Apical Electrogenic $NaHCO_3$ Cotransport

A Mechanism for HCO_3 Absorption across the Retinal Pigment Epithelium

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ABSTRACT Intracellular microelectrode techniques and intracellular pH (pH_i) measurements using the fluorescent dye 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) were employed to characterize an electrogenic bicarbonate transport mechanism at the apical membrane of the frog retinal pigment epithelium (RPE). Reductions in apical concentrations of both $[HCO_3]_o$ (at constant P_{CO_2} or pH_o) or $[Na]_o$ caused rapid depolarization of the apical membrane potential (V_{ap}). Both of these voltage responses were inhibited when the concentration of the other ion was reduced or when 1 mM diisothiocyano-2-2 disulfonic acid stilbene (DIDS) was present in the apical bath. Reductions in apical $[HCO_3]_o$ or $[Na]_o$ also produced a rapid acidification of the cell interior that was inhibited by apical DIDS. Elevating pH_i at constant P_{CO_2} (and consequently $[HCO_3]_i$) by the addition of apical NH_4 (20 mM) produced an immediate depolarization of V_{ap}. This response was much smaller when either apical $[HCO_3]_o$ or $[Na]_o$ was reduced or when DIDS was added apically. These results strongly suggest the presence of an electrogenic $NaHCO_3$ contransporter at the apical membrane. Apical DIDS rapidly depolarized V_{ap} by 2–3 mV and decreased pH_i (and $[HCO_3]_i$), indicating that the transporter moves $NaHCO_3$ and net negative charge into the cell. The voltage dependence of the transporter was assessed by altering V_{ap} with transepithelial current and then measuring the DIDS-induced change in V_{ap}. Depolarization of V_{ap} increased the magnitude of the DIDS-induced depolarization, whereas hyperpolarization decreased it. Hyperpolarizing V_{ap} beyond -114 mV caused the DIDS-induced voltage change to reverse direction. Based on this reversal potential, we calculate that the stoichiometry of the transporter is 1.6–2.4 (HCO_3/Na).

INTRODUCTION

The retinal pigment epithelium (RPE) is a single layer of cells that forms an important part of the blood–retina barrier and provides the major transport pathway

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responsible for the exchange of fluid, ions, and metabolites between the photoreceptors and the choroidal blood supply. The RPE transports fluid in the retina-to-choroid direction (Miller et al., 1982; Tsuboi, 1987), and this is believed to be an important factor in retinal adhesion (Marmor et al., 1980). In bullfrog RPE, a major driving force for fluid absorption is active HCO_3 transport (Hughes et al., 1984, 1988) but the membrane mechanisms that mediate transepithelial HCO_3 movement have not yet been identified. These transporters are likely to be important in two ways: (*a*) for driving fluid out of the extracellular space that the separates the photoreceptor outer segments and the apical membrane and (*b*) for controlling the pH in this space.

It has been shown in several species that the apical membrane potential of the RPE is sensitive to changes in extracellular bicarbonate concentration (Lasansky and De Fisch, 1966; Miller and Steinberg, 1977*a*; Steinberg et al., 1978; Tsuboi et al., 1986; Joseph and Miller, 1986). In the simplest case, this effect could be due to a HCO_3 conductance in the apical membrane (Miller and Steinberg, 1977*a*; Saito and Wright, 1984; Kaila and Voipio, 1987), or it could be mediated by an electrogenic $NaHCO_3$ cotransporter similar to those found in the basolateral membrane of other epithelia (Boron and Boulpaep, 1983; Curci et al., 1987). The observed voltage responses might also be produced, at least in part, by other mechanisms, such as pH-sensitive channels or pumps.

In the present study, we characterized the electrogenic HCO_3 transport pathway using conventional electrophysiological techniques and fluorescence microscopy measurements. We provide evidence that the apical membrane of the RPE has an electrogenic $NaHCO_3$ transport mechanism that moves Na, HCO_3, and net negative charge into the cell under control conditions and is inhibited by diisothiocyano-2-2 disulfonic acid stilbene (DIDS). From its apparent reversal potential of -114 mV, we calculate the HCO_3/Na stoichiometry of this transporter to have an upper bound of 2.4 and a lower bound of 1.6. The voltage dependence of this transporter has important physiological implications because the RPE responds electrically to changes in retinal activity (Steinberg et al., 1985). Some of this work has been presented previously in a preliminary form (Hughes et al., 1987*a*; Adorante et al., 1988).

METHODS

Medium-size bullfrogs, *Rana catesbeiana,* of both sexes were obtained from Central Valley Biologicals (Clovis, CA) or Western Scientific (Sacramento, CA) and maintained in running tap water at room temperature for at least 1 wk before use. The frogs were fed live crickets daily during captivity. The RPE-choroid was isolated from dark-adapted eyes as previously described and mounted as a flat sheet in a chamber that allowed the two sides of the tissue to be perfused independently (Hughes et al., 1988). Solutions were delivered to the chamber by gravity via CO_2-impermeable tubing (Saran; Clarkson Equipment and Controls, Detroit, MI). Separating the solution reservoirs and chamber was an open manifold (~250 μl) that served to maintain a constant hydrostatic head during solution changes (Joseph and Miller, manuscript submitted for publication). Since solution changes were made at the manifold, there was a 10–40-s delay before the dead space cleared and the new solution reached the tissue. In all figures, the horizontal bar indicates when solution changes were made at the manifold.

The composition of the apical perfusion solutions is listed in Table I. All chemicals were

obtained from Sigma Chemical Co. (St. Louis, MO). Bicarbonate-containing solutions were gassed with 95% O_2/5% CO_2, except where noted in text. Solutions buffered with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) were gassed with 100% O_2. The pH of solutions 1, 3, 5, 6, 8, 9, 11, and 12 was 7.4 ± 0.01, solutions 2, 7, and 10 had a pH of 6.4 ± 0.02, and the pH of solution 4 was 7.8 ± 0.02. Unless otherwise noted in text, the basal side of the isolated RPE-choroid was perfused continuously with solution 1.

Electrophysiology

Conventional microelectrodes were drawn from thick-walled, fiber-filled borosilicate glass tubing having an inside diameter of 0.5 mm and an outer diameter of 1 mm (Omega Dot; Glass Company of America, Bargaintown, NJ). Micropipettes were pulled on a horizontal puller (model P-77; Sutter Instrument Co., San Francisco, CA) and had a tip resistance of 80–120 MΩ when filled with 150 mM KCl.

The techniques used for electrophysiological recordings with conventional microelectrodes were similar to those used in previous studies (Hughes et al., 1988). All experiments were

TABLE I

Solutions

Solution	Na	K	*N*-methyl-D-glucamine	NH_4	Ca	Mg	Cl	HCO_3	Cyclamate	HEPES	Glucose
						mM					
1	110.0	2.0	—	—	1.8	1.0	90.1	27.5	—	—	10.0
2	110.0	2.0	—	—	1.8	1.0	90.1	2.75	24.75	—	10.0
3	110.0	2.0	—	—	1.8	1.0	62.6	27.5	27.5	—	10.0
4	110.0	2.0	—	—	1.8	1.0	62.6	55.0	—	—	10.0
5	110.0	2.0	—	—	1.8	1.0	90.1	—	17.5	10.0	10.0
6	—	2.0	110.0	—	1.8	1.0	90.1	27.5	—	—	10.0
7	—	2.0	110.0	—	1.8	1.0	90.1	2.75	24.75	—	10.0
8	90.0	2.0	—	20.0	1.8	1.0	90.1	27.5	—	—	10.0
9	—	2.0	90.0	20.0	1.8	1.0	90.1	27.5	—	—	10.0
10	90.0	2.0	—	20.0	1.8	1.0	90.1	2.75	24.75	—	10.0
11	102.0	10.0	—	—	1.8	1.0	90.1	27.5	—	—	10.0
12	111.8	0.2	—	—	1.8	1.0	90.1	27.5	—	—	10.0

carried out in a newly designed chamber (Joseph and Miller, manuscript submitted for publication) that allowed more rapid exchange of perfusion fluid (10–20 chamber vol/min). In both the apical and basal compartments of the recording chamber, a calomel electrode made electrical contact with the perfusion solution via a Ringer-agar bridge. In Na-substitution experiments, chlorided silver wires were used as reference electrodes to avoid liquid junction potentials. The transepithelial potential (TEP) (apical-side positive) was recorded differentially between the two calomel electrodes. Membrane potentials were recorded using micropipette electrodes reference to either the apical or basal calomel electrode. When referred to the apical calomel electrode, the voltage measured was the apical membrane potential, V_{ap}, and when referred to the basal calomel electrode, the voltage measured was the basal membrane potential, V_{ba}. The transepithelial resistance (R_t) and the apparent ratio of the apical to basolateral membrane resistance, R_{ap}/R_{ba} (*a* value), were obtained by passing 2-μA current pulses across the tissue and monitoring the voltage responses across the apical and basolateral membranes (Miller and Steinberg, 1977*a*; Hughes et al., 1988). All voltage signals were digitized at a rate of 4 Hz and stored on a microcomputer for subsequent analysis. In several figures

(Figs. 2, 5, 6, and 8 *A*), a slight ripple is present in the voltage records. This is due to the digital subtraction of superimposed voltage deflections caused by the bipolar current pulses used to measure R_t and the apparent a value.

Cell pH Measurement

The RPE-choroid was mounted in a specially designed chamber on the stage of an inverted microscope (Diaphot-TMD; Nikon, Inc., Garden City, NY) equipped for epifluorescent illumination. This chamber allowed constant perfusion of the apical and basolateral bathing solutions and the measurement of TEP and R_t. The RPE cells were brought into focus through a water immersion objective lens (Achromat 40/0.75; Carl Zeiss, Inc., Thornwood, NY) with a working distance of 1.6 mm. This objective faced the apical bathing solution, which was 0.35 mm deep, and covered with a glass coverslip ≈0.1 mm thick.

