in CO2 production and its release into the SRS.

| Inhibitor/condition | 2.62 mM basal bath [HCO3−]−induced pH response |
|---------------------|------------------------------------------------|
| Apical              | Basal                                           |
| DIDS                | △pH    | Control | w/ inhibitor | Recovery | *P* | n  |
|                     |       | ∆pH     |            |          |     |    |
|                     |       | −0.20 ± 0.04 | −0.09 ± 0.05 | −0.12 ± 0.03 | <0.05 | 5  |
|                     | H+ flux | −6.5 ± 1.2 | −3.6 ± 2.7 | −4.3 ± 1.3 | <0.05 | 5  |
| Na-free             | Basal | DIDS     | Na-free     |
|                     | △pH    | −0.21 ± 0.04 | −0.08 ± 0.01 | −0.14 ± 0.02 | <0.05 | 5  |
|                     | H+ flux | −7.2 ± 1.7 | −2.8 ± 0.7 | −4.9 ± 0.8 | <0.05 | 5  |
| amiloride           | △pH    | −0.22 ± 0.03 | −0.28 ± 0.05 | −0.21 ± 0.04 | <0.05 | 5  |
|                     | H+ flux | −7.1 ± 1.3 | −8.3 ± 1.6 | −5.7 ± 1.4 | <0.05 | 5  |
| bumetanide          | △pH    | −0.20 ± 0.03 | −0.21 ± 0.03 | −0.22 ± 0.03 | <0.05 | 4  |
| ouabain             | △pH    | −0.18 ± 0.02 | −0.20 ± 0.02 | −0.23 ± 0.03 | <0.05 | 3  |
|                     | H+ flux | −5.1 ± 2.1 | −5.4 ± 3.2 | −5.7 ± 2.7 | <0.05 | 3  |
| DIDS                | △pH    | −0.19 ± 0.03 | −0.23 ± 0.03 | −0.21 ± 0.01 | <0.05 | 7  |
|                     | H+ flux | −5.0 ± 1.1 | −6.0 ± 1.1 | −5.3 ± 0.9 | <0.05 | 7  |
| DZA                 | △pH    | −0.17 ± 0.01 | −0.18 ± 0.03 | −0.18 ± 0.02 | <0.05 | 4  |
|                     | H+ flux | −5.2 ± 0.5 | −5.1 ± 0.7 | −5.6 ± 0.4 | <0.05 | 4  |
| 13% CO2             | △pH    | −0.21 ± 0.05 | −0.20 ± 0.03 | −0.24 ± 0.04 | <0.05 | 9  |
|                     | H+ flux | −6.0 ± 1.3 | −6.1 ± 2.3 | −6.5 ± 1.4 | <0.05 | 9  |
| 1% CO2              | △pH    | −0.24 ± 0.08 | −0.20 ± 0.06 | −0.20 ± 0.06 | <0.05 | 5  |
|                     | H+ flux | −7.2 ± 1.8 | −10.7 ± 1.9 | −6.0 ± 1.3 | <0.05 | 5  |

aBlank cells indicate that control Ringer was perfused into the corresponding bath.
bH+ flux has units of mM × min−1, and all values are reported as mean ± SD.
cThe basal bath Δ[HCO3−]-induced pH response in control Ringer was compared to the pH response in the presence of the inhibitor/condition; *P* < 0.05 is considered significant by Student’s *t* test.

This CO2 load would be mainly dissipated by the choroidal blood supply because it features a very high flow rate (Alm and Bill, 1987) and a relatively short diffusion path from the inner segments (Oyster, 1999). We perfuse 13% CO2-equilibrated Ringer to the hfRPE apical membrane to mimic our estimate of the in vivo increase in CO2 that occurs in the SRS after the transition from light to dark (from 5 to 10 ± 3%; see Appendix 1). The present results show that in hfRPE, CO2 is more effectively transported across the apical membrane compared with the
Apical membrane CO2 and HCO3 transport

Earlier studies in frog and bovine RPE provide strong electrophysiological evidence for the electrogenic Na/2HCO3 cotransporter at the apical membrane (Hughes et al., 1989; Kenyon et al., 1997). In addition, pNBC1 has been immunolocalized to the apical membrane of rat RPE (Bok et al., 2001). In hRPE, we demonstrate apical Na/2HCO3 cotransport activity with the following experimental observations: (1) Apical DIDS acidified the cell and decreased TEP, consistent with the inhibition of electrogenic Na/2HCO3 cotransport into the cell. In addition, these DIDS-induced responses were reversed in the presence of low HCO3 Ringer (10-fold) in the apical bath—apical DIDS alkalinized the cell and increased TEP. (2) Decreasing apical bath [HCO3] decreased TEP, consistent with Na/2HCO3 efflux across the apical membrane, and this response was essentially abolished by apical DIDS.

