

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 13%; 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/⁺nHCO₃ cotransporter. The activity of this transporter was increased by elevating apical bath CO₂ and was reduced by dorzolamide. Increasing apical bath CO₂ also increased intracellular Na from 15.7 ± 3.3 to 24.0 ± 5.3 mM (*n* = 6; *P* < 0.05) by increasing apical membrane Na uptake. The CO₂-induced acidification also inhibited the basolateral membrane Cl/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.

INTRODUCTION

The retinal pigment epithelium (RPE) is a polarized monolayer of cells that forms a part of the blood–retina barrier in the back of the vertebrate eye. This epithelium separates the choroidal blood supply from the extracellular or subretinal space (SRS) that surrounds the retinal photoreceptors. By transporting ions and fluid from the SRS to the choroid (Hughes et al., 1998; Maminishkis et al., 2002), the RPE plays a critical role in maintaining the volume and chemical composition of the SRS. Large amounts of CO₂ and H₂O are deposited into the SRS due to the high metabolic activity of the photoreceptors (Wangsa-Wirawan and Linsenmeier, 2003). The high choroidal blood flow, ≈1,200 ml/min/100 g (Alm and Bill, 1987), provides a sink for the removal of these metabolites. Failure to remove CO₂ would result in acidosis detrimental to retinal function (Sillman et al., 1972; Meyertholen et al., 1986; Takahashi et al., 1993). In addition, abnormal accumulation of fluid in the SRS can cause retinal detachment and degeneration (Fisher et al., 2005).

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 (hRPE)). 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).

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Abbreviations used in this paper: AQP1, aquaporin 1; CA, carbonic anhydrase; CPE, choroid plexus epithelium; CSF, cerebrospinal fluid; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; DZA, dorzolamide; E_{NBC}, reversal potential of NBC; hRPE, human fetal retinal pigment epithelium; J_v, steady-state fluid absorption rate; pH_i, intracellular pH; RPE, retinal pigment epithelium; R_t, total tissue resistance; SRS, subretinal space; TEP, transepithelial potential.

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The close physiological relationship between HCO_3^- and fluid transport was demonstrated in frog RPE, where steady-state fluid absorption was reduced by $\approx 70\%$ 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 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 $\text{CO}_2/\text{HCO}_3^-$ and fluid 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 $\ddot{\text{U}}$ ssing chamber (exposed surface area of 7.1 mm^2). The $\ddot{\text{U}}$ ssing chamber was mounted on the stage of an axiovert-200 microscope (Carl Zeiss, Inc.) equipped with a 20 \times 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 $\text{ml} \times \text{min}^{-1}$. 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):

$$k_{440} = \frac{I_{440}^{t=n+0.5} - I_{440}^{t=n}}{\Delta t \times I_{440}^{t=n}} \times 100,$$

where ($I_{440}^{t=n+0.5} - I_{440}^{t=n}$) is the difference in BCECF fluorescence intensity between $\Delta t = 0.5$ -min intervals. From 30 experiments, the average k_{440} was $-2.21 \pm 0.50\% \times \text{min}^{-1}$.

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 $\ddot{\text{U}}$ ssing chamber. Fluorescence signals corresponding to intracellular Na concentration ($[\text{Na}]_i$) were obtained by alternating 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 $[\text{Na}]$ and 10 μM gramicidin were perfused simultaneously to both the apical and basal baths. The fluorescence ratio for each $[\text{Na}]_o$ 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 $\ddot{\text{U}}$ ssing 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,

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

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 $R_T \geq 200 \Omega \times \text{cm}^2$.

Estimating intrinsic buffering capacity

The intrinsic buffering capacity (β_i 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: $\beta_i = -93.4pH_i^3 + 2150.4pH_i^2 + 16483.6pH_i + 42065.6$ for $pH_i < 7.35$, and for $7.35 \leq pH_i \leq 7.7$, $\beta_i = 9.06$. The total buffering capacity (β_{total}) was then calculated with the equation, $\beta_{total} = \beta_i + \beta_{HCO_3} = \beta_i + 2.3[HCO_3]_i$. $[HCO_3]_i$ was estimated from the Henderson-Hasselbalch equation with the assumption that intracellular CO_2 level is 5%. H^+ flux was determined by multiplying β_{total} by an estimate of the initial dpH_i/dt determined from the pH_i response.

Steady-state fluid transport measurements

hRPE 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 hRPE monolayer were measured by injecting bipolar currents via Ag/AgCl pellet electrodes that were connected to the solution baths with agar bridges (4% wt/vol). All fluid transport experiments were performed in a Steri-Cult CO_2 incubator (Thermo Fisher Scientific) at $37^\circ C$ and 5% CO_2 . After a 15–30-min incubation in control Ringer (5% CO_2), steady-state J_v , TEP, and R_T were reached and recorded. Next, the incubator CO_2 control was set to 13%, and both bathing solutions were completely replaced with 13% CO_2 -equilibrated Ringer. After waiting another 15–30 min for J_v , TEP, and R_T to reach steady state, the values of these parameters were recorded.

Ringer solutions and chemicals

The ionic composition of the control Ringer solution was based on MEM solution (Sigma-Aldrich). Control CO_2/HCO_3 -buffered solution (pH 7.51 with 5% CO_2 , pH 7.09 with 13% CO_2 , and pH 8.20 with 1% CO_2) contains (in mM): $143.7 Na^+$, $126.1 Cl^-$, $26.2 HCO_3^-$, $5 K^+$, $1 H_2PO_4^-$, $0.5 Mg^{2+}$, $1.8 Ca^{2+}$, 2 taurine, and 5 glucose. Low Cl (1 mM) Ringer (pH 7.5 when equilibrated with 5% CO_2) was prepared by replacing all Cl^- salts with gluconate salts (except for $MgCl_2$). The high gluconate concentration (128.7 mM) in the low Cl Ringer requires consideration of the strong Ca^{2+} - and Mg^{2+} -chelating ability of gluconate. By using the stability constants of Ca and Mg gluconate (Furia, 1972), the estimated free $[Ca^{2+}]$ and $[Mg^{2+}]$ in the low Cl Ringer are 0.6 and 0.3 mM, respectively. As a test, the low Cl Ringer was supplemented with additional Ca^{2+} (to 5.7 mM) and Mg^{2+} (to 0.84 mM) to give free $[Ca^{2+}]$ and $[Mg^{2+}]$ of 1.8 and 0.5 mM, respectively. This adjustment did not significantly affect the low basal bath $[Cl^-]$ -induced pH_i , TEP,

and R_T responses. Low HCO_3 Ringer (2.62 mM HCO_3 ; pH 6.5 when equilibrated with 5% CO_2) was prepared by replacing 23.58 mM $NaHCO_3$ with equimolar Na gluconate. Ca^{2+}/Mg^{2+} -free Ringer was made by replacing all $CaCl_2$ and $MgCl_2$ with 4.6 mM NMDG-Cl. Sucrose was added to Ringer solutions to achieve osmolality of 300 ± 5 mOsm. 0.5 mM probenecid was added to all Ringer solutions used in pH_i and Na imaging experiments to slow dye leakage from the hRPE. In fluid transport experiments, hRPE culture media (300 mOsm and without any additives) equilibrated in 1, 5, or 13% CO_2 was used. DZA hydrochloride was purchased from U.S. Pharmacopeia. 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) and nigericin were purchased from EMD. All other chemicals were purchased from Sigma-Aldrich.

All values within this report are presented as means \pm SD. Statistical significance was evaluated by using the Student's *t* test (paired, two tailed). A *p*-value of <0.05 is considered statistically significant.

RESULTS

Apical or basal CO_2 -induced pH_i responses in hRPE

Fig. 2 A shows that increasing CO_2 from 5 to 13% in the apical or basal baths acidified the hRPE by ≈ 0.25 and ≈ 0.04 , respectively. Data from 13 experiments show that 13% apical CO_2 decreased pH_i by 0.23 ± 0.03 , from 7.37 ± 0.05 to 7.14 ± 0.06 ; in contrast, the 13% basal bath CO_2 -induced acidification ($\Delta pH_i = 0.03 \pm 0.01$) was almost eightfold smaller. Similarly in Fig. 2 B, decreasing CO_2 from 5 to 1% in the apical or basal baths alkalinized the hRPE 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 hRPE preparations, 13% apical CO_2 also caused significantly larger acidification ($\Delta pH_i = 0.29 \pm 0.04$) than 13% basal CO_2 ($\Delta pH_i = 0.03 \pm 0.02$; $n = 4$). This difference in the apical/basolateral CO_2 -induced pH_i response is even more pronounced in bovine RPE choroid preparations. No pH_i response to 13% basal CO_2 was

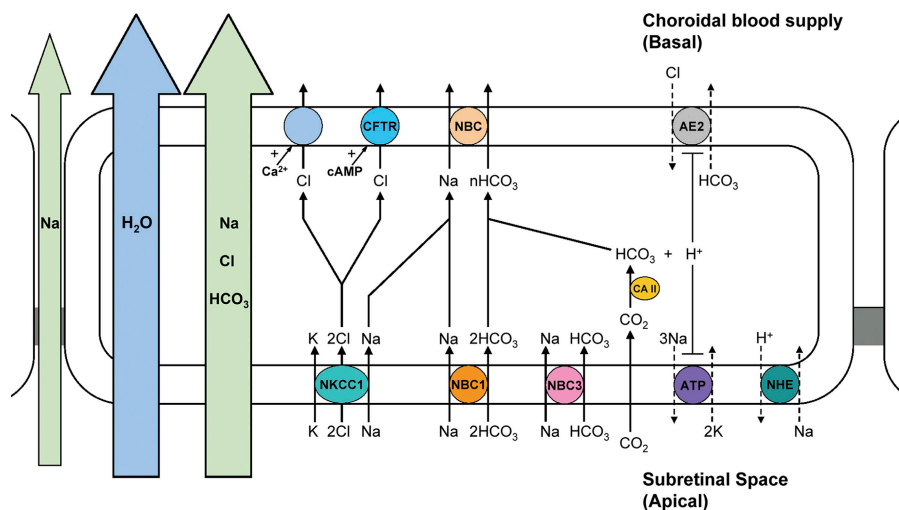


Figure 1. 13% apical CO_2 increases net solute and fluid absorption across the RPE. The transporters and channels depicted in this model are adopted from earlier studies of frog, bovine, human, and cultured human RPE. Apical membrane proteins: Na/K ATPase, Na/K/2Cl cotransporter (NKCC1), Na/H exchanger (NHE), and Na/2HCO₃ cotransporter (NBC1). Basolateral membrane proteins: Ca²⁺-activated Cl channels, cAMP-sensitive CFTR, Cl/HCO₃ exchanger (AE2), and Na/nHCO₃ cotransporter (NBC). Increasing apical CO_2 increases Na (Cl + HCO₃) and fluid absorption from the SRS to the choroid.

