Extracellular HCO_3^- Dependence of Electrogenic Na/HCO_3 Cotransporters Cloned from Salamander and Rat Kidney

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abstract   We studied the extracellular $[HCO_3^-]$ dependence of two renal clones of the electrogenic Na/HCO_3 cotransporter (NBC) heterologously expressed in *Xenopus* oocytes. We used microelectrodes to measure the change in membrane potential (ΔV_m) elicited by the NBC cloned from the kidney of the salamander *Ambystoma tigrinum* (akNBC) and by the NBC cloned from the kidney of rat (rkNBC). We used a two-electrode voltage clamp to measure the change in current (ΔI) elicited by rkNBC. Briefly exposing an NBC-expressing oocyte to $HCO_3^-/$ CO_2 (0.33–99 mM HCO_3^-, pH_o 7.5) elicited an immediate, DIDS (4,4-diisothiocyanatostilbene-2,2-disulfonic acid)-sensitive and Na^+-dependent hyperpolarization (or outward current). In ΔV_m experiments, the apparent K_m for HCO_3^- of akNBC (10.6 mM) and rkNBC (10.8 mM) were similar. However, under voltage-clamp conditions, the apparent K_m for HCO_3^- of rkNBC was less (6.5 mM). Because it has been reported that $SO_3^=/HSO_3^-$ stimulates $Na/$ HCO_3 cotransport in renal membrane vesicles (a result that supports the existence of a $CO_3^=$ binding site with which $SO_3^=$ interacts), we examined the effect of $SO_3^=/HSO_3^-$ on rkNBC. In voltage-clamp studies, we found that neither 33 mM $SO_4^=$ nor 33 mM $SO_3^=/HSO_3^-$ substantially affects the apparent K_m for HCO_3^-. We also used microelectrodes to monitor intracellular pH (pH_i) while exposing rkNBC-expressing oocytes to 3.3 mM $HCO_3^-/0.5\%$ CO_2. We found that $SO_3^=/HSO_3^-$ did not significantly affect the DIDS-sensitive component of the pH_i recovery from the initial CO_2-induced acidification. We also monitored the rkNBC current while simultaneously varying $[CO_2]_o$, pH_o, and $[CO_3^=]_o$ at a fixed $[HCO_3^-]_o$ of 33 mM. A Michaelis-Menten equation poorly fitted the data expressed as current versus $[CO_3^=]_o$. However, a pH titration curve nicely fitted the data expressed as current versus pH_o. Thus, rkNBC expressed in *Xenopus* oocytes does not appear to interact with $SO_3^=$, HSO_3^-, or $CO_3^=$.

key words: *Xenopus* oocytes • intracellular pH • extracellular pH • sulfite • carbonate

INTRODUCTION

Since its first description in the renal proximal tubule of the salamander *Ambystoma tigrinum* (Boron and Boulpaep, 1983), the electrogenic Na/HCO_3 cotransporter has been functionally identified in a wide variety of cell types (for reviews, see Boron and Boulpaep, 1989; Boron et al., 1997). After the expression cloning of the electrogenic Na/HCO_3 cotransporter (NBC)[1] from *Ambystoma* kidney (Romero et al., 1997a), closely related cDNAs have been cloned from human kidney (Burnham et al., 1997), rat kidney (Romero et al., 1998), and human pan-

creas and heart (Abuladze et al., 1998a; Choi et al., 1999). An in situ hybridization study showed the presence of NBC mRNA in the renal proximal tubule of the rabbit (Abuladze et al., 1998b). Immunocytochemical studies with polyclonal NBC antibodies have localized the NBC protein to the basolateral membrane of the *Ambystoma*, rat, and rabbit renal proximal tubule (Schmitt et al., 1999), rat epididymis (Jensen et al., 1999), and human pancreatic duct (Marino et al., 1999).