The pH-sensitive dye 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF; Molecular Probes, Inc., Eugene, OR) was used along with a ratioing fluorimetry technique (Heiple and Taylor, 1982) to measure the fluorescence intensity ratio of intracellular dye (Rink et al., 1982). BCECF has a peak excitation at 504 nm that is pH sensitive, and an isoexcitation point at 436 nm, where fluorescence excitation is independent of pH (Alpern, 1985). These properties of the dye were used to measure the fluorescence intensity ratio (Ex) 480 nm/440 nm, which is linear over a wide pH range (6.4–7.5) and invariant with respect to changes in optical path length and dye concentration (Heiple and Taylor, 1982). The excitation wavelength was alternated at 1 Hz between 440 and 480 nm using interference filters with a bandwidth of 10 nm (Omega Optical, Inc., Brattleboro, Vermont) and the epifluorescent emission was measured between 520 and 560 nm. The voltage and fluorescence signals were digitized and stored on a microcomputer for later analysis. A more detailed description of this technique and the data acquisition system is available from Dr. Chester Regan, 203 S. Coler Ave. No. 3, Urbana, IL 61801.

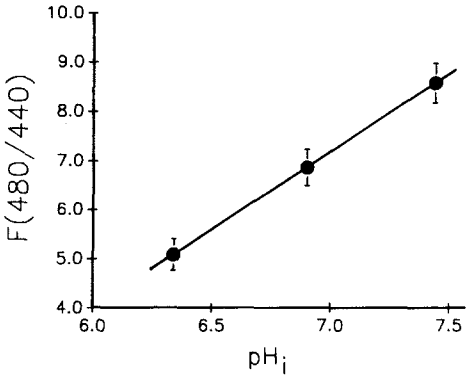

FIGURE 1. In situ calibration curve for intracellular BCECF. Closed circles represent the mean steady-state fluorescence ratio for five tissues. Error bars represent SEM. F, fluorescence.

At the beginning of each experiment, fluorescence was measured from the unloaded tissue and was later subtracted from the total emission signal. Control Ringer (Table I, solution 1) containing 13 μM of the acetomethyl derivative of BCECF (BCECF-AM) was then perfused through the apical bath for 20–30 min (flow rate > 50 chamber vol/min). Fluorescence was monitored continuously during this time. The impermeant dye (BCECF) was presumably generated intracellularly by the action of cytoplasmic esterase (Rink et al., 1982). After this period of BCECF loading, control Ringer was perfused through the chamber for 10–15 min to wash out the extracellular dye.

It seemed possible that some of the fluorescence signal originated from cells located in the

choroid on the basal side of the tissue. This could have occurred by leakage of dye around the edge of the tissue or through the junctional complex that surrounds each cell. This possibility was tested by perfusing the dye (BCECF-AM) into the basal chamber and continuously monitoring the fluorescence. This procedure should produce a choroidal signal much larger than that due to leakage from the apical side. After 25 min the extracellular dye was washed out from the basal chamber. For comparison, we then apically perfused the same tissue with dye, again for 25 min. This produced a normal signal, which was 13 times larger than that produced by basal loading. Therefore, the contribution from the choroidal cells must be considerably <7% of the total signal.

At the end of each experiment a calibration was performed by the method of Thomas et al. (1979). The calibration solutions contained 10 mM HEPES-NaOH, 105 mM KCl (to match the RPE intracellular K activity; Oakley et al., 1978; La Cour et al., 1986; Hughes et al., 1988), 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 13 μM nigericin (Sigma Chemical Co.), a K-H antiporter. The pH of these solutions was varied by HCl addition, and under these conditions intracellular and extracellular pH should be equal. The in situ calibration curve for five tissues is shown in Fig. 1.

During and after BCECF perfusion, TEP and R_t remained constant. The loading of BCECF did not alter the TEP and R_t responses to apical changes of $[Na]_o$, $[K]_o$, $[HCO_3]_o$, NH_4, or DIDS. This indicates that incorporation of dye did not alter the transport or electrical properties of the RPE.

RESULTS

Response of V_{ap} to Changes in Apical $[HCO_3]_o$

Fig. 2 *A* shows a typical membrane voltage response to a decrease in bicarbonate concentration outside the apical membrane. Decreasing apical $[HCO_3]_o$ from 27.5 to 2.75 mM at constant P_{CO_2} caused V_{ap} to rapidly depolarize and then slowly repolarize after reaching a peak. When apical $[HCO_3]_o$ was returned to 27.5 mM, V_{ap} underwent a sharp hyperpolarization and then slowly repolarized back to its initial level. These results are compatible with the presence at the apical membrane of a simple bicarbonate conductance but as shown below, they are actually mediated by a more complex transport mechanism.

Presumably, the initial V_{ap} depolarization was a direct result of the change in the transmembrane HCO_3 driving force on an electrogenic transport mechanism. The slower repolarization that followed the peak depolarization was probably due to secondary effects on the cotransporter and/or other transport pathways caused, for example, by changes in pH_i, $[HCO_3]_i$, or $[Na]_i$ (see below). Although limited by secondary effects, the initial peak voltage changes most likely reflect the changes in activity of the HCO_3-sensitive electrogenic transporter.

Similar but smaller voltage changes also occurred at the basal membrane and these were mainly the result of current shunted from the apical membrane via the paracellular pathway (Miller and Steinberg, 1977*a*).

The effect of increasing apical $[HCO_3]_o$ from 27.5 to 55 mM at constant P_{CO_2} is shown in Fig. 2 *B*. The increase in apical $[HCO_3]_o$ caused V_{ap} to rapidly hyperpolarize and then slowly repolarize to a value more negative than the control value. When the apical perfusion solution was returned to 27.5 mM HCO_3, V_{ap} underwent a sharp depolarization and then repolarized to the control level.

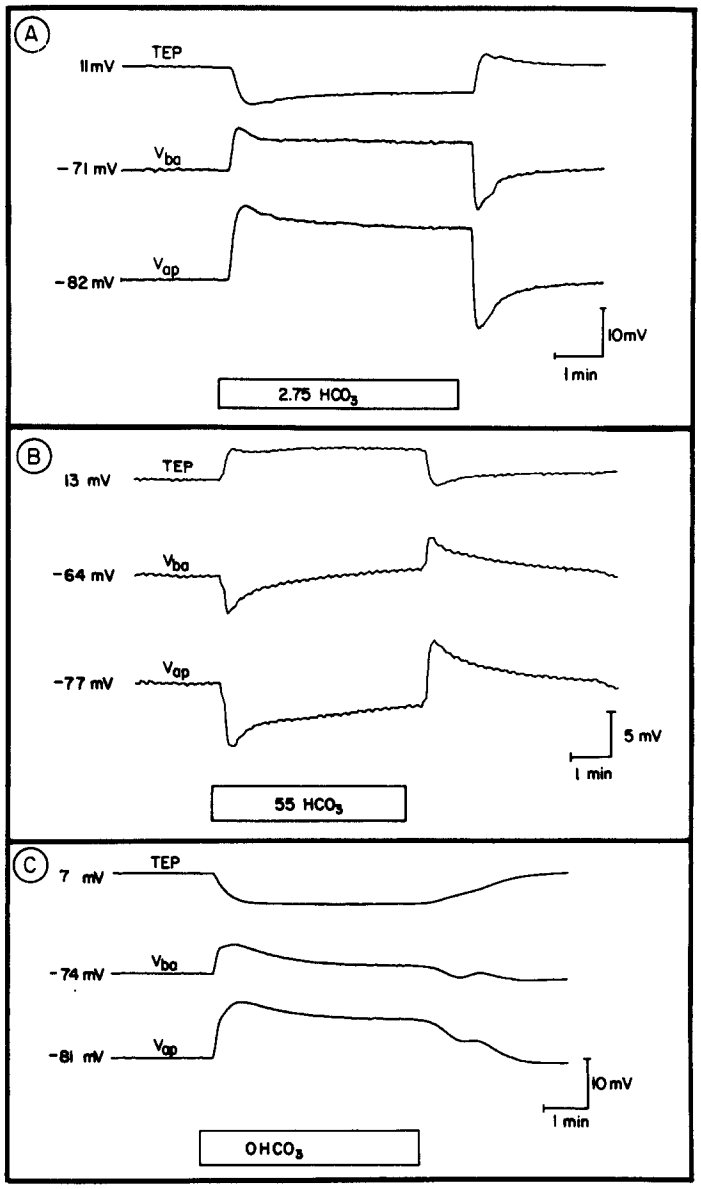

FIGURE 2. Effects of changing apical $[HCO_3]_o$. TEP, V_{ba}, and V_{ap} denote transepithelial potential, basal membrane potential, and apical membrane potential, respectively. Values in front of each trace indicate voltages at the beginning of the record. (*A*) Reducing apical $[HCO_3]_o$ 10-fold at constant P_{CO_2}, caused a rapid depolarization of V_{ap} followed by a partial repolarization. See Table II. Horizontal bar at bottom indicates when control Ringer (solution 1, Table I) was replaced with 2.75 mM HCO_3 Ringer (solution 2) at the manifold (see Methods). (*B*) Increasing apical $[HCO_3]_o$ from 27.5 (solution 3) to 55 mM (solution 4) at constant P_{CO_2} caused a rapid hyperpolarization, followed by a partial repolarization. See Table II. (*C*) Reducing apical $[HCO_3]_o$ at constant pH caused a rapid depolarization of V_{ap}. Horizontal bar indicates when HCO_3-buffered Ringer (solution 1) was replaced with HEPES-buffered Ringer gassed with 100% O_2 (solution 5). See Table III.