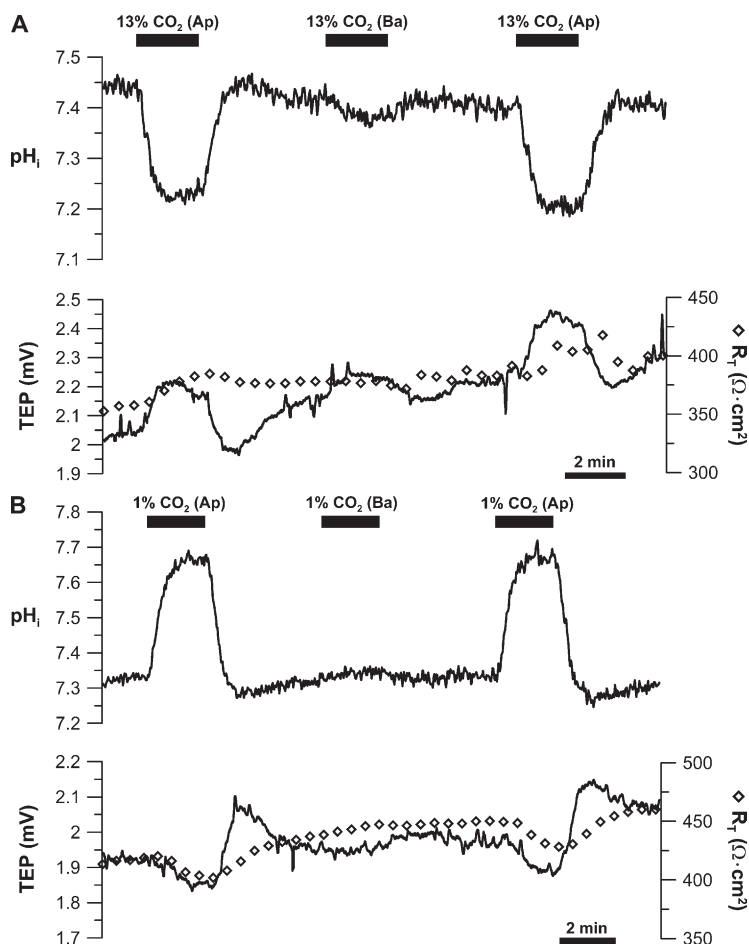


Figure 2. CO₂ flux across the apical and basolateral membranes. (A) 13% CO₂-equilibrated Ringer was perfused into the apical or basal bath. (B) 1% equilibrated Ringer was perfused into the apical or basal bath. The solid bars above the graph represent the beginning and end of a solution change (from control Ringer). The alteration in Ringer composition or the addition of drugs is indicated on the label above the solid bars. The time bar applies to pH_i, TEP, and R_T measurements and is located under the TEP/R_T panels. In each experiment, pH_i, TEP, and R_T were measured simultaneously.

observed, but a significant acidification was produced by 13% apical CO₂ ($\Delta\text{pH}_i = 0.39 \pm 0.09$; $n = 6$).

Tight junctions physically separate the RPE apical and basolateral membranes. Disruption of this barrier, by removing bath Ca²⁺ and Mg²⁺, could provide a paracellular pathway for movement of CO₂ from the basal bath to the apical membrane. To test this notion, we increased basal bath CO₂ (from 5 to 13%) 15 min after the removal of Ca²⁺ and Mg²⁺ from both bathing solutions. Upon Ca²⁺ and Mg²⁺ removal, R_T rapidly decreased at a rate of $17.3 \pm 7.3 \Omega \times \text{cm}^2 \times \text{min}^{-1}$ ($n = 5$). However, after 15 min in Ca²⁺- and Mg²⁺-free Ringer, the 13% basal bath CO₂-induced acidification was not significantly different than control (0.02 ± 0.01 vs. 0.02 ± 0.01 ; $n = 5$; $P > 0.05$), suggesting that basal bath CO₂ does not enter the apical membrane by diffusing across the tight junctions. When Ca²⁺ and Mg²⁺ were restored to the solution baths, R_T slowly recovered at a rate of $11.9 \pm 4.0 \Omega \times \text{cm}^2 \times \text{min}^{-1}$, but the recovery rate decreases significantly after ≈ 15 –20 min, and R_T recovered by only $\approx 40\%$.

Apical membrane electrogenic Na/2HCO₃ cotransporter in hRPE

We tested apical Na/2HCO₃ cotransport activity by adding apical DIDS and comparing the resultant pH_i and

TEP responses in control Ringer (26.2 mM HCO₃) versus low HCO₃ Ringer (2.62 mM HCO₃) in the apical bath (Fig. 3 A). Data from six experiments showed that in control Ringer, apical DIDS acidified the cell by 0.05 ± 0.02 and decreased TEP by 1.59 ± 0.63 mV, whereas adding apical DIDS in low apical bath [HCO₃] alkalinized the cell by 0.04 ± 0.01 and transiently increased TEP by 0.30 ± 0.15 mV; these responses are reversible. The apical DIDS-induced pH_i and TEP responses are consistent with the inhibition of an electrogenic HCO₃-dependent mechanism, whose activity can be reversed by a 10-fold reduction in apical bath [HCO₃].

To evaluate the potency of 0.5 mM of apical DIDS in the inhibition of the apical membrane Na/2HCO₃ cotransporter, we decreased apical bath [HCO₃] 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 $\Delta[\text{HCO}_3]$ -induced TEP response by sevenfold, from 2.1 ± 0.2 to 0.3 ± 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/2HCO₃ cotransporter activity. Surprisingly, the apical bath $\Delta[\text{HCO}_3]$ -induced acidification ($\Delta\text{pH}_i = 0.10 \pm 0.02$) was

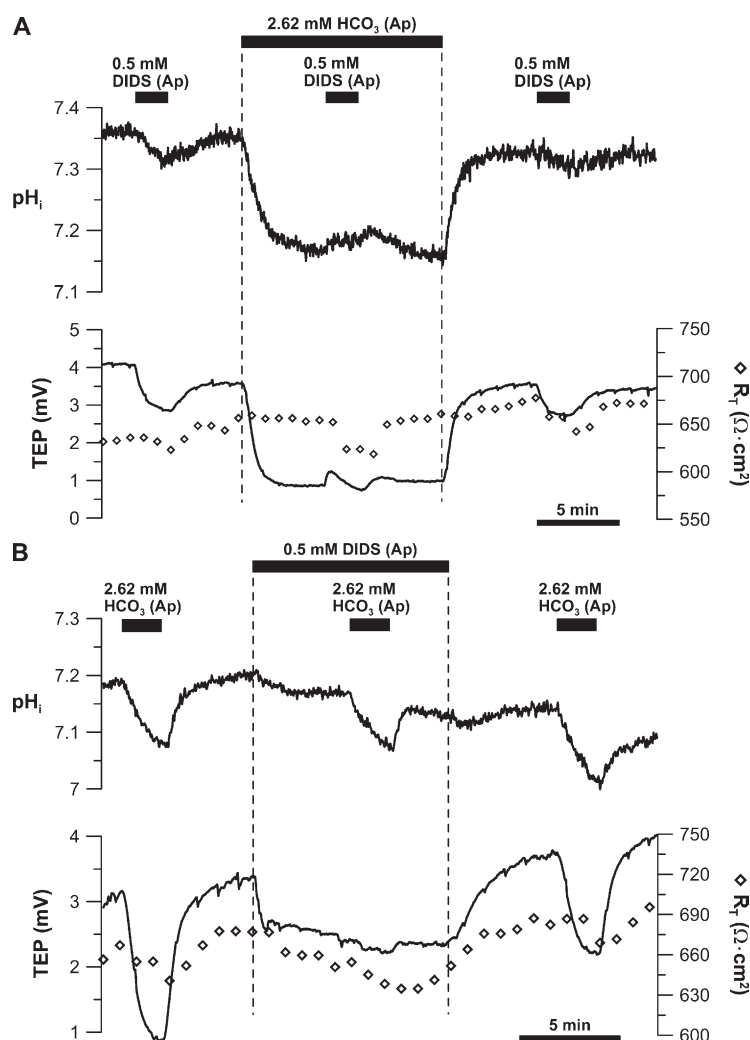


Figure 3. DIDS-sensitive Na/2HCO₃ cotransporter at the apical membrane. (A) 0.5 mM DIDS was added to the apical bath to obtain initial control response (pH_i, TEP, and R_T). The DIDS-induced response was then obtained in the presence of low (2.62 mM) HCO₃ Ringer in the apical bath. After washout with control Ringer, DIDS was added to the apical bath to obtain the final control response. (B) Low HCO₃ (2.62 mM) Ringer was perfused into the apical bath to obtain initial control response. The low basal bath [HCO₃]-induced response was then obtained in the presence of 0.5 mM of apical DIDS. After DIDS washout, low basal bath [HCO₃]-induced control response was obtained. Solid bars above the graphs represent solution changes from control Ringer as described in the legend to Fig. 2.

not significantly affected by DIDS ($\Delta\text{pH}_i = 0.09 \pm 0.01$; $n = 3$; $P > 0.05$), 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 pH_i 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 pH_i and TEP responses in control Ringer ($\Delta\text{pH}_i = 0.05 \pm 0.02$; $\Delta\text{TEP} = 1.52 \pm 0.33$ mV) were the same as that in 13% CO₂-equilibrated Ringer ($\Delta\text{pH}_i = 0.05 \pm 0.02$; $\Delta\text{TEP} = 1.57 \pm 0.67$ mV; $P > 0.05$). Similarly, the apical DIDS-induced pH_i and TEP responses in control Ringer ($\Delta\text{pH}_i = 0.05 \pm 0.02$; $\Delta\text{TEP} = 1.66 \pm 0.59$ mV) were the same as that in 1% CO₂-equilibrated Ringer ($\Delta\text{pH}_i = 0.06 \pm 0.02$; $\Delta\text{TEP} = 1.31 \pm 0.78$ mV; $n = 5$; $P > 0.05$). To further test the pH_i 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 ($\Delta\text{pH}_i = 0.22 \pm 0.03$) was the same as control ($\Delta\text{pH}_i = 0.22 \pm 0.02$; $n = 4$; $P > 0.05$). Collectively, these results lead to the conclusion that apical Na/2HCO₃ cotransport activity is not affected by apical CO₂-induced alterations in pH_i.