The electrogenic Na/HCO_3 cotransporter plays the major role in HCO_3^- reabsorption by the renal proximal tubule (Alpern, 1985; Yoshitomi et al., 1985). Several groups have determined the apparent K_m for HCO_3^- $[K_m(HCO_3^-)]$ of the Na/HCO_3 cotransporter, as naturally expressed in cells. Working on monkey kidney epithelial (BSC-1) cells, Jentsch et al. (1985) measured DIDS (4,4-diisothiocyanatostilbene-2,2-disulfonic acid)-sensitive $^{22}Na^+$ uptake and estimated an apparent K_m (HCO_3^-) of 7–14 mM for extracellular HCO_3^- at a $[Na]_o$ of 151 mM. Later, they demonstrated an inverse relationship between $K_m(HCO_3^-)$ and $[Na^+]_o$ (Jentsch et al., 1986). Akiba et al. (1986), in a study of $^{22}Na^+$ fluxes in basolateral membrane vesicles from rabbit kidney cortex, obtained an apparent $K_m(HCO_3^-)$ of 10 mM at a $[Na]_o$ of 8 mM. Using a fluorescent probe thought to react with an amino acid near

[1]*Abbreviation used in this paper:* NBC, electrogenic Na/HCO_3 cotransporter.

the substrate-binding site of NBC in solubilized membrane proteins from rabbit renal basolateral vesicles, Stim et al. (1994) obtained an apparent $K_m(HCO_3^-)$ of 15 mM. The only study of the intracellular HCO_3^- dependence of NBC is that of Gross and Hopfer (1998). These authors measured short-circuit current in an apically permeabilized monolayer of rat proximal-tubule (SKPT-0193) cells grown on filters. They obtained an apparent $K_m(HCO_3^-)$ of 19 mM ($[Na^+]_i = 10$ mM, $V_{hold} = -60$ mV).

The cloning of NBC has made it possible to address the physiology of Na/HCO_3 cotransport heterologously expressed in *Xenopus* oocytes. Under the conditions of our experiments, no other acid–base transporters are active in the oocyte. In the present study, in which we expressed *Ambystoma* kidney NBC (akNBC) or rat kidney NBC (rkNBC) in *Xenopus* oocytes, we had two major goals. The first was to determine the apparent K_m of the two NBCs for extracellular HCO_3^- under conditions of net HCO_3^- influx. This uptake of HCO_3^- is in the direction opposite the net HCO_3^- efflux that normally occurs in the renal proximal tubule. Our approach was to measure the change in membrane potential (ΔV_m) or change in current (ΔI) (under voltage-clamp conditions) as we added varying levels of HCO_3^-/CO_2 to the extracellular solution at a constant pH.

Our second goal was to examine the possibility that NBC can transport sulfite ($SO_3^=$), bisulfite (HSO_3^-), or carbonate ($CO_3^=$); $SO_3^=$ and HSO_3^- would presumably substitute for $CO_3^=$ and HCO_3^-, respectively. An earlier study with microelectrodes and $^{22}Na^+$ fluxes provided no evidence that $SO_3^=$ substitutes for $CO_3^=$ or HCO_3^- on the electrogenic Na/HCO_3 cotransporter of cultured bovine corneal endothelial cells (Jentsch et al., 1986). However, a later $^{22}Na^+$-uptake study on basolateral membrane vesicles isolated from rabbit kidney cortex led to the hypothesis that the electrogenic Na/HCO_3 cotransporter has a binding site for $CO_3^=$, and that $SO_3^=$ can substitute for $CO_3^=$ (Soleimani and Aronson, 1989). In our experiments, we examined the effect of $SO_3^=/HSO_3^-$ on both the apparent $K_m(HCO_3^-)$ and on the pH_i changes caused by rkNBC heterologously expressed in *Xenopus* oocytes. We found that $SO_3^=/HSO_3^-$ had no significant effect on either, making it unlikely that rkNBC, as expressed by itself in oocytes, transports either $SO_3^=$ or HSO_3^-. To investigate a potential role for $CO_3^=$, we monitored rkNBC current while simultaneously varying $[CO_2]_o$, pH_o, and $[CO_3^=]_o$ at a constant $[HCO_3^-]_o$. Based on the data from these experiments, we suggest that increased pH_o stimulates NBC with a pK of 7.5, but that NBC does not interact with $CO_3^=$.