TABLE II

Effect of Changes in Apical $[HCO_3]$ at Constant P_{CO_2}

Condition	V_{ap}	V_{ba}	TEP	a	R_t
		mV			$\Omega \cdot cm^2$
27.5 mM HCO_3	−79.2 ± 0.8	−69.6 ± 0.5	9.5 ± 1.0	0.41 ± 0.03	266 ± 26
2.75 mM HCO_3	−66.8 ± 0.6	−62.5 ± 0.6	4.2 ± 1.0	0.82 ± 0.01	270 ± 29
$\lvert\Delta\rvert$	12.4 ± 0.6‖	7.1 ± 0.3‖	5.3 ± 0.6‖	0.41 ± 0.08‖	4 ± 5*
	(14)	(14)	(14)	(9)	(9)
27.5 mM HCO_3	−77.5 ± 3.1	−67.2 ± 2.8	10.3 ± 1.8	0.51 ± 0.04	291 ± 14
55 mM HCO_3	−84.4 ± 3.0	−71.6 ± 2.8	12.8 ± 2.3	0.37 ± 0.03	283 ± 13
$\lvert\Delta\rvert$	6.9 ± 0.3‖	4.4 ± 0.8‡	2.5 ± 0.5‡	0.14 ± 0.01‖	8 ± 4*
	(4)	(4)	(4)	(5)	(5)

Data shown are means ± SEM obtained under steady-state conditions for 27.5 mM HCO_3, at the peak voltage after a change to 2.75 or 55 mM HCO_3, or absolute differences ($\lvert\Delta\rvert$). Numbers in parentheses indicate number of experiments. *Not significant; ‡$P < 0.02$; ‖$P < 0.001$.

The results of several similar experiments are summarized in Table II. A 10-fold reduction in apical $[HCO_3]_o$ produced a peak V_{ap} depolarization that averaged 12 ± 0.2 mV (±SEM, $n = 14$), whereas increasing apical $[HCO_3]_o$ twofold caused a peak V_{ap} hyperpolarization that averaged 6.9 ± 0.3 mV ($n = 4$). Table II also shows that changes in apical $[HCO_3]_o$ significantly altered the ratio of apical to basolateral membrane resistances (a value). The a value doubled when apical $[HCO_3]_o$ was reduced by a factor of 10 and decreased by 21% when apical $[HCO_3]_o$ was doubled.

These results agree with those reported previously (Miller and Steinberg, 1977*a*) and are compatible with the presence at the apical membrane of an electrogenic bicarbonate transport mechanism. Since the alterations in apical $[HCO_3]_o$ were made at constant P_{CO_2} (Fig. 2, *A* and *B*), there were accompanying changes in extracellular pH that could have affected a membrane conductance to bring about the observed voltage changes. To address this possibility, we replaced the HCO_3/CO_2-buffered Ringer (solution 1) at constant extracellular pH with nominally HCO_3 and CO_2-free Ringer (solution 5). This caused V_{ap} to undergo a biphasic change in voltage whose magnitude and time course was similar to that produced by the reduction in apical $[HCO_3]_o$ at constant P_{CO_2} (Fig. 2 *C*). The results of several similar experiments are summarized in Table III. Removing apical $[HCO_3]_o$ at constant pH produced a peak depolarization that averaged 13.9 ± 1.2 mV (mean ± SEM, $n = 5$). As

TABLE III

Effect of Changes in Apical $[HCO_3]$ at Constant pH

Condition	V_{ap}	V_{ba}	TEP	a	R_t
		mV			$\Omega \cdot cm^2$
27.5 mM HCO_3	−83.6 ± 1.7	−74.5 ± 0.7	9.0 ± 1.5	0.40 ± 0.04	238 ± 35
0 mM HCO_3	−69.7 ± 0.6	−68.4 ± 0.5	1.2 ± 0.8	1.26 ± 0.16	252 ± 31
$\lvert\Delta\rvert$	13.9 ± 1.2‖	6.1 ± 0.3‖	7.8 ± 1.0‡	0.85 ± 0.12‡	13 ± 6*
	(5)	(5)	(5)	(4)	(4)

Symbols and protocol are the same as in Table II. *Not significant; ‡$P < 0.01$; ‖$P < 0.001$.

in the reduction of $[HCO_3]_o$ at constant P_{CO_2}, HCO_3 removal resulted in a significant increase in the apparent a value.

When apical $[HCO_3]_o$ was decreased at either constant pH or constant P_{CO_2} (Fig. 2, *A* and *C*), an inward-directed proton electrochemical gradient was established and, if the apical membrane had a large proton conductance, this could have contributed to the observed voltage responses. Therefore, the effects of extracellular pH changes were examined on RPE-choroid preparations bathed on both sides with HEPES-buffered Ringer equilibrated with 100% O_2 (solution 5). Decreasing the pH of the apical solution from 7.4 to 6.4 caused a 2–4 mV depolarization of V_{ap} ($n = 3$), whereas increasing the apical pH from 7.4 to 8.4 hyperpolarized V_{ap} by ~2 mV ($n = 3$; not shown). These results strongly suggest that changes in extracellular pH make a relatively minor contribution to the HCO_3-induced voltage changes.

K Conductance and Na/K Pump Contributions to the HCO_3 Response

The RPE apical membrane contains two other mechanisms that determine V_{ap}, a relatively large K conductance ($T_k > 0.5$) and an electrogenic Na/K pump (Miller

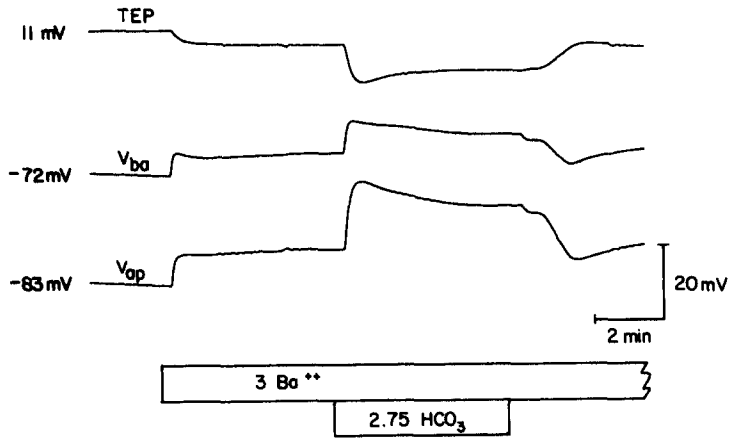

FIGURE 3. Effects of barium on the HCO_3-induced voltage response. Apical barium (3 mM) depolarized V_{ap} due to the blockage of the apical membrane K conductance. The subsequent reduction of apical $[HCO_3]_o$ from 27.5 to 2.75 mM (solutions 1 and 2) had its usual effect on V_{ap}. Abbreviations as in Fig. 2.

and Steinberg, 1977*b*; Miller et al., 1978). It is possible that one or both of these mechanisms could underlie the voltage changes that immediately follow alterations in apical $[HCO_3]_o$. For example, decreasing apical $[HCO_3]_o$ could cause a pH-dependent decrease in K conductance, which would shift V_{ap} away from E_K and thereby depolarize V_{ap}. To exclude any possible contribution from the K conductance, we measured the voltage response to changes in apical $[HCO_3]_o$ before and after blocking the K conductance with barium (Hughes et al., 1988; Joseph and Miller, 1986). Fig. 3 shows that the apical perfusion of 3 mM $BaCl_2$ (substituted for 4.5 mM NaCl) caused V_{ap} to depolarize by ~8 mV. This concentration of barium is

sufficient to block the apical membrane voltage response to step changes in apical $[K]_o$ (Griff et al., 1985; Hughes et al., 1988). The second part of Fig. 3 shows that a 10-fold reduction in apical $[HCO_3]_o$ caused V_{ap} to depolarize by ~18 mV. The magnitude of this HCO_3 response is significantly larger than the control response for the same tissue (10 mV; not shown) and this is probably due to a barium-induced increase in apical membrane resistance (Hughes et al., 1988). Therefore, the initial phase of the HCO_3-induced voltage change is not mediated by the apical K conductance.

It is also possible that changes in apical $[HCO_3]_o$ lead to intracellular ion activity changes that modulate Na/K pump rate to produce the observed voltage changes. This possibility was tested by examining the voltage response to changes in apical $[HCO_3]_o$ in the presence of apical ouabain. The inset of Fig. 4 shows that apical ouabain (0.1 mM) caused a rapid depolarization of V_{ap}, which is due to inhibition of

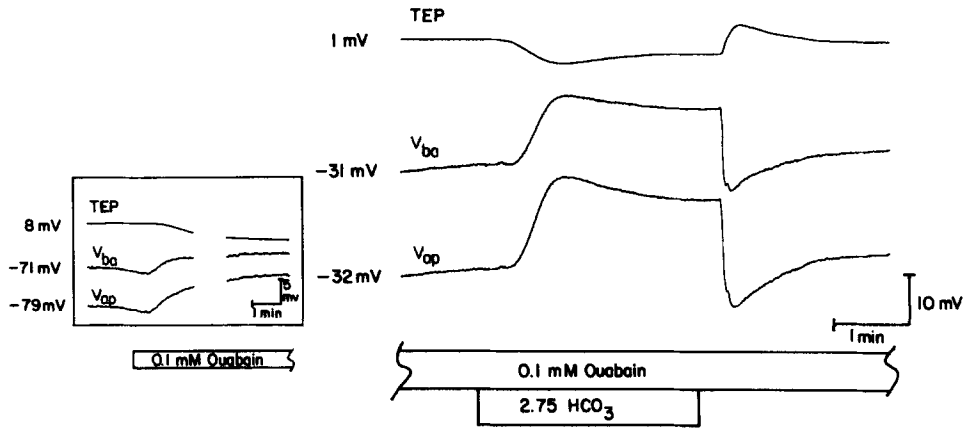

FIGURE 4. Effects of ouabain on the HCO_3-induced voltage response. (*Inset*) Perfusion of ouabain (0.1 mM) to the apical side depolarized V_{ap} due to the inhibition of the electrogenic Na/K pump. The reduction in apical $[HCO_3]_o$ from 27.5 to 2.75 mM 30 min later produced a normal voltage response. Abbreviations as in Fig. 2.

the Na/K pump current (Miller et al., 1978). Approximately 30 min later, when the membrane voltages had decreased even further, a 10-fold decrease in apical $[HCO_3]_o$ caused V_{ap} to transiently depolarize by ~17 mV. Since the amplitude of the HCO_3-induced voltage change was undiminished by ouabain, we conclude that the Na/K pump does not underlie the voltage response to changes in apical $[HCO_3]_o$.[1]

Effect of HCO_3 Transport Inhibitors

The results presented thus far are compatible with the presence of an electrogenic bicarbonate transport system at the apical membrane. To characterize this mecha-

[1] Additional experiments were performed with solutions in which apical $[K]_o$ was lowered from 2 to 0.1 mM to inhibit the Na/K pump (Oakley et al., 1978; Miller and Steinberg, 1982). These maneuvers, which were performed in the presence of 1 mM Ba in order to block the K channels, did not reduce the size of the HCO_3 responses.

nism more fully, we investigated the effect of several inhibitors of HCO_3 transport. Apical acetazolamide (0.1 mM), which inhibits an electrogenic HCO_3 transport mechanism in the proximal tubule (Biagi and Sohtell, 1986), had no effect on the HCO_3-induced voltage response in the RPE (not shown). On the other hand, the disulfonic stilbene DIDS significantly attenuated the response to changes in apical $[HCO_3]_o$.