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 pH_i 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 pH_i response from 0.11 ± 0.01 to 0.19 ± 0.01 ($P < 0.01$). The effect of DZA on the pH_i and TEP responses was partially

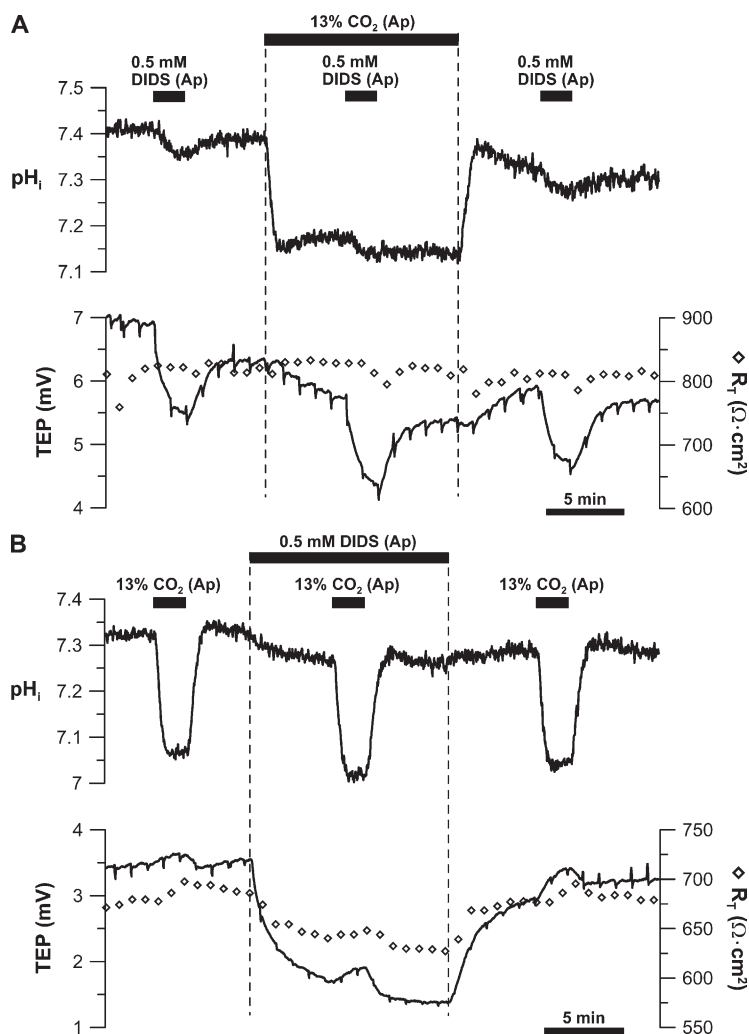


Figure 4. Effect of apical bath CO₂ on apical membrane Na/2HCO₃ cotransporter. (A) 0.5 mM DIDS was added to the apical bath to obtain initial control response. The DIDS-induced response was then obtained in the presence of 13% apical bath CO₂. After washout with control Ringer, DIDS was added to the apical bath to obtain the final control response. (B) 13% CO₂-equilibrated 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, 13% apical CO₂-induced control response was obtained. Solid bars above the graphs represent solution changes from control Ringer as described in the legend to Fig. 2.

reversible after a 5-min washout in control Ringer ($\Delta TEP = 1.27 \pm 0.46$ mV; $\Delta pH_i = 0.17 \pm 0.02$). The reduced apical bath $\Delta[HCO_3^-]$ -induced TEP response in the presence of DZA indicates inhibition of apical Na/2HCO₃ cotransport activity. On the other hand, the apical bath $\Delta[HCO_3^-]$ -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 hfrPE

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 ($\Delta pH_i = 0.18 \pm 0.05$) was abolished by 0.5 mM of basal DIDS ($\Delta pH_i = 0.02 \pm 0.01$; $n = 3$; $P < 0.05$), but this effect was not reversible. Next, we tested the pH_i dependence of the Cl/HCO₃ exchanger by comparing the basal bath $\Delta[Cl^-]$ -induced pH_i response in 5 versus 13% apical bath CO₂ (Fig. 7 A). The steady-

state pH_i in 5 and 13% apical bath CO₂ differed significantly, which required us to use the total buffering capacity of the hfrPE to calculate equivalent H⁺ fluxes. In the presence of 13% CO₂-equilibrated Ringer in the apical bath, the basal bath $\Delta[Cl^-]$ -induced change in H⁺ flux was 2.3 ± 1.0 mM \times min⁻¹, approximately fourfold smaller than the H⁺ flux in 5% CO₂ (9.0 ± 4.5 mM \times 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 $\Delta[Cl^-]$ -induced proton flux was 27.4 ± 10.8 mM \times min⁻¹, or approximately fivefold larger than the flux in 5% CO₂ (5.9 ± 6.5 mM \times min⁻¹; $n = 5$; $P = 0.01$). These experiments indicate that the DIDS-sensitive basolateral membrane Cl/HCO₃ exchanger in hfrPE is pH_i dependent.

Basolateral membrane electrogenic Na/nHCO₃ cotransport in hfrPE

In confluent monolayers of hfrPE, reducing basal bath [HCO₃⁻] 10-fold (5% CO₂) acidified the cells by 0.20 ± 0.05 , with an equivalent H⁺ flux of 6.2 ± 1.5 mM \times min⁻¹

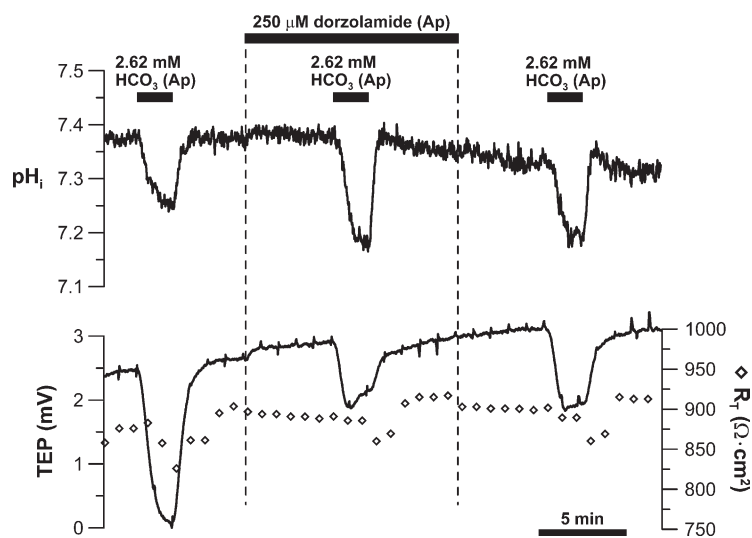


Figure 5. CA II dependence of apical membrane Na/HCO₃ cotransporter. Low HCO₃ (2.62 mM) Ringer was perfused into the apical bath to record the initial control response. This maneuver was repeated in the presence of 250 μM of apical DZA. After DZA washout, low apical bath [HCO₃]-induced control response was obtained. Solid bars above the graphs represent solution changes from control Ringer as described in the legend to Fig. 2.

($n = 45$), and increased TEP by 1.18 ± 0.60 mV ($n = 53$; Figs. 8–12 and Tables I and II). This TEP response is consistent with a basolateral membrane depolarization resulting from electrogenic Na/nHCO₃ cotransport activity. We evaluated the DIDS sensitivity of this basolateral membrane Na/nHCO₃ cotransporter by comparing the basal bath Δ [HCO₃]-induced pH_i and TEP responses in the presence of 0.5 mM of basal DIDS to that in control (Fig. 8). In five experiments, basal DIDS reduced the basal bath Δ [HCO₃]-induced acidification from 0.20 ± 0.04 to 0.09 ± 0.05 ($P < 0.05$), and reduced the TEP response from 1.41 ± 0.69 to 0.42 ± 0.29 mV ($P < 0.05$). The inhibitory effect of DIDS on the pH_i and TEP responses was irreversible after a 5-min washout with control Ringer (Δ pH_i = 0.12 ± 0.03 ; Δ TEP = 0.56 ± 0.25 mV; $P > 0.05$).