The presence of CA II and several apical membrane-bound CAs (e.g., CAs IV, IX, XII, and XIV) (Nagelhus et al., 2005) support the notion of HCO3-mediated CO2 transport from the SRS into the RPE via the electrogenic Na/2HCO3 cotransporter. However, the following experiments suggest that increasing apical bath CO2 does not stimulate apical Na/2HCO3 cotransport: (1) apical DIDS caused pHi and TEP responses in 5, 1, or 13% apical bath CO2 that were statistically indistinguishable, and (2) the magnitude of 13% apical CO2-induced acidification was unaffected by apical DIDS. This is probably because the fast CO2/HCO3 equilibration across the apical membrane prevented a change in the HCO3 gradient. Besides NBC1 (SLC4A4; Gene ID 8671), a DIDS-insensitive and electroneutral Na/HCO3 cotransporter, NBC3/NBCn1 (SLC4A7; Gene ID 9497) is also highly expressed at the apical membrane of human RPE (Wang, F.E., and S.S. Miller. 2007. Profiling MicroRNA (miRNA) Expression in Human Retina, Retinal Pigment Epithelium (RPE), and Choroid; Zhi, C.G., F.E. Wang, T. Banzon, S. Jalickee, R. Fariss, A. Maminishkis, and S.S. Miller. 2007. Membrane-Bound Carbonic Anhydrases in Human Fetal Retinal Pigment Epithelial Cells (hRPE)). Our observation that apical DIDS had little effect on the apical bath [HCO3]-induced acidification suggests that NBC3 is highly active in the RPE. However, this does not indicate that NBC1 has a lower activity than NBC3 because NBC1 is electrogenic and is therefore limited by both the membrane voltage and HCO3 gradient. In contrast, NBC3 is limited only by the HCO3 gradient. Thus, the relative activities of NBC1 and NBC3 cannot be accurately evaluated by comparing the apical bath Δ[HCO3]-induced pH responses in the presence versus absence of apical DIDS.

Apical membrane processes increase the effective apical surface area of native frog RPE by ≈30-fold relative to the basolateral surface area (Miller and Steinberg, 1977; Maminishkis et al., 2006). Increasing apical bath CO2 promotes CA II–dependent HCO3 formation in the cytosol that can be transported across the basolateral membrane by a Na/nHCO3 cotransporter. As previously shown in frog RPE, apical CO2-induced acidification inhibits the basolateral membrane Cl/HCO3 exchanger (Fig. 7 A), which can increase Cl recycling at the basolateral membrane, perhaps via CFTR or Ca2+-activated Cl channels. The combination of this effect and the CO2-induced increase in basolateral Na/nHCO3 cotransport are hypothesized to increase net Na, Cl, and HCO3 absorption, leading to the observed increase in steady-state fluid absorption across the RPE (Fig. 13).

Apical membrane CO2 and HCO3 transport

Figure 13. CO2-induced changes in fluid absorption. 5% CO2 equilibrated Ringer was added to both solution baths, and Jv was recorded with Jv, TEP, and RT at steady state. The control Ringer was then replaced with either (A) 13% CO2 or (B) 1% CO2-equilibrated Ringer in both solution baths. Jv, TEP, and RT values were recorded at steady state (15–30 min). Solid bars above the graphs represent solution changes from control Ringer as described in the legend to Fig. 2.
hindered by the mesh support and transwell filter (attached to the basal membrane). The mesh was eliminated as a possible diffusion barrier by showing that the 13% basal bath CO₂ produced the same ΔpH, with or without the mesh (unpublished data). To test the filter and its unstirred layer as a diffusion barrier, the hfRPE monolayer was uniformly damaged by mounting its apical membrane down on the mesh. This allows CO₂ from the basal bath to diffuse through the damaged areas and across the apical membrane of the RPE. If the filter was a significant barrier to CO₂, the difference between 13% apical and basal CO₂-induced ΔpH in a damaged hfRPE would be similar to that in an intact hfRPE (approximately eightfold). However, with the damaged hfRPE monolayer, the difference in CO₂-induced ΔpH, was ≈2.5-fold (n = 5), and the calculated apparent relative CO₂ permeability was 3.0 ± 1.5, suggesting that the basolateral membrane is relatively less permeable to CO₂ than the apical membrane. Besides hfRPE cultures, significant differences between the 13% apical and basal bath induced pH₁ responses were also observed in native bovine and fetal human RPE choroid preparations, thus corroborating our conclusion.