Fig. 5 shows that adding DIDS (1 mM) to the apical bathing solution caused an immediate depolarization of V_{ap}, consistent with the inhibition of a hyperpolarizing mechanism. When a 10-fold decrease in apical $[HCO_3]_o$ (constant P_{CO_2}) was made after ~5 min exposure to apical DIDS, the peak depolarization of V_{ap} was ~50% smaller than the control response in the same tissue (7 vs. 15 mV). The degree of

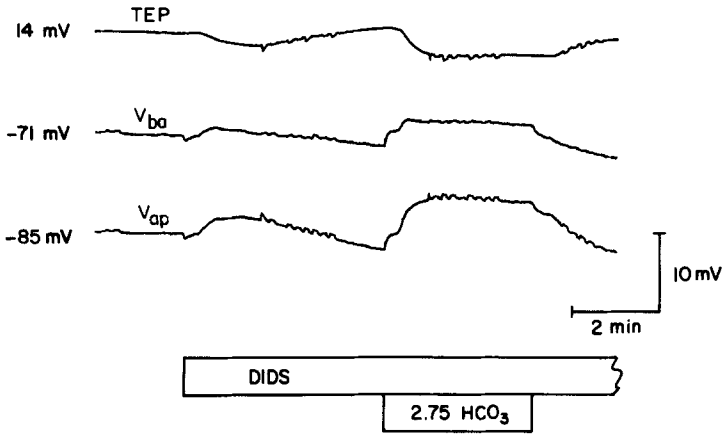

FIGURE 5. Effects of reducing apical $[HCO_3]_o$ in the presence of DIDS. This record was obtained from the same cell as that shown in Fig. 2 *A*. Perfusion of DIDS (1 mM) to the apical side depolarized V_{ap}. In the presence of DIDS, the reduction of apical $[HCO_3]_o$ from 27.5 to 2.75 mM (solutions 1 and 2) produced a smaller voltage response than control. The small depolarizations of V_{ap} and V_{ba} that occurred immediately upon switching the perfusion solution from control to DIDS Ringer were due to a hydrostatic pressure change across the tissue that increased the shunt resistance. Abbreviations as in Fig. 2.

inhibition depended on how long the tissue was exposed to DIDS. For example, in another tissue, a 5-min exposure to DIDS inhibited the voltage response to a $[HCO_3]_o$ decrease by ~60%. However, after 14 min of apical DIDS exposure, the HCO_3 response was completely blocked. DIDS was also effective in inhibiting the voltage response to increases in apical $[HCO_3]_o$. After 5 min of exposure to apical DIDS, the amplitude of the peak hyperpolarization induced by elevating apical $[HCO_3]_o$ from 27.5 to 55 mM averaged 1.3 ± 0.1 mV (n = 3), an 80% inhibition (compared with the data in Table II).

If the electrogenic HCO_3 transport mechanism is conductive, then its inhibition by DIDS should produce an increase in apical membrane resistance. This prediction was tested in the experiment illustrated in Fig. 6. The addition of apical DIDS (t = 1 min) caused rapid increases in R_t and the apparent ratio of R_{ap}/R_{ba} (a value), and the

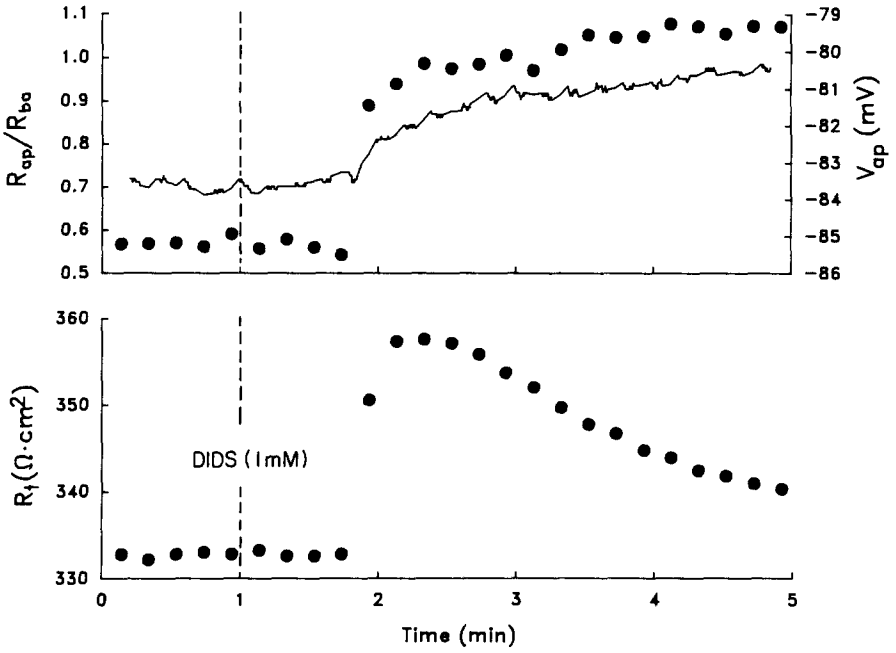

FIGURE 6. Time course of DIDS-induced changes in membrane voltage and resistance. (*Upper panel*) V_{ap} (*continuous trace*) and R_{ap}/R_{ba} (●). (*Lower panel*) R_t. Vertical interrupted line indicates the start of apical DIDS perfusion (1 mM).

time course of these resistance changes coincided with the depolarization of V_{ap}. In 10 experiments, DIDS increased the apparent a value from 0.55 ± 0.04 to 1.07 ± 0.11 ($P < 0.001$, paired t test) and increased R_t from 272 ± 23 to 288 ± 22 $\Omega \cdot cm^2$ (not significant). These results suggest that the primary effect of DIDS is to block the HCO_3-dependent apical membrane conductive mechanism (see below).

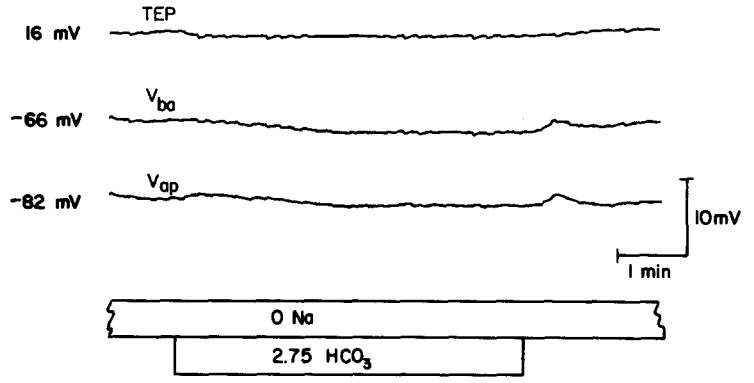

FIGURE 7. Effects of reducing apical $[HCO_3]_o$ in the absence of apical Na. Apical side had been perfused with Na-free Ringer (solution 6) for 2 min before the start of this record. Reducing apical $[HCO_3]_o$ from 27.5 to 2.75 mM (solution 7) in Na-free Ringer produced no significant voltage change. Abbreviations as in Fig. 2.