The Na dependence of the basolateral membrane HCO₃ transporter was studied by reducing basal bath [HCO₃] and measuring the resultant pH_i and TEP responses in the presence and absence of Na in both

solution baths (Fig. 9). In three experiments, Na removal reduced the basal bath Δ [HCO₃]-induced pH_i response by more than twofold (Δ pH_i = 0.08 ± 0.02) compared with control (Δ pH_i = 0.21 ± 0.04 ; $P < 0.05$). In addition, the basal bath Δ [HCO₃]-induced TEP response (Δ TEP = 1.01 ± 0.21 mV) was essentially abolished in the absence of Na (Δ TEP = 0.04 ± 0.07 mV; $P < 0.05$), and this effect was reversible. This indicates that although reducing basal bath [HCO₃] causes HCO₃ efflux via both Cl/HCO₃ exchanger and Na/nHCO₃ cotransporter, the TEP response corresponds specifically to Na/nHCO₃ cotransporter activity due to its electrogenicity and Na dependence. This allows one to distinguish the activity of the Na/nHCO₃ cotransporter from that of the Cl/HCO₃ exchanger.

Basolateral Na/nHCO₃ 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

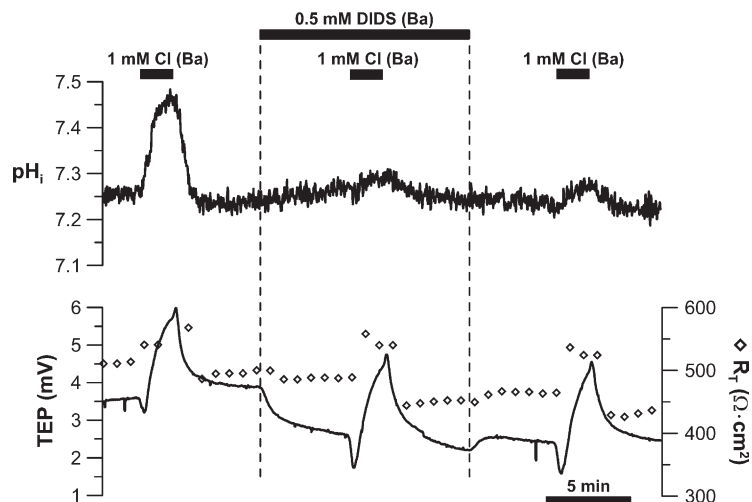


Figure 6. DIDS sensitivity of basolateral membrane Cl/HCO₃ exchanger. Low (1 mM) Cl Ringer was perfused into the apical bath to record the initial control response. This maneuver was repeated in the presence of 0.5 mM of apical DIDS. After DIDS washout, the low basal bath [Cl]-induced control response was obtained. Solid bars above the graphs represent solution changes from control Ringer as described in the legend to Fig. 2.

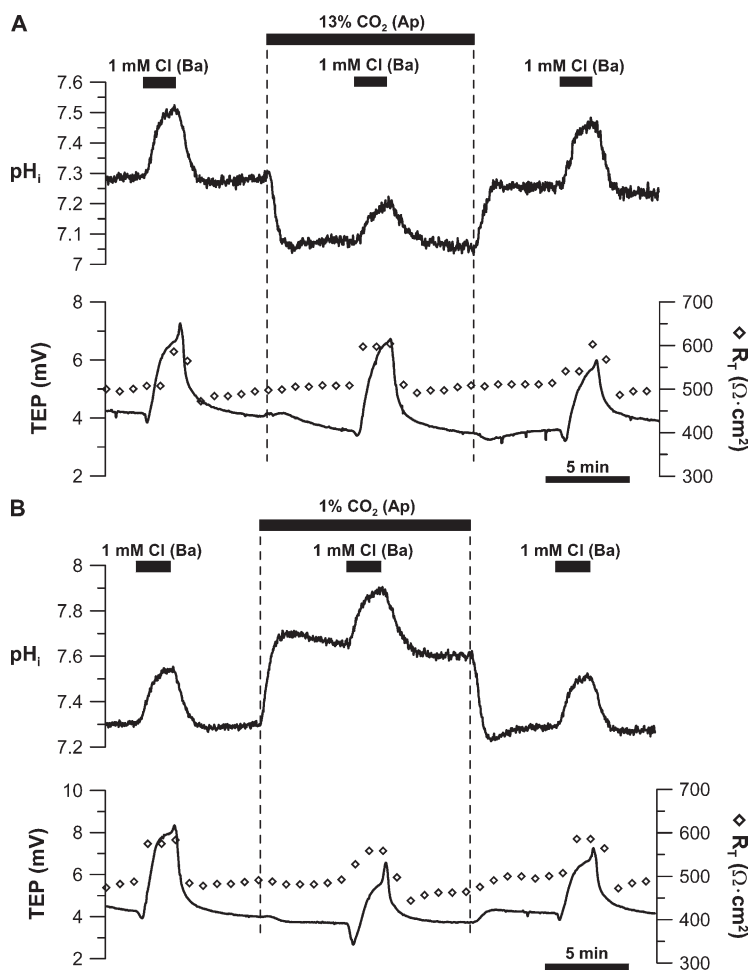


Figure 7. pH sensitivity of basolateral membrane Cl/HCO_3 exchanger. Low (1 mM) Cl Ringer was perfused into the apical bath to record the initial control response. This maneuver was then repeated in (A) 13% or (B) 1% apical bath CO_2 . After returning to control Ringer, low basal bath $[\text{Cl}]$ -induced 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/nHCO_3 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 pH_i and TEP responses caused by a 10-fold reduction in basal bath $[\text{HCO}_3]$. In five experiments, apical amiloride did not affect basal bath $\Delta[\text{HCO}_3]$ -induced TEP response ($\Delta\text{TEP} = 1.28 \pm 0.58$ vs. 1.24 ± 0.50 mV; $P > 0.05$), indicating that the apical membrane Na/H exchanger does not provide substrate for basolateral Na/nHCO_3 cotransport activity. On the other hand, the basal bath $\Delta[\text{HCO}_3]$ -induced acidification and H^+ flux was larger in the presence of apical amiloride ($\Delta p\text{H}_i = 0.28 \pm 0.05$; H^+ flux = 8.3 ± 1.6 $\text{mM} \times \text{min}^{-1}$) compared with control ($\Delta p\text{H}_i = 0.22 \pm 0.03$; H^+ flux = 7.1 ± 1.3 $\text{mM} \times \text{min}^{-1}$; $n = 5$; $P < 0.05$). This observation indicates that the Na/H exchanger normally acts to buffer cell acidification produced by HCO_3 efflux from the basolateral membrane.

Next, we inhibited the $\text{Na}/\text{K}/2\text{Cl}$ cotransporter with 200 μM of apical bumetanide, which did not affect the basal bath $\Delta[\text{HCO}_3]$ -induced $p\text{H}_i$ response ($\Delta p\text{H}_i = 0.20 \pm 0.03$ vs. 0.21 ± 0.03 ; $n = 4$; $P > 0.05$) and TEP response ($\Delta\text{TEP} = 0.89 \pm 0.28$ vs. 0.80 ± 0.18 mV; $n = 6$; $P > 0.05$). This lack of effect suggests that Na entry via the $\text{Na}/$

$\text{K}/2\text{Cl}$ cotransporter does not contribute significantly to basolateral Na/nHCO_3 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/nHCO_3 cotransporter. Adding 200 μM ouabain into the apical bath caused an acute TEP decrease ($\Delta\text{TEP} = 0.55 \pm 0.47$ mV; $n = 5$), as expected from inhibition of the Na/K ATPase. However, apical ouabain did not affect the basal bath $\Delta[\text{HCO}_3]$ -induced $p\text{H}_i$ response ($\Delta p\text{H}_i = 0.18 \pm 0.02$ vs. 0.20 ± 0.02 ; $n = 3$; $P > 0.05$) and TEP response ($\Delta\text{TEP} = 1.26 \pm 0.59$ 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/nHCO_3 cotransport activity.

The basolateral membrane Na/nHCO_3 cotransporter may be dependent on Na and HCO_3 entry from the apical membrane via the electrogenic $\text{Na}/2\text{HCO}_3$ cotransporter, as shown in Fig. 1. We tested the coupling between the apical and basolateral membrane Na/HCO_3 cotransporters by decreasing basal bath $[\text{HCO}_3]$ in the presence of 0.5 mM of apical DIDS (Fig. 10). In seven experiments, the basal bath $\Delta[\text{HCO}_3]$ -induced TEP response decreased from 0.86 ± 0.17 to 0.49 ± 0.06 mV ($P < 0.05$) in the presence of apical DIDS,

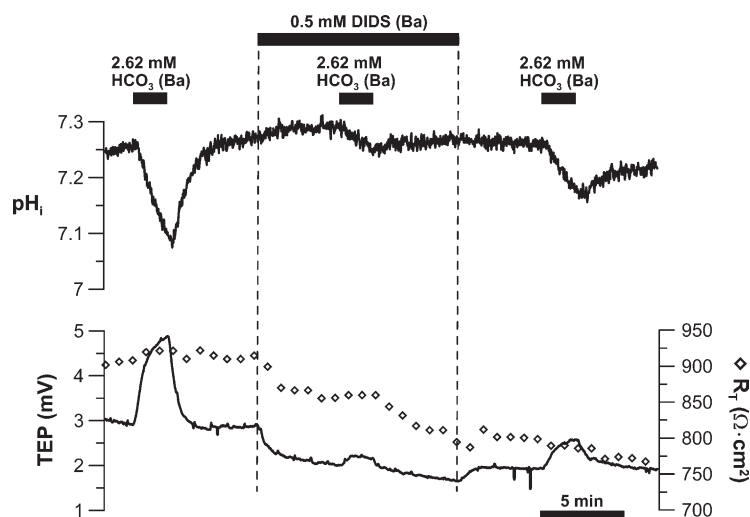


Figure 8. DIDS sensitivity of basolateral membrane Na/nHCO₃ cotransporter. Low HCO₃ (2.62 mM) Ringer was perfused into the basal bath to record the pH_i, TEP, and R_T responses, first in the absence and then in the presence of 0.5 mM of basal DIDS. After DIDS washout, low basal bath [HCO₃]-induced control 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 HCO₃ entry via the Na/2HCO₃ cotransporter reduces the HCO₃ supply that drives basolateral Na/nHCO₃ cotransport. However, apical DIDS increased the basal bath Δ [HCO₃]-induced acidification from 0.19 ± 0.03 to 0.23 ± 0.03 ($P < 0.05$; $n = 7$), and H⁺ flux from 5.0 ± 1.1 to 6.0 ± 1.1 mM \times min⁻¹ ($P < 0.05$; $n = 7$). This observation suggests that normally, apical HCO₃ entry via the Na/2HCO₃ cotransporter is a buffer that counteracts the acidification caused by HCO₃ efflux from the basolateral membrane.