As another test of the basolateral membrane CO₂ permeability, basal bath CO₂ was increased from 5 to 13% in the absence of apical flow. In this case, the 13% basal CO₂-induced ΔpH was approximately threefold greater than with continuous apical perfusion (n = 4), suggesting that stopped apical flow increases the thickness of the unstirred layer at the apical membrane surface, which limits CO₂ diffusion out of the cell and causes a larger acidification. This result indicates that the basolateral membrane has some CO₂ permeability. Alternatively, the paracellular pathway may allow small amounts of CO₂ to enter the apical membrane from the basal bath. Tight junctions were disrupted by removing all Ca²⁺ and Mg²⁺ from both solution baths, but this maneuver did not increase the 13% basal CO₂-induced acidification. Therefore, we conclude that the basolateral membrane is the main pathway for CO₂ entry from the basal bath.

A possible CO₂ transport mechanism arises from the ability of aquaporin 1 (AQP1) to function as a CO₂ channel (Cooper and Boron, 1998; Endeward et al., 2006). In cultured hfRPE cells, AQP1 mRNA is highly expressed in human RPE (Wang, F.E., and S.S. Miller. 2007. Profiling MicroRNA (miRNA) Expression in Human Retina, Retinal Pigment Epithelium (RPE), and Choroid) show high mRNA expression levels for NBC1 (SLC4A4; Gene ID 8671) and NBC4/NBCe2 (SLC4A5; Gene ID 57835), both of which are candidates for the identity of the basolateral membrane Na/nHCO₃ cotransporter in human RPE. Although this transporter’s identity is unknown, both NBC1 and NBC4 have been shown to transport Na/nHCO₃ with a 1:3 stoichiometry of 1:2 (Gross et al., 2001; Virkki et al., 2002), suggesting inward Na/nHCO₃ cotransport from the basolateral membrane. However, NBC4 transports NaHCO₃ with a 1:3 stoichiometry at the apical membrane of the choroid plexus epithelium (CPE) (Millar and Brown, 2008). Because both the RPE and the CPE derive from the neural ectoderm and share many similarities in HCO₃ transport mechanisms (Brown et al., 2004; Praetorius, 2007), it is possible that the RPE expresses NBC4 at the basolateral membrane and transports Na/nHCO₃ with a 1:3 Na:HCO₃ stoichiometry. In addition, our calculation of the reversal potential of the Na/nHCO₃ cotransporter indicates that a 1:3 stoichiometry is required for Na/nHCO₃ transport out of the cell; this calculation is

epithelia (Waisbren et al., 1994; Endeward and Gros, 2005), and the RPE may possess a basolateral membrane with a unique lipid composition that limits CO₂ flux. The influence of lipid composition and properties on gas permeability has been studied in lipid bilayers (Hill et al., 1999; Hill and Zeidel, 2000), a possibility that remains to be evaluated in RPE.

**Basolateral membrane HCO₃ transporters**

The DIDS-sensitive Cl/HCO₃ exchanger at the basolateral membrane of hfRPE was inhibited or activated under acidic (13% apical CO₂) or basic (1% apical CO₂) conditions, respectively, as in frog RPE (Lin and Miller, 1994). From our Affymetrix data, AE2 (SLC4A2; Gene ID 6522) is the only AE isoform detected in cultured fetal human RPE and in native adult and fetal human RPE. Because AE2 is known to be pH sensitive (Kurschat et al., 2006; Stewart et al., 2007), it is possibly the isoform located at the basolateral membrane of hfRPE. Because this exchanger was inhibited by 13% apical CO₂, the RPE requires an alternate HCO₃ efflux pathway at the basolateral membrane to mediate transepithelial HCO₃ absorption. The following observations indicate the presence of an electrogenic Na/nHCO₃ cotransporter at the basolateral membrane: (1) reducing [HCO₃] at the basal bath increased TEP, consistent with a depolarization of the basolateral membrane; (2) this TEP increase was significantly inhibited (≈70%) in the presence of basal DIDS; and (3) this TEP increase was completely abolished in the absence of Na in both bathing solutions.