METHODS

Preparation of Xenopus Oocytes

We prepared oocytes from *Xenopus laevis* (NASCO) by incubating small pieces of ovary for 45 min in a Ca^{2+}-free ND96 solution (pH 7.5, room temperature) containing 2 mg/ml collagenase (Type IA, #C-2674; Sigma Chemical Co.). We washed the oocytes three times for 10 min each in Ca^{2+}-free ND96, and then washed them again for an additional 20 min in Ca^{2+}-containing ND96. Until we used the oocytes, we incubated them at 18°C in OR3 media. This medium is a 1:2 dilution in water of Leibovitz's L15 Medium (41300-039; GIBCO BRL), supplemented with 50 U/ml of penicillin-streptomycin (15140-122; GIBCO BRL), 10 mM of HEPES, and titrated to pH 7.5 with NaOH. 1 d after this isolation procedure, we injected stage V or VI oocytes with either water (50 nl/cell) or 0.2 μg/μl cRNA (50 nl/cell) encoding rkNBC or akNBC. We used oocytes expressing NBC in electrophysiological experiments 3–10 d after injection.

Solutions

Table I summarizes the composition of standard solutions used in the present study. For experiments conducted in the absence of $SO_4^=$ or $SO_3^=/HSO_3^-$, our HEPES-buffered HCO_3^-/CO_2-free solution was Solution 1. This solution was noteworthy in that it contained only 7.6 mM Cl^-, but 99 mM gluconate. Our standard HCO_3^-/CO_2 Solution 2 contained 66 mM gluconate and 33 mM HCO_3^- (i.e., compared with Solution 1, 33 mM HCO_3^- replaced 33 mM gluconate) and was equilibrated with 5% CO_2, pH 7.5. We varied $[HCO_3^-]_o$ from 0.66 to 99 mM at constant pH_o by always maintaining the same ratio of $[HCO_3^-]/[CO_2]$. For example, the solution containing 16.5 mM HCO_3^- also contained 2.5% CO_2. We maintained a constant $[Cl^-]_o$ by exchanging HCO_3^- for gluconate in the solutions. The CO_2/O_2 mixtures with which we equilibrated our solutions were primary standard grade and analyzed; the mixing tolerance for the CO_2 was 1% (TechAir). In all solutions, pH was 7.5.

The solutions containing $SO_4^=$ were similar to those described above, except that we replaced 66 of the 99 mM gluconate in the HEPES-buffered solution (Solution 3 in Table I) with 33 mM $SO_4^=$ and 33 mM mannitol. In the HCO_3^-/CO_2-containing solutions, we kept $[SO_4^=]$ fixed at 33 mM, and substituted HCO_3^- for gluconate. Because the solutions contained a maximal [gluconate]$_o$ of only 33 mM, we were limited to HCO_3^- concentrations no higher than 33 mM.

The so-called "sulfite" solutions actually contained both $SO_3^=$ and HSO_3^- (pK 6.9). Thus, a pH 7.5 solution containing 33 mM "total $SO_3^=$" actually contains 26.4 mM $SO_3^=$ and 6.6 mM HSO_3^-. Similar to the situation for the $SO_4^=$ solutions, we replaced 66 of the 99 mM gluconate in the HEPES-buffered solution with 33 mM total $SO_3^=/HSO_3^-$ and 33 mM mannitol (Solution 5 in Table I). In the HCO_3^-/CO_2-containing solutions, we kept [total $SO_3^=/HSO_3^-]_o$ fixed at 33 mM, but substituted HCO_3^- for gluconate. In all solutions, pH was 7.5.