Apical CO₂-induced changes in basolateral membrane Na/nHCO₃ cotransporter activity

Optimal Na/nHCO₃ cotransporter activity requires a steady supply of HCO₃, and the inhibition of CA II with DZA should reduce Na/nHCO₃ cotransport across the basolateral membrane. This notion was tested by reducing basal bath [HCO₃] 10-fold in the presence of 250 μ M DZA in the basal bath (Fig. 11). In a total of nine experiments, DZA reduced the basal bath Δ [HCO₃]-induced TEP response by $\approx 30\%$ (from Δ TEP = $1.44 \pm$

0.80 to 0.98 ± 0.50 mV; $P < 0.01$), suggesting that CA II inhibition reduces basolateral Na/nHCO₃ cotransport activity. In contrast, DZA did not affect the basal bath Δ [HCO₃]-induced acidification (Δ pH_i = 0.18 ± 0.03) compared with control (Δ pH_i = 0.17 ± 0.01 ; $n = 4$; $P > 0.05$). This lack of effect probably occurred because the DZA-induced reduction in basolateral membrane HCO₃ efflux is counteracted by a concomitant reduction in intracellular CO₂/HCO₃ buffering capacity.

A 13% CO₂ load applied to the apical membrane should increase basolateral membrane Na/nHCO₃ cotransport activity by shifting intracellular CO₂/HCO₃ equilibrium toward the formation of HCO₃ (facilitated by CA II activity). To test this hypothesis, we made a 10-fold reduction in basal bath [HCO₃] and compared the resultant pH_i and TEP responses in 5 versus 13% apical bath CO₂ (Fig. 12 A). With 13% apical bath CO₂, the basal bath Δ [HCO₃]-induced TEP response (Δ TEP = 1.35 ± 0.78 mV) was $\approx 20\%$ higher than control (Δ TEP = 1.11 ± 0.67 mV; $n = 9$; $P < 0.05$). However, there was no change in basal bath Δ [HCO₃]-induced pH_i response in

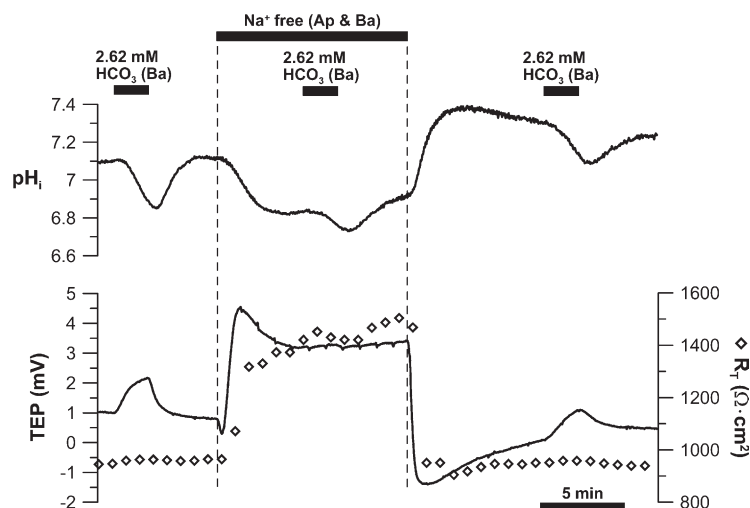


Figure 9. Na dependence of basolateral membrane Na/nHCO₃ cotransporter. Low HCO₃ (2.62 mM) Ringer was perfused into the basal bath to record the initial control response, and this maneuver was repeated in the absence of Na in both the apical and basal baths. After returning to control Ringer, low basal bath [HCO₃]-induced control response was obtained. Solid bars above the graphs represent solution changes from control Ringer as described in the legend to Fig. 2.

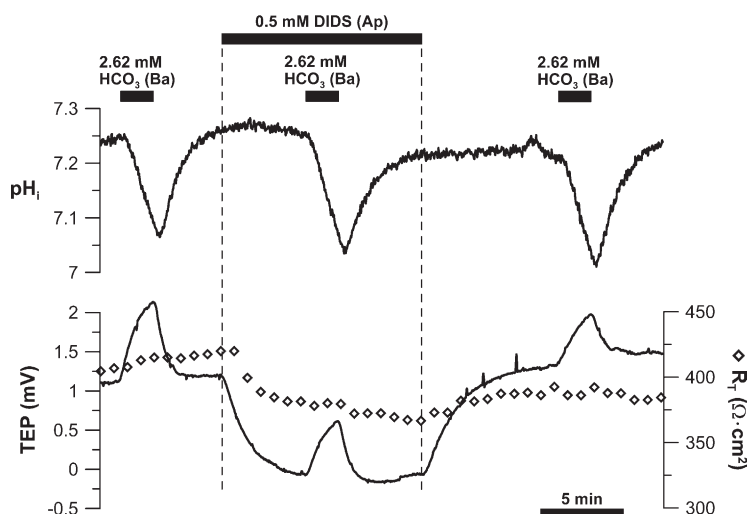


Figure 10. Linked activity of the apical and basolateral membrane Na/HCO₃ cotransporters. Low HCO₃ (2.62 mM) Ringer was perfused into the basal bath to obtain the initial control response, and this maneuver was then repeated in the presence of 0.5 mM of apical DIDS. After DIDS washout, the low basal bath [HCO₃]-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 = $6.0 \pm 1.3 \text{ mM} \times \text{min}^{-1}$) compared with control (H⁺ flux = $6.1 \pm 2.3 \text{ mM} \times \text{min}^{-1}$; $n = 9$; $P > 0.05$). Presumably, 13% apical CO₂ did not significantly alter H⁺ flux caused by basal bath $\Delta[\text{HCO}_3^-]$ because the CO₂-induced increase in HCO₃ efflux via the Na/nHCO₃ cotransporter was offset by concomitant inhibition of the pH_i-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 $\Delta[\text{HCO}_3^-]$ -induced TEP response ($\Delta\text{TEP} = 0.37 \pm 0.32 \text{ mV}$) was more than fivefold smaller than control ($\Delta\text{TEP} = 1.26 \pm 0.74 \text{ mV}$; $n = 5$, $P < 0.01$). 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 $\Delta[\text{HCO}_3^-]$ -induced equivalent H⁺ flux from 7.2 ± 1.8 to

$10.7 \pm 1.9 \text{ mM} \times \text{min}^{-1}$ ($n = 5$; $P = 0.04$). 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 $\Delta[\text{HCO}_3^-]$ -induced TEP response (Fig. 12 A), suggesting that 13% apical CO₂ activates the basolateral membrane Na/nHCO₃ cotransporter, which should decrease [Na]_i. However, 13% apical CO₂ increased [Na]_i from 15.7 ± 3.3 to $24.0 \pm 5.3 \text{ mM}$ ($n = 6$; $P < 0.05$). 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 pH_i 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 pH_i in 5 or 13% apical bath CO₂. As an additional test, we compared the magnitude of the 13% apical CO₂-induced

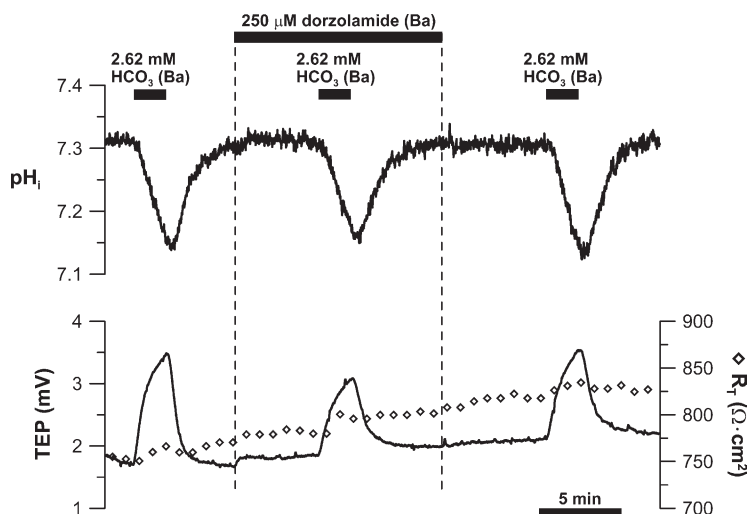


Figure 11. CA II dependence of basolateral membrane Na/nHCO₃ cotransporter. Low HCO₃ (2.62 mM) Ringer was perfused into the basal bath to record the pH_i, TEP, and R_f responses, first in the absence and then in the presence of 250 μM of basal DZA. After DZA washout, low basal bath [HCO₃]-induced control response was obtained. Solid bars above the graphs represent solution changes from control Ringer as described in the legend to Fig. 2.