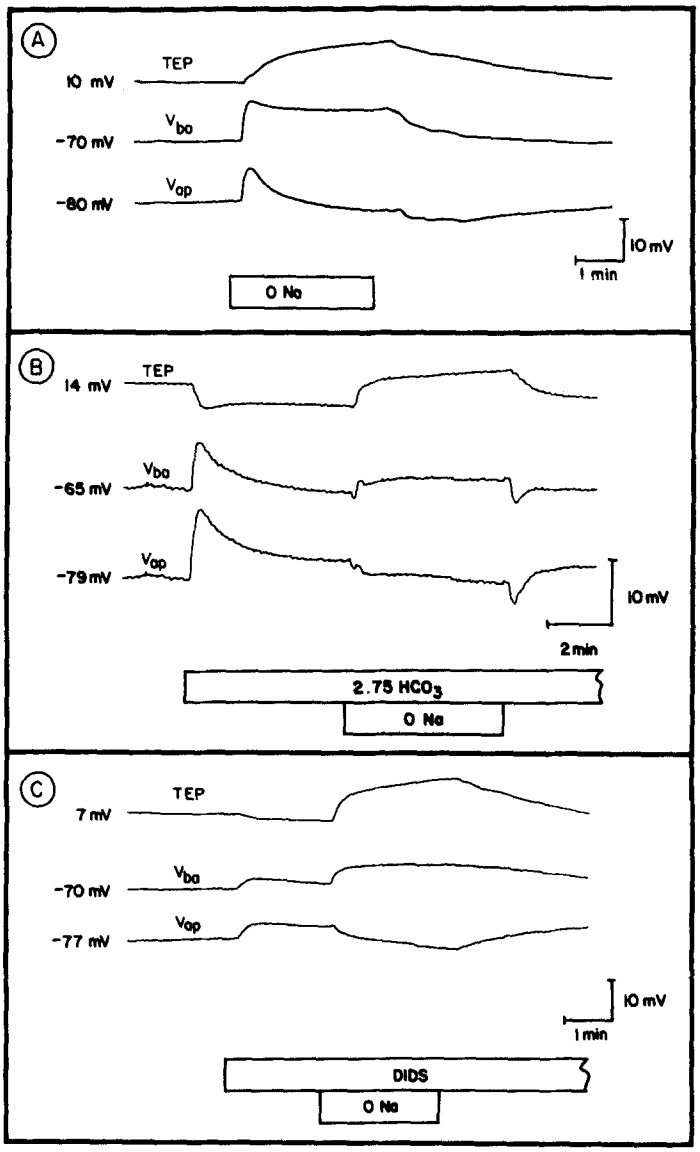

FIGURE 8. Effects of apical Na removal on membrane voltage. (*A*) Complete Na removal from the apical side in the presence of apical HCO_3 (solution 6) produced an immediate depolarization of V_{ap}. See Table IV. (*B*) The effects of low $[HCO_3]_o$ on the Na-induced voltage changes. The reduction of apical $[HCO_3]_o$ from 27.5 to 2.75 mM (solutions 1 and 2) produced typical depolarizations of V_{ap} and V_{ba}. When a Na-free solution (solution 7) was subsequently perfused to the apical side, there was no immediate depolarization of V_{ap}. (*C*) The effect of DIDS on the Na-induced voltage changes. Perfusion of apical DIDS (1 mM) depolarized V_{ap}, consistent with the inhibition of a hyperpolarizing mechanism. The perfusion of Na-free Ringer (solution 6) plus DIDS failed to cause an immediate depolarization. Abbreviations as in Fig. 2.

Ionic Dependence of the HCO_3 Response

In some cell types, it has been shown that the membrane voltage response to step changes in external $[HCO_3]_o$ is dependent on the presence of Na (Jentsch et al., 1984; Curci et al., 1987; Lopes et al., 1987). To test whether the electrogenic HCO_3 mechanism in the RPE is Na dependent, we examined the effect of apical $[HCO_3]_o$ reduction after apical Na had been removed. Fig. 7 shows that in the absence of apical Na, the apical membrane voltage response to a 10-fold reduction in apical $[HCO_3]_o$ was completely blocked, indicating that the RPE HCO_3 transporter is in fact Na dependent. Similar results were obtained in two other tissues.

If Na and HCO_3 transport are coupled in an electrogenic fashion, then several predictions can be made: (*a*) the removal of Na from the apical bathing solution should depolarize V_{ap}; (*b*) the voltage response to Na removal should require the presence of HCO_3; and (*c*) the voltage response to Na removal should be inhibited by preincubation with apical DIDS. Fig. 8 *A* shows that perfusion of the apical side of the RPE with Na-free Ringer (solution 6) caused V_{ap} to depolarize by ~8 mV and then repolarize toward the initial baseline. In nine experiments, the average peak

TABLE IV

Effect of Apical Na Removal

Condition	V_{ap}	V_{ba}	TEP	a	R_t
		mV			$\Omega \cdot cm^2$
110 mM Na	−79.6 ± 1.0	−68.2 ± 1.6	11.3 ± 1.0	0.62 ± 0.09	204 ± 8
0 mM Na	−72.0 ± 1.3	−58.6 ± 1.8	13.4 ± 1.0	1.44 ± 0.09	239 ± 15
$\lvert\Delta\rvert$	7.5 ± 0.5‡	9.6 ± 0.4‡	2.1 ± 0.2‡	0.82 ± 0.13*	36 ± 8*
	(9)	(9)	(9)	(4)	(4)

Data shown are means ± SEM obtained under steady-state conditions for 110 mM Na or at the peak voltage after a change to 0 Na. *$P < 0.02$; ‡$P < 0.001$.

depolarization of V_{ap} was 7.5 ± 0.5 mV (Table IV). The polarity of this initial voltage change is opposite to that expected for a Na diffusion potential across either the apical membrane or the paracellular pathway.[2]

The HCO_3 dependence of the 0 Na response was tested in a series of experiments similar to that illustrated in Fig. 8 *B*. The solution perfusing the apical side was first switched from control to 2.75 mM HCO_3 Ringer (solution 2), causing a transient depolarization of V_{ap}. When Na was subsequently removed from the apical bathing solution (solution 6), the initial depolarization of the apical membrane seen under

[2] Because the paracellular pathway of the RPE is highly permeable to Na (Miller and Steinberg, 1977*a*; Hughes et al., 1988), the replacement of Na in the apical bath with the relatively impermeable cation *N*-methyl-D-glucamine creates a diffusion potential that hyperpolarizes V_{ap} and depolarizes V_{ba}. Therefore, the magnitude of the V_{ap} depolarization induced by Na removal is attenuated by the Na diffusion potential across the paracellular shunt. In Fig. 8 *A*, the basal membrane depolarized even more than the apical membrane because of the combined effect of current shunted from the apical membrane and the Na diffusion potential across the shunt.

control conditions was completely absent. The hyperpolarization of V_{ap} and depolarization of V_{ba} that did occur were most likely caused by a Na diffusion potential across the shunt pathway.[2]

Fig. 8 *C* shows the effect of DIDS on the depolarization of V_{ap} induced by apical Na removal. The perfusion of control Ringer containing 1 mM DIDS to the apical side caused its usual depolarization of V_{ap}. The subsequent substitution of control Ringer with Na-free Ringer (plus DIDS) 2 min later failed to depolarize V_{ap}. Again, the voltage changes that did occur at the two membranes were in opposite directions and most likely were caused by a Na diffusion potential across the paracellular shunt pathway. This result was corroborated in two other tissues. The interdependence of Na and HCO_3 in generating voltage changes at the apical membrane, and the inhibition of both voltage responses by apical DIDS, strongly suggest an electrogenic $NaHCO_3$ cotransporter.

$NaHCO_3$ transport in some tissues is coupled to Cl in an obligatory fashion (Thomas, 1977; Boron and Russell, 1983; Guggino et al., 1983; Boron, 1985). We found, however, that normal voltage responses to changes in apical $[HCO_3]_o$ could be elicited in the RPE even after it had been bathed on both sides with Cl-free Ringer for 1 h or more ($n = 2$, not shown). Therefore, it seems unlikely that the electrogenic $NaHCO_3$ transporter in the RPE is coupled to Cl.

Origin of the DIDS-induced Change in V_{ap}

The application of apical DIDS produced an apical membrane depolarization that averaged 2.1 ± 0.2 mV (mean ± SEM; $n = 20$). Since DIDS partially blocks the apical membrane voltage responses to reduction in $[HCO_3]_o$ and $[Na]_o$ (Figs. 5 and 8 *C*), this depolarization could result directly from an inhibition of cotransporter current. It is unlikely that the DIDS-induced depolarization was generated by a change in another transport pathway, such a decrease in g_K or an increase in g_{Na} and/or g_{Cl} (Biagi, 1985; Inoue, 1985, 1986). The former possibility is eliminated because of the observation that 3 mM barium had no effect on the DIDS response (not shown). The latter possibility also seems unlikely because: (*a*) the apical membrane has very little conductance to either ion (Miller and Steinberg, 1977*a*); (*b*) E_{Na} and E_{Cl} are more positive than the resting membrane potential (Fong et al., 1988); and (*c*) DIDS appears to decrease, not increase, apical membrane conductance (Fig. 6).

If the voltage response to apical DIDS is due to inhibition of the cotransporter, then it should be possible to alter the magnitude or direction of the DIDS response by the appropriate alteration in $[Na]_o$ or $[HCO_3]_o$. Fig. 9 *A* shows that when apical HCO_3 was elevated to 55 mM, DIDS caused a 5-mV depolarization of V_{ap}. This response (5.8 ± 0.2 mV; mean ± SEM, $n = 3$) was significantly larger than the DIDS-induced depolarization in control Ringer, as would be expected if the cotransporter were stimulated by high apical $[HCO_3]$. In contrast, Fig. 9, *B* and *D*, shows that DIDS caused V_{ap} to hyperpolarize when either HCO_3 or Na were removed from the apical bathing solution ($n = 3$). These results are consistent with a reversal of the cotransporter. More importantly, Fig. 9, *C* and *E*, shows that when either substrate was removed from both bathing solutions, DIDS had no effect on V_{ap} ($n = 3$). From these results we conclude that the DIDS-induced voltage change can be attributed solely to inhibition of the $NaHCO_3$ cotransporter.

Relationship between $NaHCO_3$ Cotransport and pH_i

It seems likely that an important function of the $NaHCO_3$ cotransporter is to provide the main driving force for active HCO_3 (and fluid) absorption across the RPE (Hughes et al., 1988; Hughes et al., 1987). Therefore, this cotransporter should provide a constant influx of HCO_3 into the cell, elevating its intracellular concentration above electrochemical equilibrium. Consequently, any alteration in cotransporter rate should change $[HCO_3]_i$ and thus intracellular pH (pH_i). These expectations were tested directly by using the fluorescent dye BCECF to measure pH_i.