Our Affymetrix data on human RPE (native adult and fetal RPE, and cultured fetal RPE) (Wang, F.E., and S.S. Miller. 2007. Profiling MicroRNA (miRNA) Expression in Human Retina, Retinal Pigment Epithelium (RPE), and Choroid) show high mRNA expression levels for NBC1 (SLC4A4; Gene ID 8671) and NBC4/NBCe2 (SLC4A5; Gene ID 57835), both of which are candidates for the identity of the basolateral membrane Na/nHCO₃ cotransporter in human RPE. Although which transporter’s identity is unknown, both NBC1 and NBC4 have been shown to transport NaHCO₃ with a 1:3 stoichiometry of 1:2 (Gross et al., 2001; Virkki et al., 2002), suggesting inward Na/nHCO₃ cotransport from the basolateral membrane. However, NBC4 transports NaHCO₃ with a 1:3 stoichiometry at the apical membrane of the choroid plexus epithelium (CPE) (Millar and Brown, 2008). Because both the RPE and the CPE derive from the neural ectoderm and share many similarities in HCO₃ transport mechanisms (Brown et al., 2004; Praetorius, 2007), it is possible that the RPE expresses NBC4 at the basolateral membrane and transports Na/nHCO₃ with a 1:3 Na:HCO₃ stoichiometry. In addition, our calculation of the reversal potential of the Na/nHCO₃ cotransporter indicates that a 1:3 stoichiometry is required for Na/nHCO₃ transport out of the cell; this calculation is
based on our estimation of resting [Na], and pHi in control Ringer (see Appendix 2).

The basolateral membrane Na/nHCO3 cotransporter is more dependent on HCO3 than Na as a substrate, as supported by the following experiments: (1) Reducing basal bath [HCO3] 10-fold caused a TEP response that was reduced in the presence of apical DIDS, suggesting that inhibiting NBC1 reduced basolateral Na/nHCO3 cotransport activity. (2) The basal bath Δ[HCO3]-induced TEP response was reduced in the presence of basal DZA, suggesting that CA inhibition reduces basolateral Na/nHCO3 cotransport. DZA reduces HCO3 transport in two ways. First, DZA slows CA-mediated hydration of CO2 to HCO3. Second, DZA inhibits the apical membrane Na/2HCO3 cotransporter, as indicated by the reduction of apical bath Δ[HCO3]-induced TEP response in the presence of apical DZA. (3) The basal bath Δ[HCO3]-induced TEP response was increased in 13% apical bath CO2 and decreased in 1% apical bath CO2. This suggests that apical CO2 entry and its subsequent conversion into HCO3 is an important source of HCO3 substrate for basolateral Na/nHCO3 cotransport activity.

In addition to showing that the basolateral membrane Na/nHCO3 cotransporter is dependent on apical HCO3 supply, we also eliminated Na as a limiting substrate for basolateral Na/nHCO3 cotransport by examining three Na transport proteins at the apical membrane of the RPE (Hughes et al., 1998): (1) bumetanide-sensitive Na/K/2Cl cotransporter, (2) amiloride-sensitive Na/H exchanger, and (3) ouabain-sensitive Na/K ATPase. In hRPE, the presence of bumetanide, amiloride, or ouabain in the apical bath had no effect on the basal bath Δ[HCO3]-induced TEP responses, suggesting that these Na transport mechanisms are not linked to basolateral Na/nHCO3 cotransport activity. Collectively, our data indicate that the basolateral membrane Na/nHCO3 cotransporter is mainly driven by HCO3 supplied by NBC1-mediated Na/2HCO3 entry and CA II–mediated hydrolysis of CO2 to HCO3.

Apical Na entry pathways and CO2/HCO3-driven fluid transport

13% apical CO2 increased basolateral Na/nHCO3 cotransport, which should decrease [Na]i. However, Na imaging experiments showed that 13% apical CO2 increased [Na]i, suggesting that Na enters the RPE via apical membrane Na transport processes. Although the Na/H exchanger can be activated by intracellular acidification (Aronson et al., 1982; Dunham et al., 2004), the following experiments show that 13% apical CO2 does not activate the Na/H exchanger: (1) the magnitude of the 13% apical CO2-induced acidification was unaffected in the presence of apical amiloride, and (2) adding 1 mM amiloride into the apical bath did not affect the steady-state pH, in 5 or 13% apical bath CO2. These observations indicate that the Na/H exchanger could not have contributed to the CO2-induced [Na]i increase. This lack of participation might have occurred for three reasons: (1) the 13% CO2-induced acidification was too small; (2) there was no change in the proton gradient across the Na/H exchanger; and (3) the 13% CO2-equilibrated Ringer is acidic relative to control (pH 7.09 vs. 7.5), and the low extracellular pH may have inhibited the Na/H exchanger (Aronson et al., 1983). We ruled out the first possibility with a 10-mM NH4 prepulse that caused only 0.1 decrease in pHi (n = 4) but still showed the characteristic Na/H exchanger–mediated pHi recovery; in comparison, 13% apical CO2 acidified the cell by >0.2 pH units. In addition, we showed that reducing basal bath [HCO3] acidified the cell by only 0.2 but was able to activate the Na/H exchanger. Na/2HCO3 entry via NBC1 was eliminated as a possible cause of the 13% apical CO2-induced [Na]i increase because: (1) the apical DIDS-induced pHi and TEP responses were the same in 5 or 13% apical bath CO2, and (2) the magnitude of the 13% apical CO2-induced pHi response was unaltered in the presence of apical DIDS.