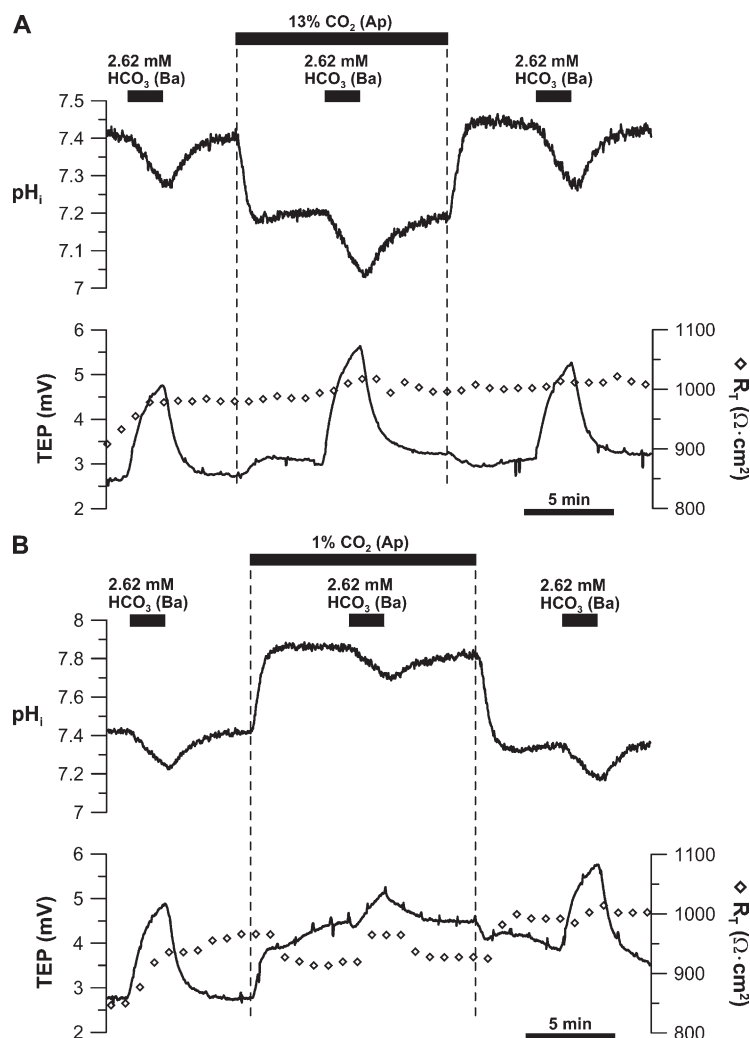


Figure 12. Effect of apical bath CO_2 on basolateral membrane Na/nHCO_3 cotransporter. Low HCO_3^- (2.62 mM) Ringer was perfused into the basal bath to record the initial control response, and this maneuver was repeated in (A) 13% or (B) 1% apical bath CO_2 . After returning to control Ringer, low basal 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.

acidification in the presence or absence of 1 mM amiloride in the apical bath. In four experiments, amiloride did not affect the 13% apical CO_2 -induced acidification ($\Delta\text{pH}_i = 0.23 \pm 0.01$) compared with control ($\Delta\text{pH}_i = 0.22 \pm 0.02$; $P > 0.05$). These experiments indicate that 13% apical CO_2 -induced acidification did not activate the Na/H exchanger.

CO_2 - and HCO_3^- -mediated fluid absorption across RPE

We tested the role of the apical membrane $\text{Na}/2\text{HCO}_3^-$ cotransporter in fluid transport by adding 0.5 mM DIDS into the apical bath and measuring the resultant change in J_v . In four experiments, 0.5 mM of apical DIDS decreased J_v by more than twofold (from 16.7 ± 4.8 to $7.7 \pm 3.7 \mu\text{l} \times \text{cm}^{-2} \times \text{hr}^{-1}$; $P < 0.05$), suggesting that HCO_3^- transport mediates a major component of fluid absorption across the RPE apical membrane. At the basolateral membrane, HCO_3^- transporters and several Cl^- channels are DIDS sensitive. Not surprisingly, the addition of 0.5 mM DIDS to the basal bath decreased J_v from 20.3 ± 8.2 to $11.2 \pm 6.0 \mu\text{l} \times \text{cm}^{-2} \times \text{hr}^{-1}$ ($n = 9$; $P < 0.05$).

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 ≈ 5 to $9 \mu\text{l} \times \text{cm}^{-2} \times \text{hr}^{-1}$, and Fig. 13 B shows that 1% CO_2 decreased J_v from ≈ 7 to $2.5 \mu\text{l} \times \text{cm}^{-2} \times \text{hr}^{-1}$. In four experiments, increasing CO_2 from 5 to 13% in both solution baths increased J_v by more than twofold (from 2.8 ± 1.6 to $6.7 \pm 2.3 \mu\text{l} \times \text{cm}^{-2} \times \text{hr}^{-1}$; $n = 5$; $P < 0.05$). 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 ± 3.9 to $3.4 \pm 1.1 \mu\text{l} \times \text{cm}^{-2} \times \text{hr}^{-1}$; $n = 4$; $P < 0.05$).

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 ≈ 70 mm Hg in the choriocapillaris to ≈ 0 mm Hg at the photoreceptor cilium, which

TABLE I
Summary of basal bath $\Delta[\text{HCO}_3^-]$ -induced pH_i responses

Inhibitor/condition ^a		2.62 mM basal bath $[\text{HCO}_3^-]$ -induced pH_i response ^b					<i>n</i>
		Data	Control	w/inhibitor	Recovery	P ^c	
Apical	Basal	ΔpH_i	-0.20 ± 0.04	-0.09 ± 0.05	-0.12 ± 0.03	<0.05	5
	DIDS	H^+ flux	-6.5 ± 1.2	-3.6 ± 2.7	-4.3 ± 1.3	<0.05	
Na-free	Na-free	ΔpH_i	-0.21 ± 0.04	-0.08 ± 0.01	-0.14 ± 0.02	<0.05	3
		H^+ flux	-7.2 ± 1.7	-2.8 ± 0.7	-4.9 ± 0.8	<0.05	
amiloride		ΔpH_i	-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	
bumetanide		ΔpH_i	-0.20 ± 0.03	-0.21 ± 0.03	-0.22 ± 0.03	>0.05	4
		H^+ flux	-6.9 ± 1.8	-6.8 ± 0.9	-7.6 ± 1.6	>0.05	
ouabain		ΔpH_i	-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	
DIDS		ΔpH_i	-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	
	DZA	ΔpH_i	-0.17 ± 0.01	-0.18 ± 0.03	-0.18 ± 0.02	>0.05	4
		H^+ flux	-5.2 ± 0.3	-5.1 ± 0.7	-5.6 ± 0.4	>0.05	
13% CO_2		ΔpH_i	-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	
1% CO_2		ΔpH_i	-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	

^aBlank cells indicate that control Ringer was perfused into the corresponding bath.

^b H^+ flux has units of $\text{mM} \times \text{min}^{-1}$, and all values are reported as mean \pm SD.

^cThe basal bath $\Delta[\text{HCO}_3^-]$ -induced pH_i response in control Ringer was compared to the pH_i response in the presence of the inhibitor/condition; $P < 0.05$ is considered significant by Student's *t* test.

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 CO_2 production and its release into the SRS.

This CO_2 load would be mainly dissipated by the chorioidal 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% CO_2 -equilibrated Ringer to the hRPE apical membrane to mimic our estimate of the in vivo increase in CO_2 that occurs in the SRS after the transition from light to dark (from 5 to $10 \pm 3\%$; see Appendix 1). The present results show that in hRPE, CO_2 is more effectively transported across the apical membrane compared with the

TABLE II
Summary of basal bath $\Delta[\text{HCO}_3^-]$ -induced TEP responses

Inhibitor/condition ^a		2.62 mM basal bath $[\text{HCO}_3^-]$ -induced TEP response (mV) ^b				<i>n</i>
		Control	w/ inhibitor	Recovery	P ^c	
Apical	Basal					5
	DIDS	1.41 ± 0.69	0.42 ± 0.29	0.56 ± 0.25	<0.05	
Na-free	Na-free	1.01 ± 0.21	0.04 ± 0.07	0.79 ± 0.38	<0.05	3
		1.28 ± 0.58	1.24 ± 0.50	1.23 ± 0.40	>0.05	
amiloride		0.89 ± 0.28	0.80 ± 0.18	0.97 ± 0.29	>0.05	6
		1.26 ± 0.59	1.17 ± 0.49	1.19 ± 0.51	>0.05	
ouabain		0.86 ± 0.16	0.49 ± 0.07	0.63 ± 0.15	<0.05	6
		1.44 ± 0.80	0.98 ± 0.50	1.06 ± 0.55	<0.05	
DIDS	DZA	1.11 ± 0.67	1.35 ± 0.78	1.07 ± 0.67	<0.05	9
		1.26 ± 0.74	0.37 ± 0.32	0.99 ± 0.59	<0.05	

^aBlank cells indicate that control Ringer was perfused into the corresponding bath.

^bAll values are reported as mean \pm SD.

^cThe basal bath $\Delta[\text{HCO}_3^-]$ -induced TEP response in control Ringer was compared to the TEP response in the presence of the inhibitor/condition; $P < 0.05$ is considered significant by Student's *t* test.

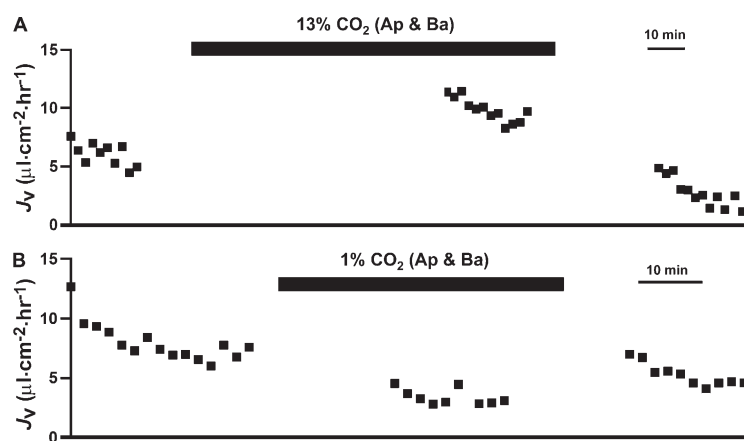


Figure 13. CO_2 -induced changes in fluid absorption. 5% CO_2 equilibrated Ringer was added to both solution baths, and J_v was recorded with J_v , TEP, and R_T at steady state. The control Ringer was then replaced with either (A) 13% CO_2 or (B) 1% CO_2 -equilibrated Ringer in both solution baths. J_v , TEP, and R_T 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.

basolateral membrane probably because of a difference in exposed surface area (Miller and Steinberg, 1977; Maminishkis et al., 2006). Increasing apical bath CO_2 promotes CA II-dependent HCO_3^- formation in the cytosol that can be transported across the basolateral membrane by a Na/nHCO_3 cotransporter. As previously shown in frog RPE, apical CO_2 -induced acidification inhibits the basolateral membrane Cl/HCO_3^- exchanger (Fig. 7 A), which can increase Cl recycling at the basolateral membrane, perhaps via CFTR or Ca^{2+} -activated Cl channels. The combination of this effect and the CO_2 -induced increase in basolateral Na/nHCO_3 cotransport are hypothesized to increase net Na , Cl , and HCO_3^- absorption, leading to the observed increase in steady-state fluid absorption across the RPE (Fig. 13).