To determine the pH_o or $[CO_3^=]_o$ dependence of the NBC current, we used solutions containing a constant 33 mM HCO_3^-. We varied pH_o from 9.2 to 6.2 by equilibrating with gas mixtures having $[CO_2]$ values of 0.1–100%; as a result, $[CO_3^=]_o$ varied from ~3.5 μM to ~3.5 mM. Our standard 33 mM $HCO_3^-/5\%$ CO_2 (Solution 2 in Table I) contained ~70 μM $CO_3^=$ at pH 7.5.

In all solutions, $[Cl^-]$ was 7.6 mM, osmolarity was 225 mOsm, and temperature was 22°C. We delivered solutions continuously at a rate of 7 ml/min through Tygon (Tygon Norton Co.) tubing, which has a low permeability to CO_2.

Voltage and pH-sensitive Microelectrodes

V_m measurements. In some experiments, we used the change in membrane potential (ΔV_m) elicited by switching from a HEPES-buffered solution to a HCO_3^-/CO_2-buffered solution as an index of the electrogenic flux mediated by NBC. We made the voltage microelectrodes by pulling borosilicate glass capillary tubing,

Composition of Standard Solutions[‡]

Component	1 standard HEPES	2 standard HCO_3^-	3 standard SO_4 + HEPES	4 standard SO_4 + HCO_3^-	5 standard SO_3 + HEPES	6 standard SO_3 + HCO_3^-
	mM	*mM*	*mM*	*mM*	*mM*	*mM*
Na^+	104	104	104	104	97.4	97.4
K^+	2	2	2	2	2	2
Mg^{2+}	1	1	1	1	1	1
Ca^{2+}	1.8	1.8	1.8	1.8	1.8	1.8
Total cations, meq	111.6	111.6	111.6	111.6	105	105
Cl^-	7.6	7.6	7.6	7.6	7.6	7.6
Gluconate$^-$	99	66	33	0	33	0
HCO_3^-	0	33	0	33	0	33
$SO_4^=$	0	0	33	33	0	0
$SO_3^=$	0	0	0	0	26.4	26.4
HSO_3^-	0	0	0	0	6.6	6.6
$HEPES^-$	5	5	5	5	5	5
Total anions, meq	111.6	111.6	111.6	111.6	105	105
Mannitol	0	0	33	33	33	33
HEPES (neutral)	5	5	5	5	5	5
pH	7.5	7.5	7.5	7.5	7.5	7.5

[‡]Solutions containing 33 mM HCO_3^- were equilibrated with 5% CO_2.

1.16 mm i.d. $\times$ 2.0 mm o.d. (GC200F-10; Warner Instruments Corp.) on a microelectrode puller (P-97; Sutter Instrument Co.), and then filling with 3 M KCl. The electrodes had resistances of 1–10 MΩ.

pH$_i$ measurements. In some experiments, we used the rate of pH$_i$ increase (dpH$_i$/dt) as an index of the flux of HCO_3^- into oocytes expressing rkNBC. We made the pH microelectrodes using the same glass as described above, using an approach described previously (Siebens and Boron, 1987; Nakhoul et al., 1998). We silanized the glass by exposing it to vapors of *bis*-(dimethylamino)-dimethylsilane (14755; Fluka Chemical Corp.). We filled the tips of these electrodes with hydrogen-ionophore-I (Cocktail B, #918882/1; Fluka Chemical Corp.) and back-filled the electrodes with a solution containing 15 mM NaCl, 230 mM NaOH, and 40 mM KH$_2$PO$_4$, pH 7.0. The pH microelectrodes had slopes of −54 to −59 mV per pH unit between pH values of 6.0 and 8.0. They had resistances of up to 100 MΩ. The voltage- and pH-sensitive microelectrodes were connected to high-impedance electrometers (FD223; World Precision Instruments, Inc.). The bath reference electrode was a calomel reference electrode (1362079; Fisher Scientific). We corrected for bath junction potentials.