In alveolar epithelium, Na/K ATPase activity is reduced by CO2-induced acidification (Briva et al., 2007), suggesting the possibility of a similar effect in RPE. In frog RPE, 13% CO2-induced acidification activated the Na/K/2Cl cotransporter after inhibition of the basolateral membrane Cl/HCO3 exchanger and the subsequent reduction in intracellular [Cl] (Edelman et al., 1994). Both the 13% apical CO2-induced inhibition of the Na/K ATPase and activation of the Na/K/2Cl cotransporter can increase [Na]i, thus providing Na entry pathway across the apical membrane that can mediate solute-driven fluid absorption. But these mechanisms remain to be evaluated.

In bovine RPE, net active Cl absorption is mediated by the Na/K/2Cl cotransporter at the apical membrane (Edelman et al., 1994) and by Ca2+-activated and cAMP/PKA-dependent CFTR Cl channels at the basolateral membrane (Joseph and Miller, 1991; Báleik et al., 1995; Hughes et al., 1998). Evidence for the expression and basolateral membrane localization of CFTR in hRPE has been presented (Blang et al., 2003). The 13% apical CO2-induced activation of the Na/K/2Cl cotransporter and inhibition of the Cl/HCO3 exchanger would both increase net Cl absorption across the RPE.

HCO3 transport also plays a significant role in RPE fluid transport. Steady-state fluid absorption was decreased by =50% after the addition of apical DIDS, indicating that the DIDS-sensitive NBC1 mediates HCO3-driven fluid transport. The presence of NBC3 at the apical membrane suggests that it also contributes to HCO3-mediated fluid transport. In addition, DZA or acetazolamide decreases steady-state fluid absorption across hRPE in vitro (Zhi, C.G., F.E. Wang, T. Banzon, S. Jalickee, R. Fariss, A. Maminishkis, and S.S. Miller. 2007. Membrane-Bound Carbonic Anhydrases in
Human Fetal Retinal Pigment Epithelial Cells (hfRPE)). These observations are corroborated in the present experiments by the DZA-induced inhibition of NBC1 at the apical membrane and the Na/nHCO3 cotransporter at the basolateral membrane. Interestingly, animal models and clinical studies showed that systemically administered acetazolamide increases fluid absorption across the RPE (Wolfensberger, 1999). It has been proposed that acetazolamide increases RPE fluid absorption in vivo by affecting membrane-bound CAs at the basolateral membrane, but there are no known membrane-bound CAs at the basolateral membrane of native RPE (Zhi, C.G., F.E. Wang, T. Banzon, S. Jalickee, R. Fariss, A. Maminishkis, and S.S. Miller. 2007. Membrane-Bound Carbonic Anhydrases in Human Fetal Retinal Pigment Epithelial Cells (hfRPE)). In addition, acetazolamide readily permeates RPE basolateral membrane, which would reduce fluid absorption by inhibiting cytosolic CA II, as observed in vitro. Further experiments involving interactions between the distal retina, choroid, and RPE are required to reconcile the difference in the effect of CA inhibitors on RPE fluid transport in vivo and in vitro.

HCO3−-mediated solute and fluid transport in the CPE

Both the RPE and the CPE develop from neural ectoderm; therefore, it is not surprising to find many similarities in the solute transport mechanisms of these two epithelia (Hughes et al., 1998; Brown et al., 2004; Praetorius, 2007). As demonstrated in this study, HCO3− transport mediates net solute and fluid absorption in human RPE. This is supported by experiments where acetazolamide or DZA reduced steady-state fluid absorption by ≈50% in hfRPE cultures (Zhi, C.G., F.E. Wang, T. Banzon, S. Jalickee, R. Fariss, A. Maminishkis, and S.S. Miller. 2007. Membrane-Bound Carbonic Anhydrases in Human Fetal Retinal Pigment Epithelial Cells (hfRPE)). Similarly in the CPE, HCO3− transport is an important mediator of cerebrospinal fluid (CSF) production (Saito and Wright, 1983, 1984); CSF secretion is inhibited by basal DIDS (Deng and Johanson, 1989). In addition, acetazolamide reduces CSF secretion by ≈40% (Vogh et al., 1987). Acetazolamide is used to prevent cerebral edema at high altitudes (Wright et al., 2008) and to reduce CSF pressure in children with hydrocephalus (Cowan and Whitelaw, 1991). The inhibitory effect of acetazolamide on CSF secretion led to the notion that CO2 entry into the CPE from the blood plasma and the subsequent hydration of CO2 into HCO3− stimulates NaHCO3 secretion across the apical membrane. This conclusion is supported by experiments in cat CPE, where ≈40% of Na secretion is attributed to CA II–mediated HCO3− formation from CO2 (Vogh and Maren, 1975). Perhaps not surprising, this mechanism of CO2-driven HCO3− transport is also found in the RPE.