Apical membrane CO_2 and HCO_3^- transport

Earlier studies in frog and bovine RPE provide strong electrophysiological evidence for the electrogenic $\text{Na}/2\text{HCO}_3^-$ 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 $\text{Na}/2\text{HCO}_3^-$ cotransport activity with the following experimental observations: (1) Apical DIDS acidified the cell and decreased TEP, consistent with the inhibition of electrogenic $\text{Na}/2\text{HCO}_3^-$ cotransport into the cell. In addition, these DIDS-induced responses were reversed in the presence of low HCO_3^- Ringer (10-fold) in the apical bath—apical DIDS alkalinized the cell and increased TEP. (2) Decreasing apical bath $[\text{HCO}_3^-]$ decreased TEP, consistent with $\text{Na}/2\text{HCO}_3^-$ 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 HCO_3^- -mediated CO_2 transport from the SRS into the RPE via the electrogenic $\text{Na}/2\text{HCO}_3^-$ cotransporter. However, the following experiments suggest that increasing apical bath CO_2 does not stimulate apical $\text{Na}/2\text{HCO}_3^-$ cotransport: (1)

apical DIDS caused pH_i and TEP responses in 5, 1, or 13% apical bath CO_2 that were statistically indistinguishable, and (2) the magnitude of 13% apical CO_2 -induced acidification was unaffected by apical DIDS. This is probably because the fast $\text{CO}_2/\text{HCO}_3^-$ equilibration across the apical membrane prevented a change in the HCO_3^- gradient. Besides NBC1 (SLC4A4; Gene ID 8671), a DIDS-insensitive and electroneutral Na/HCO_3^- 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 $\Delta[\text{HCO}_3^-]$ -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 HCO_3^- gradient. In contrast, NBC3 is limited only by the HCO_3^- gradient. Thus, the relative activities of NBC1 and NBC3 cannot be accurately evaluated by comparing the apical bath $\Delta[\text{HCO}_3^-]$ -induced pH_i 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). Electron micrography of hRPE provides evidence for similar structures in hRPE (Maminishkis et al., 2006) and supports the notion of a relatively larger apical surface area. This difference suggests a possible basis for the approximately eightfold difference in the ΔpH_i produced by altering CO_2 (from 5 to 13%) in the apical versus basal bath, which allowed us to calculate a 10-fold difference between the relative CO_2 permeability of the apical and basolateral membranes of hRPE cultures (see Appendix 1). However, in hRPE cultures, CO_2 entry from the basal bath may be

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 CO_2 produced the same ΔpH_i with or without the mesh (unpublished data). To test the filter and its unstirred layer as a diffusion barrier, the hRPE monolayer was uniformly damaged by mounting its apical membrane down on the mesh. This allows CO_2 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 CO_2 , the difference between 13% apical and basal CO_2 -induced ΔpH_i in a damaged hRPE would be similar to that in an intact hRPE (approximately eightfold). However, with the damaged hRPE monolayer, the difference in CO_2 -induced ΔpH_i was ≈ 2.5 -fold ($n = 5$), and the calculated apparent relative CO_2 permeability was 3.0 ± 1.5 , suggesting that the basolateral membrane is relatively less permeable to CO_2 than the apical membrane. Besides hRPE cultures, significant differences between the 13% apical and basal bath induced pH_i responses were also observed in native bovine and fetal human RPE choroid preparations, thus corroborating our conclusion.

As another test of the basolateral membrane CO_2 permeability, basal bath CO_2 was increased from 5 to 13% in the absence of apical flow. In this case, the 13% basal CO_2 -induced ΔpH_i 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 CO_2 diffusion out of the cell and causes a larger acidification. This result indicates that the basolateral membrane has some CO_2 permeability. Alternatively, the paracellular pathway may allow small amounts of CO_2 to enter the apical membrane from the basal bath. Tight junctions were disrupted by removing all Ca^{2+} and Mg^{2+} from both solution baths, but this maneuver did not increase the 13% basal CO_2 -induced acidification; therefore, we conclude that the basolateral membrane is the main pathway for CO_2 entry from the basal bath.

A possible CO_2 transport mechanism arises from the ability of aquaporin 1 (AQP1) to function as a CO_2 channel (Cooper and Boron, 1998; Endeward et al., 2006). In cultured hRPE 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). In addition, AQP1 was also detected exclusively at the apical membrane of hRPE (unpublished data), corroborating an earlier study on rat RPE (Stamer et al., 2003). However, 1 mM pCMBS (nonspecific AQP1 inhibitor) did not block or inhibit CO_2 transport across the hRPE apical membrane ($n = 3$), inconsistent with the AQP1 hypothesis. CO_2 barriers have been discovered at the apical membranes of gastric cells and colon

epithelia (Waisbren et al., 1994; Endeward and Gros, 2005), and the RPE may possess a basolateral membrane with a unique lipid composition that limits CO_2 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 HCO_3^- transporters

The DIDS-sensitive $\text{Cl}^-/\text{HCO}_3^-$ exchanger at the basolateral membrane of hRPE was inhibited or activated under acidic (13% apical CO_2) or basic (1% apical CO_2) 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 hRPE. Because this exchanger was inhibited by 13% apical CO_2 , the RPE requires an alternate HCO_3^- efflux pathway at the basolateral membrane to mediate transepithelial HCO_3^- absorption. The following observations indicate the presence of an electrogenic $\text{Na}^+/\text{nHCO}_3^-$ cotransporter at the basolateral membrane: (1) reducing $[\text{HCO}_3^-]$ at the basal bath increased TEP, consistent with a depolarization of the basolateral membrane; (2) this TEP increase was significantly inhibited ($\approx 70\%$) 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 $\text{Na}^+/\text{nHCO}_3^-$ cotransporter in human RPE. Although this cotransporter's identity is unknown, both NBC1 and NBC4 have been shown to transport $\text{Na}^+:\text{HCO}_3^-$ with a stoichiometry of 1:2 (Gross et al., 2001; Virkki et al., 2002), suggesting inward $\text{Na}^+/\text{HCO}_3^-$ cotransport from the basolateral membrane. However, NBC4 transports $\text{Na}^+:\text{HCO}_3^-$ 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 HCO_3^- transport mechanisms (Brown et al., 2004; Praetorius, 2007), it is possible that the RPE expresses NBC4 at the basolateral membrane and transports $\text{Na}^+/\text{nHCO}_3^-$ with a 1:3 $\text{Na}^+:\text{HCO}_3^-$ stoichiometry. In addition, our calculation of the reversal potential of the $\text{Na}^+/\text{nHCO}_3^-$ cotransporter indicates that a 1:3 stoichiometry is required for $\text{Na}^+/\text{nHCO}_3^-$ transport out of the cell; this calculation is

based on our estimation of resting $[\text{Na}]_i$ and pH_i in control Ringer (see Appendix 2).

The basolateral membrane Na/nHCO_3 cotransporter is more dependent on HCO_3 than Na as a substrate, as supported by the following experiments: (1) Reducing basal bath $[\text{HCO}_3]$ 10-fold caused a TEP response that was reduced in the presence of apical DIDS, suggesting that inhibiting NBC1 reduced basolateral Na/nHCO_3 cotransport activity. (2) The basal bath $\Delta[\text{HCO}_3]$ -induced TEP response was reduced in the presence of basal DZA, suggesting that CA inhibition reduces basolateral Na/nHCO_3 cotransport. DZA reduces HCO_3 transport in two ways. First, DZA slows CA-mediated hydration of CO_2 to HCO_3 . Second, DZA inhibits the apical membrane $\text{Na}/2\text{HCO}_3$ cotransporter, as indicated by the reduction of apical bath $\Delta[\text{HCO}_3]$ -induced TEP response in the presence of apical DZA. (3) The basal bath $\Delta[\text{HCO}_3]$ -induced TEP response was increased in 13% apical bath CO_2 and decreased in 1% apical bath CO_2 . This suggests that apical CO_2 entry and its subsequent conversion into HCO_3 is an important source of HCO_3 substrate for basolateral Na/nHCO_3 cotransport activity.

In addition to showing that the basolateral membrane Na/nHCO_3 cotransporter is dependent on apical HCO_3 supply, we also eliminated Na as a limiting substrate for basolateral Na/nHCO_3 cotransport by examining three Na transport proteins at the apical membrane of the RPE (Hughes et al., 1998): (1) bumetanide-sensitive $\text{Na}/\text{K}/2\text{Cl}$ 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 $\Delta[\text{HCO}_3]$ -induced TEP responses, suggesting that these Na transport mechanisms are not linked to basolateral Na/nHCO_3 cotransport activity. Collectively, our data indicate that the basolateral membrane Na/nHCO_3 cotransporter is mainly driven by HCO_3 supplied by NBC1-mediated $\text{Na}/2\text{HCO}_3$ entry and CA II-mediated hydrolysis of CO_2 to HCO_3 .