Two-Electrode Oocyte Voltage Clamp

We voltage clamped oocytes using a two-electrode voltage clamp (OC-725B Oocyte Clamp; Warner Instrument Corp.). We impaled cells with microelectrodes filled with 3 M KCl (resistance = 0.3–1.0 MΩ). The holding potential (V_{hold}) was −60 mV. The currents were filtered at 20 Hz (four-pole Bessel filter).

Data Acquisition

The pH$_i$, V_m, and I$_{out}$ data were recorded digitally on 80486-based personal computer. The analogue-to-digital converter (ADC-30; Contec Microelectronics U.S.A., Inc.) sampled the V_m and pH$_i$ data at a rate of 0.4 Hz, and sampled the current data at a rate of

1 Hz. Software for data acquisition and analysis, as well as for fitting of the data, was developed in our laboratory.

Statistics and Data Analysis

We determined rates of pH$_i$ change (dpH$_i$/dt) by fitting a line to pH$_i$ versus time data using a linear least-squares method. All average dpH$_i$/dt, ΔV_m, and ΔI data are reported as mean $\pm$ SEM. For ratios, we present the averages as log-normal means. The statistical significance of log-normal data was determined using an unpaired Student's *t* test.

In analyzing ΔV_m (or ΔI) data obtained in the absence of $SO_4^=$ or $SO_3^=$/HSO_3^- (i.e., when gluconate and HCO_3^- were the major anions), we normalized absolute values of ΔV_m (or ΔI) obtained under "test" conditions to bracketing values of ΔV_m (or ΔI) obtained under "standard" conditions of 33 mM HCO_3^-. As noted in the discussion, the simplest equation that adequately fitted our data was a model having a Michaelis-Menten dependence on $[HCO_3^-]_o$, plus a linear component:

$$v = \frac{[HCO_3^-]}{[HCO_3^-] + K_m} v_{max} + \alpha[HCO_3^-], \qquad (1)$$

where v is an absolute value of the velocity of the reaction (i.e., ΔV_m or ΔI) at each value of $[HCO_3^-]$, v_{max} is the maximum velocity, and α is a constant. We can rearrange Eq. 1 to obtain α as follows (Eq. 2):

$$\alpha = \frac{v}{[HCO_3^-]} - \frac{v_{max}}{[HCO_3^-] + K_m}. \qquad (2)$$

Under our standard conditions of $[HCO_3^-] = [HCO_3^-]_{std} = 33$ mM, α becomes:

$$\alpha = \frac{v_{std}}{[HCO_3^-]_{std}} - \frac{v_{max}}{[HCO_3^-]_{std} + K_m}. \qquad (3)$$

Substituting Eq. 3 into Eq. 1 yields:

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$$v = [\text{HCO}_3^-] \cdot v_{\max}\left(\frac{1}{[\text{HCO}_3^-] + K_m} - \frac{1}{[\text{HCO}_3^-]_{std} + K_m}\right)$$
$$+ \frac{[\text{HCO}_3^-]}{[\text{HCO}_3^-]_{std}} \cdot v_{std}. \tag{4}$$

We define the normalized velocity (v^*) to be the ratio of the observed velocity to the velocity under standard conditions (i.e., $v^* = v/v_{std}$). Substituting this definition of v^* into Eq. 4, we have:

$$v^* = \frac{v}{v_{std}} = [\text{HCO}_3^-] \cdot v^*_{\max}$$
$$\cdot \left(\frac{1}{[\text{HCO}_3^-] + K_m} - \frac{1}{[\text{HCO}_3^-]_{std} + K_m}\right) + \frac{[\text{HCO}_3^-]}{[\text{HCO}_3^-]_{std}}, \tag{5}$$

where the normalized $v_{\max}$ is defined as $v^*_{\max} = v_{\max}/v_{std}$. We used a nonlinear least-squares curve fitting approach to obtain $v^*_{\max}$ and K_m, and then computed the normalized $\alpha^* = \alpha/v_{std}$, using the following equation:

$$\alpha^* = \frac{\alpha}{v_{std}} = \frac{1}{[\text{HCO}_3^-]_{std}} - \frac{v^*_{\max}}{[\text{HCO}_3^-]_{std} + K_m}. \tag{6}$$