Despite many similarities, the RPE normally absorbs Na (Cl + HCO3−) and fluid, whereas the CPE secretes Na (Cl + HCO3−) and fluid that helps form CSF. As in the RPE, the CPE expresses Na/HCO3 cotransporters at both the apical and basolateral membranes. However, the most striking difference is that both NBC1/NBCe1 and NBC3/NBCn1 in the RPE are expressed at the apical membrane (Zhi, C.G., F.E. Wang, T. Banzon, S. Jalickee, R. Fariss, A. Maminishkis, and S.S. Miller. 2007. Membrane-Bound Carbonic Anhydrases in Human Fetal Retinal Pigment Epithelial Cells (hfRPE)), whereas in the CPE, these two transporters are expressed at the basolateral membrane (Brown et al., 2004; Praetorius, 2007). This difference suggests that NBC4, which is found at the apical membrane of the CPE (Millar and Brown, 2008), may be the unidentified Na/nHCO3 cotransporter at the basolateral membrane of the RPE. We hypothesize that the difference in the membrane location of these HCO3 transporters (i.e., NBC1, NBC3, and NBC4) in the RPE and CPE is the basis for their difference in HCO3− and fluid transport direction.

If the CO2-permeability difference of the apical and basolateral membranes of the RPE also manifests in the CPE, what is its functional significance? In the central nervous system, metabolic CO2 produced by the brain is released into the CSF and subsequently neutralized by HCO3− secreted from the CPE. We hypothesize that the CPE has a relatively lower CO2 permeability at the apical membrane than at the basolateral membrane, and this property would promote CA II–mediated HCO3− secretion across the apical membrane. This possibility remains to be evaluated.

Physiological implications

Upon dark adaptation, oxygen consumption in the retina increases (Kimble et al., 1980; Medrano and Fox, 1995; Cringle et al., 2002; Yu and Cringle, 2002), thus generating and depositing more CO2 and H2O into the SRS. Both CO2 and H2O generation can be estimated from the rate of oxygen consumption measured in situ in cat and nonhuman primate eyes (Wangsa-Wirawan and Linsenmeier, 2003). Our calculations (see Appendix 1) provide an estimate of CO2 production in adult human photoreceptors of ≈0.29 and 0.54 mmol × hr−1 in light and dark, respectively. Considering that SRS [CO2] is ≈2 mM, impaired CO2 transport across the RPE could cause significant SRS or RPE acidification resulting in photoreceptor or RPE cell death. In addition, oxidative phosphorylation in the adult retina produces water at a rate of ≈0.5 μl × cm−2 × hr−1 in light and 0.9 μl × cm−2 × hr−1 in dark adapted eyes. Because glycolysis in the retina accounts for ≈95% of its total glucose consumption (Winkler et al., 2008), the combined retinal water production by aerobic respiration and glycolysis is calculated to be 3.6 and 6.5 μl × cm−2 × hr−1 in the light and dark, respectively. The CO2-induced changes in ion
transport in the RPE is one of many events that follows the transition from light to dark in vivo. Others include: (1) an increase in SRS [K+] from ≈3 to 5 mM, (2) the decrease in SRS [Ca^{2+}], and (3) the decrease in SRS pH (Steinberg et al., 1983; Borgula et al., 1989; Livsey et al., 1990; Yamamoto et al., 1992; Gallemore et al., 1994). Dark adaptation decreases SRS volume in situ (Li et al., 1994a,b). In addition, in a rat model of retinal reattachment (Maminishkis et al., 2002), fluid clearance from the SRS was faster in the dark-adapted eye (Maminishkis, A., personal communication), suggesting that steady-state fluid absorption across the RPE is higher in the dark.