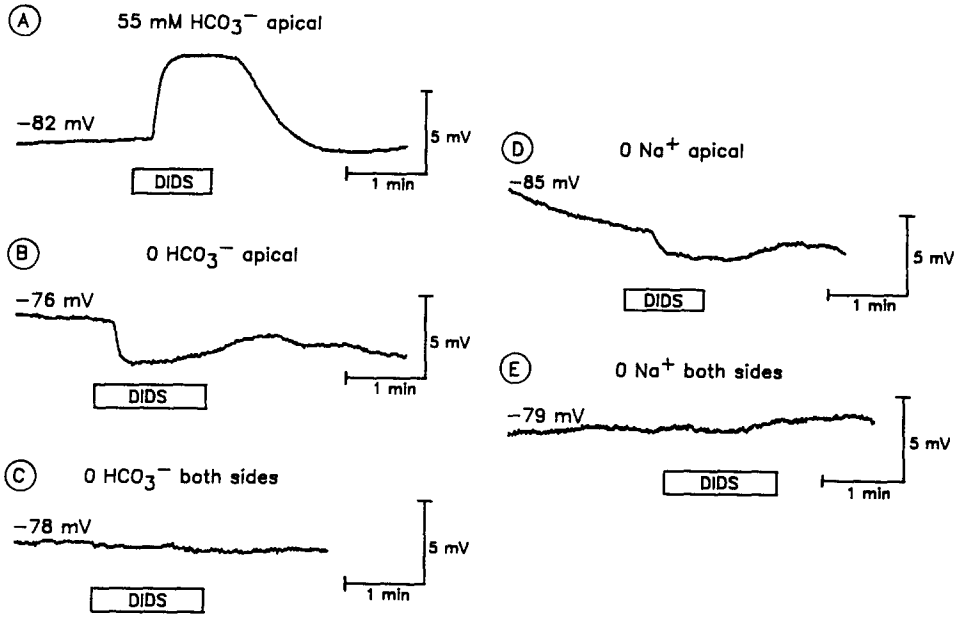

FIGURE 9. HCO_3 and Na dependence of the DIDS-induced change in V_{ap}. The effect of apical DIDS (1 mM) was examined under the following conditions: (*A*) 55 mM apical $[HCO_3]_o$ (basal $[HCO_3]$ = 27.5 mM); (*B*) nominally HCO_3-free apical side (basal $[HCO_3]_o$ = 27.5 mM); (*C*) nominally HCO_3-free both sides; (*D*) Na-free apical side (basal $[Na]_o$ = 110 mM); and (*E*) Na-free both sides. Values above each trace indicate V_{ap} at the beginning of the recording.

Fig. 10 shows simultaneous recordings of (*A*) pH_i and (*B*) TEP and R_t. In control Ringer, pH_i was 7.21 ± 0.03 (mean ± SEM; n = 20). Removal of apical Na or a 10-fold reduction in apical $[HCO_3]_o$ (constant P_{CO_2}) produced normal changes in TEP (compare with Figs. 2 *A* and 8 *A*) and also caused rapid and reversible acidification of the cell interior. Both of these pH_i responses were inhibited by the application of apical DIDS (1 mM). In the absence of DIDS, the initial rates of acidification (measured over the first 30 s) after Na removal or HCO_3 reduction averaged 0.49 ± 0.04 (mean ± SEM; n = 6) and 0.64 ± 0.01 (n = 7) pH U/min, respectively. After 15 min of exposure to DIDS, these rates of acidification were reduced by 80–90%. In the case of Na removal, the initial rate of acidification was reduced to 0.05 ± 0.01 pH

U/min (n = 4) and in two tissues, the acidification rate produced by HCO_3 reduction was decreased to 0.10 and 0.15 pH U/min.

It is worth noting that DIDS itself caused a rapid acidification of the cell interior (0.11 ± 0.06 pH U/min; n = 5). This result and the ion substitution experiments summarized above strongly support the notion of a $NaHCO_3$ cotransporter that moves HCO_3 and net negative charge into the cell.

Effect of NH_4

Extracellular NH_4 addition causes intracellular alkalinization due to the nonionic diffusion of NH_3 (Boron and De Weer, 1976), and in the presence of CO_2, this will

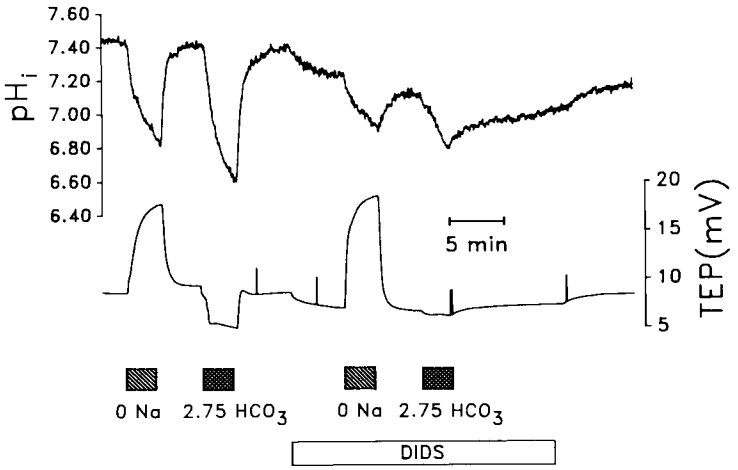

FIGURE 10. Effect of apical Na and HCO_3 reduction on intracellular pH. Simultaneous recordings of intracellular pH (*upper panel*) and TEP (*lower panel*). Horizontal bars indicate when the solution bathing the apical membrane was changed from control Ringer (solution 1) to Na-free Ringer (solution 6), 2.75 mM HCO_3 Ringer at constant P_{CO_2} (solution 2), or these same solutions plus 1 mM DIDS. The basolateral membrane was bathed with control Ringer throughout the course of the experiment. The upward deflections in the TEP record are voltage responses to 1 μA DC pulses across the tissue used for the determination of R_t. In control Ringer, R_t was 210 Ω·cm^2.

cause an increase in $[HCO_3]_i$. Thus, NH_4 can be used to investigate the effects of changes in $[HCO_3]_i$ and/or pH_i on the electrogenic $NaHCO_3$ transporter. Fig. 11 shows that elevating NH_4 in the apical bath leads to a rapid alkalinization, 0.2 pH U, followed by a slower acidification. In seven tissues, the peak pH_i increase averaged 0.25 ± 0.03 (±SEM). Since these pH_i increases took place at constant P_{CO_2}, one can calculate that $[HCO_3]_i$ increased from 17 to 31 mM. On thermodynamic grounds, this increase in $[HCO_3]_i$ would be expected to reduce the net inward driving force on the electrogenic $NaHCO_3$ cotransporter and thereby depolarize the apical membrane and decrease the TEP (see bottom of Fig. 11).

A typical intracellular voltage response to apical NH_4 is shown in the first part of Fig. 12 *A*. NH_4 (20 mM, solution 8) caused V_{ap} to depolarize rapidly by ≈13 mV and then partially repolarize. When control Ringer was restored, V_{ap} underwent a sharp hyperpolarization and then slowly repolarized toward its initial level. In 11 experiments, the peak depolarization produced by 20 mM NH_4 averaged 16.3 ± 0.8 mV (Table V).[3]

If the NH_4-induced depolarization of V_{ap} is mainly due to the slowing of the electrogenic cotransporter, then one would expect the voltage response to NH_4 to be reduced by the reduction of apical HCO_3 or Na or by the addition of apical DIDS. The second part of Fig. 12 *A* shows that the NH_4 response can be inhibited by the reduction of apical $[HCO_3]_o$ at constant P_{CO_2}. In this cell, the peak of the NH_4-induced apical membrane depolarization was reduced by 50% to 7 mV after apical $[HCO_3]_o$ was lowered to 2.75 mM. Fig. 12 *B* shows that in the absence of apical Na, a 20 mM pulse of NH_4 (solution 9) produced a relatively small depolarization of V_{ap}. In five experiments on three cells, the mean peak depolarization produced by NH_4 in the absence of apical Na was 3.6 ± 0.7 mV (≈78% inhibition). Finally, the initial

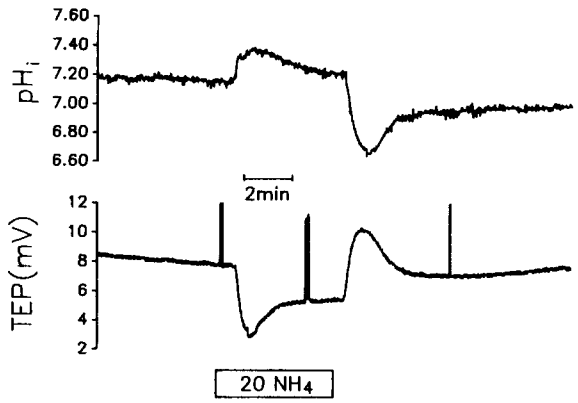

FIGURE 11. Effect of NH_4 on intracellular pH. Simultaneous recordings of intracellular pH (*upper panel*) and TEP (*lower panel*). Horizontal bars indicate when the solution bathing the apical membrane was changed from control Ringer (solution 1) to 20 mM NH_4 Ringer (solution 8).

voltage response to NH_4 was significantly inhibited by DIDS, as illustrated in Fig. 12 *C*. After preincubation with apical DIDS (1 mM) for 5 min, the addition of apical NH_4 caused V_{ap} to rapidly depolarize by ≈6 mV and then slowly depolarize another 10 mV over the next few minutes. The mechanism(s) underlying the slower, secondary depolarization has not been studied but probably depends on changes in pH_i (see Fig. 11). In four experiments on DIDS-treated tissues, the initial peak depolarization induced by NH_4 averaged 6.0 ± 0.6 mV, a 62% smaller response than that obtained in the absence of DIDS. Inhibition of the NH_4-induced V_{ap} depolarization transient by Na or HCO_3 reduction or by DIDS addition strongly suggests that this voltage response is mediated by the apical membrane electrogenic $NaHCO_3$ cotransporter.

[3] This depolarization is not due to the permeation of NH_4 through the apical K conductance because the voltage response to NH_4 was not diminished by barium (2 mM).