In analyzing normalized ΔI data obtained in the presence of sulfate or sulfite, ($[\text{HCO}_3^-]_o$ between 0 and 33 mM), we fitted the data with a normalized version of a function similar to Eq. 1, except that we assumed that α^* was fixed to the same value obtained from the curve fit of the data obtained in the absence of sulfate or sulfite (see Table III). In this case, the equation for normalized data is:

$$v^* = \frac{v}{v_{std}} = \frac{[\text{HCO}_3^-]_{std} + K_m}{[\text{HCO}_3^-] + K_m} \cdot \frac{[\text{HCO}_3^-]}{[\text{HCO}_3^-]_{std}}$$
$$\cdot \{1 - (\alpha^*)[\text{HCO}_3^-]_{std}\} + (\alpha^*)[\text{HCO}_3^-], \tag{7}$$

where $\alpha^* = 0.00577$ mM^{-1}. After obtaining K_m by curve fitting, we obtained the value of $v^*_{\max}$ using Eq. 8:

$$v^*_{\max} = \{1 - (\alpha^*)[\text{HCO}_3^-]_{std}\} \cdot \frac{[\text{HCO}_3^-]_{std} + K_m}{[\text{HCO}_3^-]_{std}}. \tag{8}$$

RESULTS

$[\text{HCO}_3^-]_o$ Dependence of akNBC, Based on Changes in V_m

Effect of adding 33 mM HCO_3^-/5% CO_2 on pH_i and V_m. Fig. 1 A illustrates the results of an experiment on a water-injected (i.e., control) oocyte. As described previously (Romero et al., 1997a), switching the extracellular solution from one buffered with HEPES to one buffered with HCO_3^-/CO_2, at a constant pH_o, causes a slow and sustained fall in pH_i, as well as a slowly developing depolarization. These changes in pH_i and V_m are fully reversible. Fig. 1 B illustrates the results of a similar experiment, but performed on an oocyte injected 3 d earlier with cRNA encoding rkNBC. Although there is a modest recovery of pH_i from the initial CO_2-induced acidification, the major difference between this experiment and the one in Fig. 1 A is that applying HCO_3^-/CO_2 elicited an immediate hyperpolarization of 85 mV. This hyperpolarization partially decayed over the course of 12 min. The slow pH_i recovery and the large
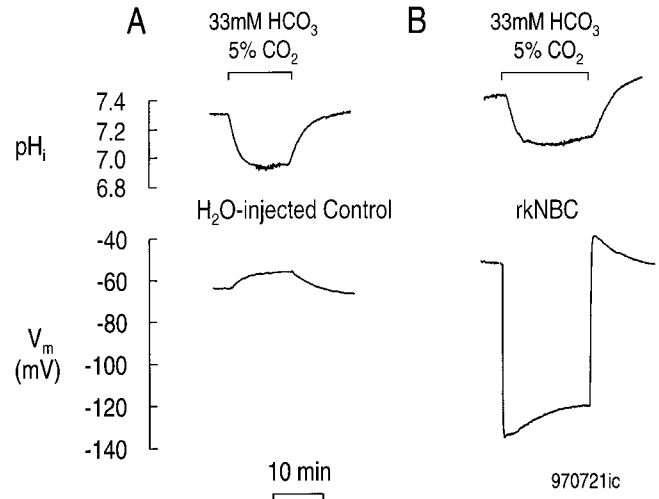

Figure 1. Membrane potential and pH_i of *Xenopus laevis* oocytes during a superfusion of 33 HCO_3^-/5% CO_2 solution. (A) Water-injected oocyte. The CO_2/HCO_3^- solution is Solution 2 in Table I. Typical of six experiments. (B) Oocyte expressing rkNBC. Typical of nine experiments. pH_o 7.5, 22°C.

hyperpolarization are both consistent with the electrogenic influx of Na^+ and HCO_3^-. Previous work has shown that pretreating akNBC-expressing oocytes with DIDS blocks both the pH_i recovery and the V_m changes (Romero et al., 1997a).