In the dark-adapted eye, the high oxidative metabolism in the inner segments of the photoreceptors generates CO2 and H2O that are deposited into the SRS. The RPE uses the limited CO2 diffusion at the basolateral membrane to drive Na, Cl, and HCO3 transport across the RPE, which increases solute-driven fluid transport. This mechanism not only prevents CO2 accumulation in the SRS, but it also removes water from the vicinity of the photoreceptors. This helps maintain the proper anatomical relationship between the photoreceptors and the RPE apical membrane, thus avoiding retinal detachment and photoreceptor degeneration (Stone et al., 1999; Wickham et al., 2006; Nakazawa et al., 2007).

APPENDIX 1

Relative CO2 permeability

\[
\frac{d[CO_2]}{dt} = D \cdot (CO_{2,in} - CO_{2,out})
\]

Differentiating the CO2/HCO3 equilibrium constant,

\[
\frac{d[CO_2]}{dt} = \frac{[HCO_3^-]}{K_a} \cdot \frac{d[H^+]}{dt}
\]

Combining the above equations gives

\[
\frac{[HCO_3^-]}{K_a} \cdot \frac{d[H^+]}{dt}_{Ap} = D_{Ap} \cdot (CO_{2,in} - CO_{2,out})
\]

\[
\frac{[HCO_3^-]}{K_a} \cdot \frac{d[H^+]}{dt}_{Ba} = D_{Ba} \cdot (CO_{2,in} - CO_{2,out})
\]

The relative permeability (P) of CO2 at the apical versus the basolateral membrane is

\[
P = \frac{D_{Apical}}{D_{Basal}} = \frac{d[H^+]_{Ap}}{d[H^+]_{Ba}}
\]

where D is the diffusion coefficient and \( \frac{d[H^+]_{Ap}}{dt} \) and \( \frac{d[H^+]_{Ba}}{dt} \) are the H+ fluxes caused by perfusing 13% CO2-equilibrated Ringer to the apical and basal bath, respectively. The H+ fluxes were obtained by multiplying the 13% CO2-induced dpH/dt with the total buffering capacity of the hRPE. Based on these considerations, the relative permeability of apical versus basolateral membrane of hRPE to CO2 is 9.9 ± 4.4 (n = 7).

Retinal water production by aerobic respiration

In the dark, outer retina O2 consumption (Wangsa-Wirawan and Linsenmeier, 2003) is 4.2 ± 0.5 ml O2 x 100g^-1 min^-1. In the light, outer retina O2 consumption (Wangsa-Wirawan and Linsenmeier, 2003) is 2.3 ± 0.6 ml O2 x 100g^-1 min^-1. Wet weight of human retina (Bhosale and Bernstein, 2005) is 5.44 g. Oxygen consumption in the dark (density of oxygen at 36.9°C is 0.039 mmol/ml):

\[
0.042 \mu l \cdot mg^{-1} \cdot hr^{-1} \times 5.44 g \times 0.0393 mM \cdot ml^{-1} = 0.54 mmol O_2 \cdot hr^{-1}
\]

Oxygen consumption in the light:

\[
0.023 \mu l \cdot mg^{-1} \cdot hr^{-1} \times 5.44 g \times 0.0393 mM \cdot ml^{-1} = 0.29 mmol O_2 \cdot hr^{-1}
\]

In aerobic respiration, one molecule of water is generated for every molecule of oxygen consumed. Therefore, water generated in the dark is:

\[
9.72 \mu l \cdot mg^{-1} \cdot hr^{-1} \times 18 mg \cdot mmol^{-1} = 0.54 mmol H_2O \cdot hr^{-1}
\]

Water generated in the light is:

\[
5.22 \mu l \cdot mg^{-1} \cdot hr^{-1} \times 18 mg \cdot mmol^{-1} = 0.29 mmol H_2O \cdot hr^{-1}
\]

Assuming that the entire retina surface is 10.94 cm^2 (http://webvision.med.utah.edu/), the total rate of fluid generated by the retina through aerobic respiration in the dark is:

\[
\frac{9.72 \mu l \cdot H_2O \cdot hr^{-1}}{10.94 \ cm^2} = 0.89 \mu l \ H_2O \cdot cm^{-2} \cdot hr^{-1}
\]

In the light:

\[
\frac{5.31 \mu l \cdot H_2O \cdot hr^{-1}}{10.94 \ cm^2} = 0.48 \mu l \ H_2O \cdot cm^{-2} \cdot hr^{-1}
\]

Total retinal water production in the light and dark

For every glucose molecule that undergoes aerobic respiration, six molecules of CO2 are produced. Therefore, glucose consumption by aerobic respiration in the dark is:
0.54 mmol·hr⁻¹×\frac{1}{6CO₂} = 0.09 mmol·hr⁻¹