Relationship between Apical Membrane Potential and the DIDS-induced Voltage Change

If membrane voltage is a determinant of the overall driving force for this electrogenic transporter, then it should be possible to alter or even reverse the direction of net transport by the appropriate variation in membrane voltage. To test this hypothesis, we altered the apical membrane voltage by passing DC current across the epi-

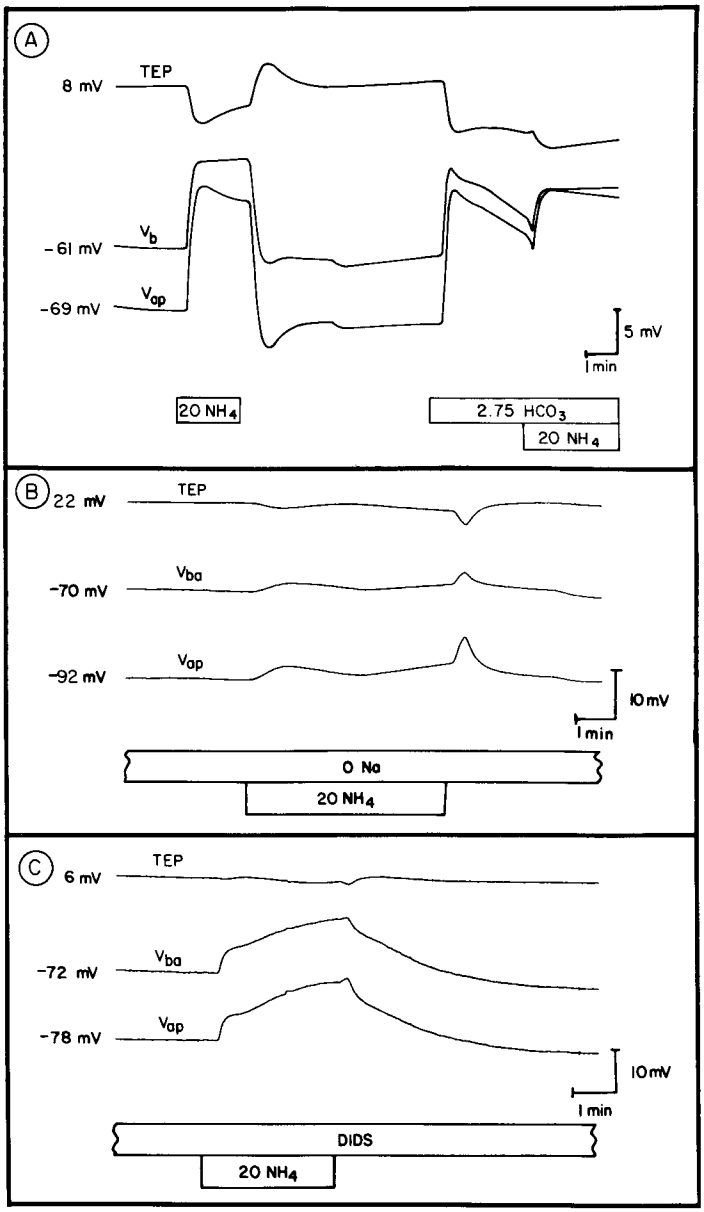

FIGURE 12

TABLE V

Effects of Apical NH_4

Condition	V_{ap}	V_{ba}	TEP	a	R_t
	mV				$\Omega \cdot cm^2$
Control	−82.4 ± 1.3	−73.6 ± 1.0	8.8 ± 0.9	0.51 ± 0.05	226 ± 15
20 NH_4	−66.1 ± 1.0	−63.4 ± 1.0	2.7 ± 0.7	1.38 ± 0.10	222 ± 18
$\lvert\Delta\rvert$	16.3 ± 0.8‡	10.1 ± 0.3‡	6.2 ± 0.7‡	0.87 ± 0.05‡	4 ± 6*

Data shown are means ± SEM obtained under control conditions or at the peak voltage after a change to 20 NH_4 Ringer. *Not significant; ‡$P < 0.001$.

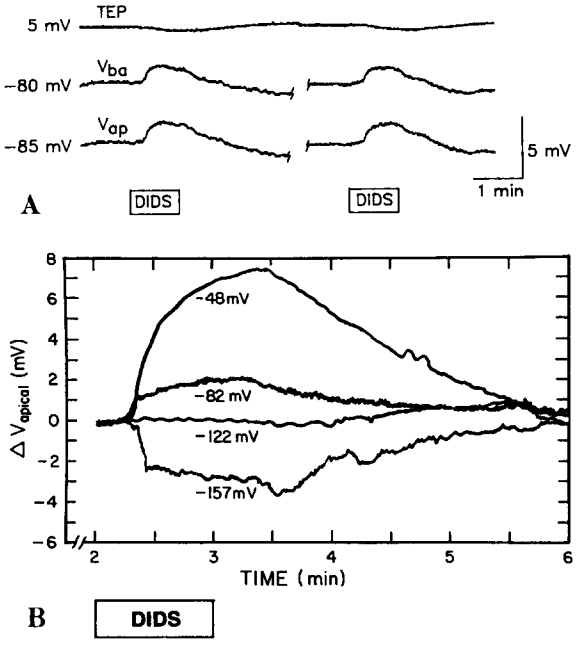

FIGURE 13. Reproducibility of DIDS-induced voltage changes. Two successive 1-min exposures to 1 mM apical DIDS produced nearly identical voltage responses. (*B*) DIDS-induced voltage changes recorded from a single cell under open-circuit conditions and while apical membrane was polarized by transepithelial current clamp. Baselines of these traces have been superimposed to facilitate comparison. Abbreviations as in Fig. 2.

FIGURE 12. (*Opposite*) The effects of apical $[NH_4]_o$ on membrane voltage. (*A*) The first horizontal bar indicates the substitution of control Ringer (solution 1) with 20 mM NH_4 Ringer (solution 8) on the apical side. NH_4 produced a rapid depolarization of V_{ap}, followed by a partial repolarization. See Table V. Next, apical $[HCO_3]_o$ was decreased from 27.5 to 2.75 mM (solution 2), and the apical side was again exposed to 20 mM NH_4 (solution 10). The amplitude of the NH_4-induced depolarization was significantly reduced. (*B*) The effects of apical $[NH_4]_o$ in the absence of apical Na. Apical side had been perfused with Na-free Ringer (solution 6) for 5 min before the start of this record. In the absence of apical Na, an increase in $[NH_4]$ to 20 mM (solution 9) failed to produce a significant depolarization of V_{ap}. (*C*). Effects of increasing apical $[NH_4]_o$ in the presence of DIDS. Apical side had been perfused with control Ringer plus DIDS for 3 min before the start of this record. The perfusion of NH_4 Ringer (20 mM; solution 8) plus DIDS produced a rapid depolarization of V_{ap} that was ~60% smaller than that observed in the absence of DIDS (compare with Fig. 10). Abbreviations as in Fig. 2.

thelium, either inward across the apical membrane (hyperpolarizing) or outward across the apical membrane (depolarizing).[4] These current steps were kept on for several minutes in order to maintain a steady level of membrane hyperpolarization or depolarization before DIDS was applied. The degree to which changes in apical membrane voltage altered the $NaHCO_3$ transport rate was assessed by measuring the magnitude of the DIDS-induced voltage change at each level of polarization. In these experiments, the exposure of the tissue to DIDS was limited to 1 min. This length of time was chosen because it was long enough for the DIDS-induced change in voltage to reach its peak value and yet short enough to ensure reversibility. Fig. 13 *A* shows two sequential DIDS responses from the same cell. These two responses are practically indistinguishable, indicating that the effects of short exposures to apical DIDS are reversible and that the degree of inhibition is constant from one response to the next.

Fig. 13 *B* shows four DIDS responses recorded from a single cell with the apical

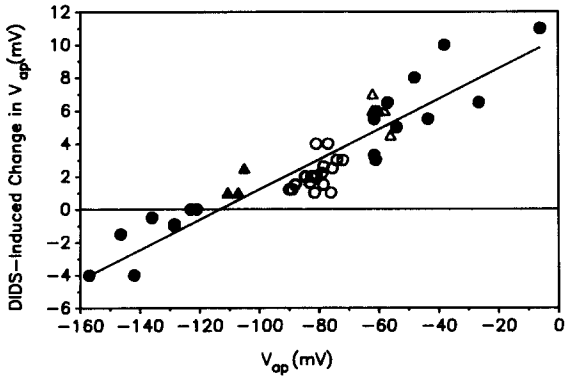

FIGURE 14. Relation between the DIDS-induced change in V_{ap} and the initial value of V_{ap}. DIDS responses were recorded either under open-circuit conditions (○), while V_{ap} was depolarized by 10 mM $[K]_o$ (solution 11, △) or hyperpolarized by 0.2 mM $[K]_o$ (solution 12, ▲), or while V_{ap} was polarized by transepithelial current clamp (●). The straight line indicates the best fit of the data by least squares analysis. R^2 = 0.84; X intercept = −114 mV.

membrane potential first at open circuit and then at three current-clamped potentials. The baselines of these traces have been superimposed to facilitate comparison. In the open circuit condition, V_{ap} of this cell was −82 mV and apical DIDS caused a 2-mV depolarization. When V_{ap} was depolarized to −48 by transepithelial current clamp, DIDS caused a larger depolarization of 7.5 mV. On the other hand, when V_{ap} was hyperpolarized to −122 mV, DIDS had essentially no effect on V_{ap}, and when V_{ap} was driven even more negative to −157 mV, the DIDS-induced voltage change reversed polarity and was −2.5 mV.

Fig. 14 summarizes the results of similar experiments in which V_{ap} was polarized by either transepithelial current (●) or by elevating apical $[K]_o$ to 10 mM (△) or

[4] These transepithelial currents also polarized the basolateral membrane in the opposite direction and could have influenced the DIDS response. This possibility seems unlikely because similar DIDS-induced voltage changes were produced when both membranes were polarized in the same direction by alterations in apical $[K]_o$ (Fig. 14).

reducing it to 0.2 mM (▲). There is a clear correlation between membrane voltage and the DIDS-induced voltage response. When V_{ap} was depolarized, the DIDS-induced depolarization tended to be larger than it was at the resting potential (○). Conversely, when V_{ap} was hyperpolarized, the voltage response tended to be smaller than it was at resting potential. Most importantly, the DIDS-induced voltage change reversed polarity and became negative-going at ~ -114 mV. This reversal potential is most likely the equilibrium potential of the cotransporter (see below).