Effect on V_m of adding graded levels of HCO_3^-/CO_2 at a constant pH_o of 7.5. The expression level of the akNBC clone, as judged in voltage-clamp experiments (not shown), was not sufficiently high to allow us to measure NBC currents accurately at low values of $[\text{HCO}_3^-]_o$. To obtain a first approximation of the $[\text{HCO}_3^-]_o$ dependence of akNBC, we monitored changes in V_m while briefly applying extracellular solutions containing various levels of HCO_3^-/CO_2. Fig. 2 shows a typical experiment. We began with the oocyte in our standard gluconate-HEPES solution (Table I, Solution 1). We then switched to our standard gluconate-HCO_3^-/CO_2 solution, which was buffered to pH 7.50 with 33 mM HCO_3^- and 5% CO_2 (Solution 2), and determined the maximal change in V_m (ΔV_m). After returning the oocyte to the HEPES-buffered solution and waiting for V_m to stabilize, we exposed the cell to the first of five test HCO_3^-/CO_2 solutions, each having a pH of 7.50. During the rest of the experiment, we bracketed each test HCO_3^-/CO_2 pulse with a standard HCO_3^-/CO_2 pulse. To compensate for differences in the expression level of akNBC in individual oocytes, we then obtained a normalized ΔV_m by computing the ratio of the ΔV_m of the test pulse to the mean ΔV_m of the two bracketing standard pulses.

Curve fitting. As noted in the discussion, we attempted to fit the normalized akNBC data (Fig. 3, ■) with a variety of "single-enzyme" rapid-equilibrium kinetic models. Visually, none of these fits was fully satis-
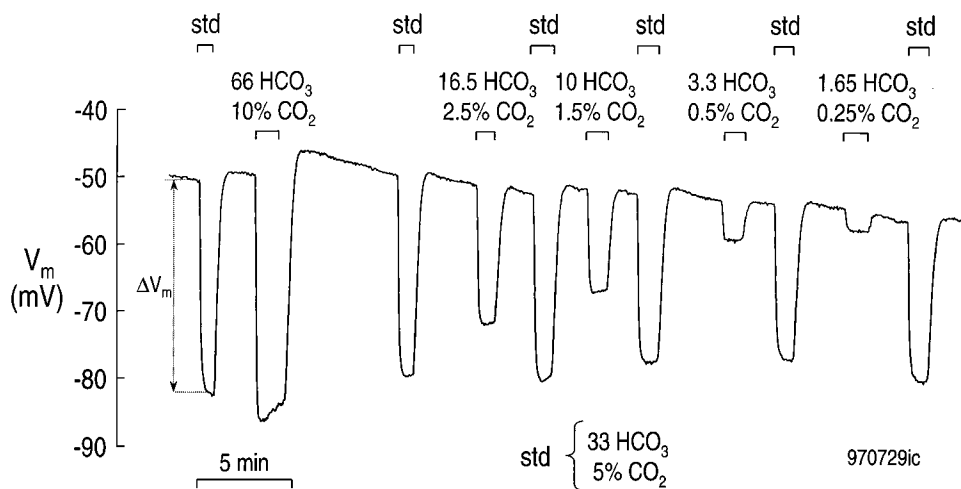
Figure 2. Membrane potential of akNBC-expressing *Xenopus laevis* oocytes during superfusion of solutions with different levels of HCO_3^-/CO_2. In our assay, we bracketed each test pulse with a pulse of the standard (std) CO_2/HCO_3^- solution (33 mM $HCO_3^-/5\%$ CO_2, Solution 2 in Table I). We normalized the ΔV_m under test conditions to the mean ΔV_m for the bracketing std pulses. The HEPES-buffered solution was Solution 1 in Table I. Typical of nine experiments.