Apical Na entry pathways and CO_2/HCO_3 -driven fluid transport

13% apical CO_2 increased basolateral Na/nHCO_3 cotransport, which should decrease $[\text{Na}]_i$. However, Na imaging experiments showed that 13% apical CO_2 increased $[\text{Na}]_i$, 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 CO_2 does not activate the Na/H exchanger: (1) the magnitude of the 13% apical CO_2 -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_i in 5 or 13% apical bath CO_2 . These observations indicate that the Na/H exchanger could not have

contributed to the CO_2 -induced $[\text{Na}]_i$ increase. This lack of participation might have occurred for three reasons: (1) the 13% CO_2 -induced acidification was too small; (2) there was no change in the proton gradient across the Na/H exchanger; and (3) the 13% CO_2 -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 NH_4 prepulse that caused only ≈ 0.1 decrease in pH_i ($n = 4$) but still showed the characteristic Na/H exchanger-mediated pH_i recovery; in comparison, 13% apical CO_2 acidified the cell by >0.2 pH units. In addition, we showed that reducing basal bath $[\text{HCO}_3]$ acidified the cell by only ≈ 0.2 but was able to activate the Na/H exchanger. $\text{Na}/2\text{HCO}_3$ entry via NBC1 was eliminated as a possible cause of the 13% apical CO_2 -induced $[\text{Na}]_i$ increase because: (1) the apical DIDS-induced pH_i and TEP responses were the same in 5 or 13% apical bath CO_2 , and (2) the magnitude of the 13% apical CO_2 -induced pH_i response was unaltered in the presence of apical DIDS.

In alveolar epithelium, Na/K ATPase activity is reduced by CO_2 -induced acidification (Briva et al., 2007), suggesting the possibility of a similar effect in RPE. In frog RPE, 13% CO_2 -induced acidification activated the $\text{Na}/\text{K}/2\text{Cl}$ cotransporter after inhibition of the basolateral membrane Cl/HCO_3 exchanger and the subsequent reduction in intracellular $[\text{Cl}]$ (Edelman et al., 1994). Both the 13% apical CO_2 -induced inhibition of the Na/K ATPase and activation of the $\text{Na}/\text{K}/2\text{Cl}$ cotransporter can increase $[\text{Na}]_i$, 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 $\text{Na}/\text{K}/2\text{Cl}$ cotransporter at the apical membrane (Edelman et al., 1994) and by Ca^{2+} -activated and cAMP/PKA-dependent CFTR Cl channels at the basolateral membrane (Joseph and Miller, 1991; Bialek et al., 1995; Hughes et al., 1998). Evidence for the expression and basolateral membrane localization of CFTR in hRPE has been presented (Blaug et al., 2003). The 13% apical CO_2 -induced activation of the $\text{Na}/\text{K}/2\text{Cl}$ cotransporter and inhibition of the Cl/HCO_3 exchanger would both increase net Cl absorption across the RPE.

HCO_3 transport also plays a significant role in RPE fluid transport. Steady-state fluid absorption was decreased by $\approx 50\%$ after the addition of apical DIDS, indicating that the DIDS-sensitive NBC1 mediates HCO_3 -driven fluid transport. The presence of NBC3 at the apical membrane suggests that it also contributes to HCO_3 -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 CO_2 and H_2O that are deposited into the SRS. The RPE uses the limited CO_2 diffusion at the basolateral membrane to drive Na, Cl, and HCO_3^- transport across the RPE, which increases solute-driven fluid transport. This mechanism not only prevents CO_2 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 CO_2 permeability

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

Differentiating the CO_2/HCO_3^- equilibrium constant,

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

Combining the above equations gives

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

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

The relative permeability (P) of CO_2 at the apical versus the basolateral membrane is

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

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% CO_2 -

equilibrated Ringer to the apical and basal bath, respectively. The H^+ fluxes were obtained by multiplying the 13% CO_2 -induced dpH_i/dt with the total buffering capacity of the hRPE. Based on these considerations, the relative permeability of apical versus basolateral membrane of hRPE to CO_2 is 9.9 ± 4.4 ($n = 7$).

Retinal water production by aerobic respiration

In the dark, outer retina O_2 consumption (Wangsa-Wirawan and Linsenmeier, 2003) is $4.2 \pm 0.5 \text{ ml } O_2 \times 100 \text{ g}^{-1} \text{ min}^{-1}$. In the light, outer retina O_2 consumption (Wangsa-Wirawan and Linsenmeier, 2003) is $2.3 \pm 0.6 \text{ ml } O_2 \times 100 \text{ g}^{-1} \text{ min}^{-1}$. 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):

$$0.042 \text{ ml } O_2 \cdot \text{g}^{-1} \text{ min}^{-1} \times 60 \text{ min} \cdot \text{hr}^{-1} \times$$

$$5.44 \text{ g} \times 0.0393 \text{ mM} \cdot \text{ml}^{-1} = 0.54 \text{ mmol } O_2 \cdot \text{hr}^{-1}$$

Oxygen consumption in the light:

$$0.023 \text{ ml } O_2 \cdot \text{g}^{-1} \text{ min}^{-1} \times 60 \text{ min} \cdot \text{hr}^{-1} \times$$

$$5.44 \text{ g} \times 0.0393 \text{ mM} \cdot \text{ml}^{-1} = 0.29 \text{ mmol } O_2 \cdot \text{hr}^{-1}$$

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

$$0.54 \text{ mmol } O_2 \cdot \text{hr}^{-1} \times 18 \text{ mg} \cdot \text{mmol}^{-1} \times$$

$$1 \mu\text{l} \cdot \text{mg}^{-1} = 9.72 \mu\text{l } H_2O \cdot \text{hr}^{-1}$$

Water generated in the light is:

$$0.29 \text{ mmol } H_2O \cdot \text{hr}^{-1} \times 18 \text{ mg} \cdot \text{mmol}^{-1} \times$$

$$1 \mu\text{l} \cdot \text{mg}^{-1} = 5.22 \mu\text{l } H_2O \cdot \text{hr}^{-1}$$

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:

$$\frac{9.72 \mu\text{l } H_2O / \text{hr}}{10.94 \text{ cm}^2} = 0.89 \mu\text{l } H_2O \cdot \text{cm}^{-2} \cdot \text{hr}^{-1}$$

In the light:

$$\frac{5.31 \mu\text{l } H_2O / \text{hr}}{10.94 \text{ cm}^2} = 0.48 \mu\text{l } H_2O \cdot \text{cm}^{-2} \cdot \text{hr}^{-1}$$

Total retinal water production in the light and dark

For every glucose molecule that undergoes aerobic respiration, six molecules of CO_2 are produced. Therefore, glucose consumption by aerobic respiration in the dark is:

$$0.54 \text{ mmol} \cdot \text{hr}^{-1} \times \frac{1 \text{ glucose}}{6 \text{ CO}_2} = 0.09 \text{ mmol} \cdot \text{hr}^{-1}$$

Glucose consumption by aerobic respiration in the light is:

$$0.29 \text{ mmol} \cdot \text{hr}^{-1} \times \frac{1 \text{ glucose}}{6 \text{ CO}_2} = 0.05 \text{ mmol} \cdot \text{hr}^{-1}$$

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:

$$0.09 \text{ mmol} \cdot \text{hr}^{-1} \times \frac{95}{5} \times \frac{2 \text{ H}_2\text{O}}{1 \text{ glucose}} \times \frac{18 \mu\text{l}}{1 \text{ mmol}} \times \frac{1}{10.94 \text{ cm}^2} = 5.6 \mu\text{l} \cdot \text{cm}^{-2} \cdot \text{hr}^{-1}$$

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

$$0.05 \text{ mmol} \cdot \text{hr}^{-1} \times \frac{95}{5} \times \frac{2 \text{ H}_2\text{O}}{1 \text{ glucose}} \times \frac{18 \mu\text{l}}{1 \text{ mmol}} \times \frac{1}{10.94 \text{ cm}^2} = 3.1 \mu\text{l} \cdot \text{cm}^{-2} \cdot \text{hr}^{-1}$$

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

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

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

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

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

CO₂ production in the light and dark

CO₂ production = O₂ consumption. CO₂ production in the dark is $4.2 \pm 0.5 \text{ ml CO}_2 \times 100 \text{ g}^{-1} \text{ min}^{-1}$, and in light is $2.3 \pm 0.6 \text{ ml O}_2 \times 100 \text{ g}^{-1} \text{ min}^{-1}$. Therefore, CO₂ production increases by 1.4–2.6-fold after transitioning from light to dark. This increase in CO₂ production translates to an increase in SRS CO₂ concentration, from 5 to $10 \pm 3\%$.

APPENDIX 2

Na/nHCO₃ cotransporter reversal potential calculation

$$E_{NBC} = \frac{2.3RT}{F(n-1)} \log \left(\frac{[Na^+]_{in}}{[Na^+]_{out}} \right) \left(\frac{[HCO_3^-]_{in}}{[HCO_3^-]_{out}} \right)^n$$

$[Na^+]_{in} = 15.7 \text{ mM}$, $[Na^+]_{out} = 143.7 \text{ mM}$, $[HCO_3^-]_{in} = 27.9 \text{ mM}$, and $[HCO_3^-]_{out} = 26.2 \text{ mM}$. n is the stoichiometry of the Na/nHCO₃ cotransporter. We calculated the reversal potential of the Na/nHCO₃ cotransporter NBC (E_{NBC}) to be -55.7 mV for a Na:HCO₃ transport stoichiometry of 1:2. In this case, E_{NBC} is more hyperpolarized than the average basolateral membrane potential ($V_B = -49.8 \pm 3.7 \text{ mV}$) (Maminishkis et al., 2006), and the Na/2HCO₃ cotransporter transports Na and HCO₃ into the cell. To transport Na/nHCO₃ out of the cell against the strong inward Na gradient in control conditions, E_{NBC} must be more depolarized than V_B , and this condition is achieved for a Na:HCO₃ transport stoichiometry of 1:3, where $E_{NBC} = -27.0 \text{ mV}$.

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