DISCUSSION

Previous work by Miller and Steinberg (1977*a*) established that the apical membrane potential of the frog RPE is affected by changes in extracellular [HCO_3]. Because HCO_3 absorption across the RPE is an important determinant of fluid transport across the RPE (Hughes et al., 1984; Hughes et al., 1987*b*), the present study was undertaken to characterize the underlying mechanism. Evidence is now provided that the apical membrane of the RPE has an electrogenic $NaHCO_3$ cotransporter that moves Na, HCO_3, and negative charge into the cell.

In agreement with a previous study (Miller and Steinberg, 1977*a*), decreases in apical $[HCO_3]_o$ caused rapid depolarization of V_{ap}, whereas increases caused hyperpolarization (Fig. 2, *A–C*). The possibility that an electrogenic $NaHCO_3$ cotransport mechanism underlies these voltage responses was suggested by four observations. First, the HCO_3-induced voltage changes were blocked by the removal of Na from the apical bath (Fig. 7), which indicates that the electrogenic HCO_3 transporter is Na dependent. Secondly, apical Na removal itself caused an immediate depolarization of V_{ap} (Fig. 8 *A*). This finding is incompatible with a simple Na conductance. Thirdly, the Na-induced voltage change was blocked by low apical $[HCO_3]_o$ (Fig. 8 *B*). Finally, the initial apical membrane depolarizations produced by $[HCO_3]_o$ or $[Na]_o$ reduction were significantly attenuated by the addition of the anion transport inhibitor DIDS (Figs. 5 and 8 *C*). These electrophysiological results, which indicate that Na and HCO_3 are transported across the apical membrane along with net negative charge, were corroborated by intracellular pH measurements (Fig. 10).

DIDS significantly increased the apparent a value and R_t, which is consistent with an increase in apical membrane resistance and provides evidence that the cotransporter is a conductive mechanism. Reducing apical $[Na]_o$ or $[HCO_3]_o$ also increased the apparent a value (Tables II–IV) and supports the notion that this cotransporter moves net (negative) charge.

The introduction of 20 mM NH_4 into the apical bath caused a rapid depolarization V_{ap} (Fig. 12 *A*). The peak NH_4 response was markedly reduced either by Na removal, HCO_3 reduction, or the addition of DIDS, suggesting that it is mediated by a slowing of the electrogenic $NaHCO_3$ transporter. Measurements of pH_i using the fluorescent dye BCECF showed that extracellular NH_4 (20 mM) alkalinizes the RPE cell interior by 0.2 pH U (Fig. 11). Since this alkalinization, which occurred at constant P_{CO_2}, must also increase $[HCO_3]_i$, it is likely that a change in the HCO_3 chemical gradient is a principal cause of the cotransporter inhibition. The present experiments do not exclude the possibility that the transporter might be also affected kinetically by the increase in pH_i.

Direction of Cotransport

The disulfonic stilbenes DIDS and SITS have been shown to inhibit electrogenic $NaHCO_3$ cotransport in a variety of tissues (Boron and Boulpaep, 1983; Jentsch et al., 1984; Biagi, 1985; Curci et al., 1987; Sasaki et al., 1987). For example, in the proximal tubule, electrogenic $NaHCO_3$ transport mediates the efflux of HCO_3 across the basolateral membrane and SITS causes both a membrane hyperpolarization and an intracellular alkalinization (Boron and Boulpaep, 1983; Lopes et al. 1987). In contrast, we found in the RPE that apical DIDS acidified the cell interior (Fig. 10) and caused a rapid depolarization of V_{ap} (Figs. 5, 6, and 8 *C*). This effect of DIDS on V_{ap} was mediated by the cotransporter since it was absent when the tissue was bathed in either Na-free of HCO_3-free solutions (Fig. 9, *C* and *E*). Therefore we conclude that this cotransporter must hyperpolarize the apical membrane by carrying Na, HCO_3, and net negative charge inward across the apical membrane.

Na or HCO_3 removal from the apical bathing solution produced V_{ap} depolarizations that were large relative to the DIDS response, presumably because these maneuvers reversed the direction of the cotransporter, whereas DIDS caused only its inhibition. Evidence that the cotransporter reverses direction when either HCO_3 or Na are removed from the apical bath is provided by the data shown in Fig. 9, *B* and *D*, where DIDS produced membrane hyperpolarizations.

Apparent Reversal Potential and Stoichiometry

The electrogenicity of the $NaHCO_3$ transporter suggests that its rate may be affected by membrane voltage as well as Na and HCO_3 chemical gradients across the apical membrane. Fig. 14 summarizes data which shows that the magnitude of the DIDS-induced voltage change is a function of V_{ap}. When V_{ap} was depolarized from its resting level, the magnitude of the DIDS-induced voltage change increased, and when it was hyperpolarized, the DIDS response became smaller. At values more hyperpolarized than −114 mV, the polarity of the DIDS-induced voltage change reversed. This reversal can be used to estimate the cotransporter's equilibrium potential, since the DIDS-induced voltage change results from inhibition of the electrogenic cotransporter.

This reversal potential estimate allowed us to calculate the stoichiometry of the $NaHCO_3$ cotransporter. From thermodynamic considerations (Aronson, 1984), it can be shown that:

$$E_{rev} = \frac{2.3RT}{F(n-1)} \log \frac{[Na]_i}{[Na]_o} \left(\frac{[HCO_3]_i}{[HCO_3]_o} \right)^n \quad (1)$$

where E_{rev} is the reversal (equilibrium) potential, n is the ratio of HCO_3 to Na ions that are carried by the cotransporter, and R, T, and F have their usual meaning. In control Ringer, $[Na]_o$ was 110 mM, $[HCO_3]_o$ was 27.5 mM. When V_{ap} was current-clamped to $\sim E_{rev}$ (−114 ± 10 mV; see Fig. 14), pH_i was ~6.9 (n = 5; Lin and Miller, unpublished observations). Assuming that intracellular and extracellular P_{CO_2} are equal (5%), we calculate $[HCO_3]_i$ to be 9 mM. Intracellular $[Na]_i$ is assumed to lie between 5 and 30 mM, a range that encompasses virtually all epithelia.

These values can be used in Eq. (1) to calculate the upper and lower bounds for

the stoichiometric ratio (n) of the cotransporter. The data imply that the HCO/Na ratio lies between 1.6 and 2.4.

Relation to Other $NaHCO_3$ Cotransport Mechanisms

Evidence for electrogenic $NaHCO_3$ cotransport mechanisms has been presented for a variety of other tissues, including amphibian and mammalian proximal tubule (Boron and Boulpaep, 1983; Guggino et al., 1983; Biagi and Sohtell, 1985; Alpern and Chambers, 1986; Sasaki et al., 1987), oxyntic cells (Curci et al., 1987) and cultured bovine corneal endothelium (Jentsch et al., 1984). These transporters mediate the net efflux of HCO_3 and Na from the cell and acidify the cell interior. The electrochemical gradient for Na across the basolateral membrane requires that the HCO_3/Na stoichiometry of these cotransporters be approximately 3:1 (Alpern and Chambers, 1986; Lopes et al., 1987). In contrast, the RPE cotransporter mediates the influx of HCO_3 and Na into the cell across the apical membrane and has an opposite effect on cell pH_i.

$NaHCO_3$ Cotransport and RPE Physiology

Fluid transport across the RPE is driven by active HCO_3 and Cl absorption (Hughes et al., 1984, 1987*b*; Tsuboi, 1987). Although no direct measurement of HCO_3 flux across the RPE has been reported to date, a considerable amount of evidence has accumulated that strongly supports the notion of net HCO_3 absorption by this epithelium. Lasansky and De Fisch (1966) used a manometric technique and measured the disappearance of HCO_3 from the solution bathing the apical membrane. Net HCO_3 absorption has also been inferred from the analysis of net ionic fluxes measured across the frog RPE under both open and short-circuit conditions (Miller and Steinberg, 1977*b*; Miller and Farber, 1984; Hughes et al., 1984). Finally, fluid absorption across the frog RPE has been shown to be significantly inhibited by the removal of HCO_3/CO_2 from the bathing media (Hughes et al., 1984).

It seems likely that transepithelial HCO_3 absorption begins with active influx through the $NaHCO_3$ cotransporter. The exit mechanism for HCO_3 across the basolateral membrane is electroneutral since V_{ba} is insensitive to changes in $[HCO_3]_o$ outside the basolateral membrane (Miller and Steinberg, 1977*a*). Recent studies provide strong evidence that this mechanism is a HCO_3/Cl exchanger (Fong et al., 1988; Edelman et al., 1988).

In the vertebrate eye, the K concentration of the subretinal space decreases from approximately 5 to 2 mM after the onset of light. This decrease in $[K]_o$ hyperpolarizes the apical membrane of the RPE, producing the RPE component of the c-wave of the DC electroretinogram (Steinberg et al., 1985). Since the cotransporter is voltage dependent (Fig. 14), one might expect the light-evoked decrease in $[K]_o$ to reduce the rate of HCO_3 influx across the apical membrane and decrease intracellular pH. These changes in $pH_i/[HCO_3]_i$ could significantly affect other RPE transport processes. Recently, we have observed in frog RPE that increases in apical $[K]_o$ from 2 to 5 mM stimulate the net secretion of Cl (Edelman et al., 1988). This Cl secretion is blocked by apical or basal DIDS and by the removal of either apical Na or HCO_3. If this secretory response is mediated by the electrogenic $NaHCO_3$ cotransporter, then it provides an example of RPE function modulated by changes

in retinal physiology. It will be interesting to learn what other retina–RPE interactions are mediated by this cotransporter.

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