Glucose consumption by aerobic respiration in the light is:

0.29 mmol·hr⁻¹×\frac{1}{6CO₂} = 0.05 mmol·hr⁻¹

Assuming that glycolysis in the retina accounts for 95% of glucose consumption in the dark (Winkler et al., 2008), the rate of water generation by glycolysis in the dark is:

\[ \frac{0.09 \text{ mmol} \cdot \text{hr}^{-1} \times \frac{95}{5} \times \frac{2H₂O}{1 \text{glucose}} \times \frac{18\mu l}{1 \text{mmol} \times 10.94 \text{ cm}^{2}} = 5.6 \mu l \cdot \text{cm}^{-2} \cdot \text{hr}^{-1} \]

The rate of water generation by glycolysis in the light is:

\[ \frac{0.05 \text{ mmol} \cdot \text{hr}^{-1} \times \frac{95}{5} \times \frac{2H₂O}{1 \text{glucose}} \times \frac{18\mu l}{1 \text{mmol} \times 10.94 \text{ cm}^{2}} = 3.1 \mu l \cdot \text{cm}^{-2} \cdot \text{hr}^{-1} \]

Total water produced by aerobic respiration and glycolysis in the dark is:

\[ 0.89 + 5.6 = 6.5 \mu l \cdot \text{cm}^{-2} \cdot \text{hr}^{-1} \]

Total water produced by aerobic respiration and glycolysis in the light:

\[ 0.48 + 3.1 = 3.6 \mu l \cdot \text{cm}^{-2} \cdot \text{hr}^{-1} \]

\( J_v \) of human RPE in vivo has been estimated using B-scan ultrasonography to be =11 \mu l \times \text{cm}^{-2} \times \text{hr}^{-1} (Chihara and Naoi, 1985), comparable to our in vitro measurements (Fig. 13).

\[ \text{CO}_2 \text{ production in the light and dark} \]

\[ \text{CO}_2 \text{ production} = O_2 \text{ consumption. CO}_2 \text{ production in the dark is 4.2 } \pm 0.5 \text{ ml CO}_2 \times 100 \text{ g}^{-1} \text{ min}^{-1}, \text{ and in light is 2.3 } \pm 0.6 \text{ ml O}_2 \times 100 \text{ g}^{-1} \text{ min}^{-1}. \text{ Therefore, CO}_2 \text{ production increases by 1.4–2.6-fold after transitioning from light to dark. This increase in CO}_2 \text{ production translates to an increase in SRS CO}_2 \text{ concentration, from 5 to 10 } \pm 3\%. \]

\[ \text{APPENDIX 2} \]

\[ \text{Na}/n\text{HCO}_3 \text{ cotransporter reversal potential calculation} \]

\[ E_{\text{NBC}} = \frac{2.3RT}{F(n - 1)} \log \left( \frac{[Na^+]_{\text{out}}}{[Na^+]_{\text{in}}} \right) \left( \frac{[HCO₃^-]_{\text{out}}}{[HCO₃^-]_{\text{in}}} \right)^n \]

\[ [Na^+]_{\text{in}} = 15.7 \text{ mM, [Na}^+\text{]}_{\text{out}} = 143.7 \text{ mM, [HCO}_₃^-\text{]}_{\text{in}} = 27.9 \text{ mM, and [HCO}_₃^-\text{]}_{\text{out}} = 26.2 \text{ mM. n is the stoichiometry of the Na}/n\text{HCO}_3 \text{ cotransporter. We calculated the reversal potential of the Na}/n\text{HCO}_3 \text{ cotransporter NBC (}E_{\text{NBC}}\text{) to be } -55.7 \text{ mV for a Na}/n\text{HCO}_3 \text{ transport stoichiometry of 1:2. In this case, } E_{\text{NBC}} \text{ is more hyperpolarized than the average basolateral membrane potential (}V_B = -49.8 \pm 3.7 \text{ mV} \text{ (Maminishkis et al., 2006), and the Na}/n\text{HCO}_3 \text{ cotransporter transports Na and HCO}_3 \text{ into the cell. To transport Na}/n\text{HCO}_3 \text{ out of the cell against the strong inward Na gradient in control conditions, } E_{\text{NBC}} \text{ must be more depolarized than } V_B, \text{ and this condition is achieved for a Na}/n\text{HCO}_3 \text{ transport stoichiometry of 1:3, where } E_{\text{NBC}} = -27.0 \text{ mV.} \]

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