factory. We also fitted our normalized ΔV_m data with a model for two enzymes catalyzing the same reaction. Although this fit was visually satisfactory (not shown), the higher of the two $K_m(HCO_3^-)$ values (i.e., ~231 mM) was far higher than the highest $[HCO_3^-]_o$ tested (i.e., 99 mM), and the standard deviation of this $K_m(HCO_3^-)$ was more than twofold higher than the K_m value. Therefore, we fitted the normalized ΔV_m akNBC data with a kinetic model for a single Michaelis-Menten process plus a linear component (Eq. 5). The result of this fit is shown as the solid curve in Fig. 3. As summarized in Table II, the apparent $K_m(HCO_3^-)$ was 10.6 mM.

[HCO_3^-]_o Dependence of rkNBC, Based on Changes in V_m

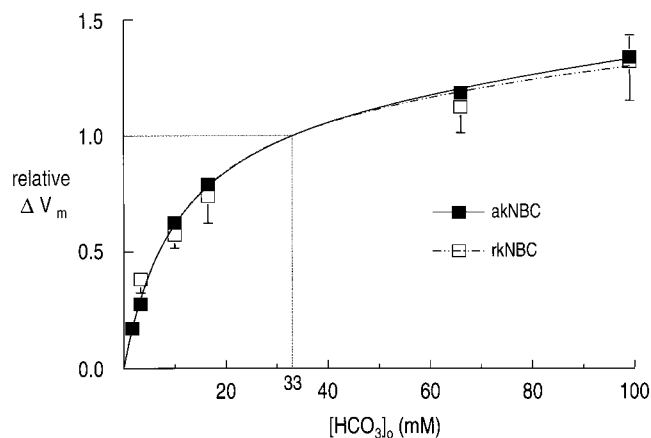To compare the HCO_3^- dependencies of rkNBC and akNBC, we used a protocol identical to that used in Fig.

Figure 3. $[HCO_3^-]_o$ dependence of akNBC and rkNBC, based on ΔV_m data. The solid curve represents the result of a nonlinear least-squares curve fit of Eq. 5 to the akNBC data (■) similar to those shown in Fig. 2. The broken curve represents the result of a similar fit to the rkNBC data (□). Each symbol represents the mean of six to nine data points, obtained in separate experiments. The vertical bars represent SEMs; the bars are omitted when they are smaller than the size of the symbol. The kinetic parameters are summarized in the first two lines of Table II.

2, except that we used oocytes expressing rkNBC rather than akNBC. The results of this series of rkNBC experiments are summarized (Fig. 3, □). The broken curve represents the result of the nonlinear least-squares fit of Eq. 5. The result is an apparent $K_m(HCO_3^-)$ of 10.8 mM, which is not different from the value obtained for akNBC (Table II).

[HCO_3^-]_o Dependence of rkNBC, Studied in Voltage-clamped Oocytes

Because NBC is voltage dependent (Heyer et al., 1999), negative shifts in V_m produced by NBC would slow the very transporter responsible for the V_m change. Because the expression of rkNBC (as judged by NBC-dependent currents obtained under voltage-clamp conditions) was much higher than for akNBC, we elected to use the voltage-clamp approach to study the $[HCO_3^-]_o$ dependence of rkNBC.

Effect of 99 mM HCO_3^- on membrane current. Fig. 4 A shows that briefly exposing a control (i.e., H_2O-injected) oocyte to a solution containing 99 mM $HCO_3^-/15\%$ CO_2 caused very little change in the membrane current ($V_{hold} = -60$ mV). However, as shown in Fig. 4 B, the same maneuver elicited an outward current of ~500 nA in an oocyte expressing rkNBC. Fig. 4 C shows the results from a second oocyte expressing rkNBC. Here, the HCO_3^-/CO_2 exposure caused almost no change in membrane current in the absence of

TABLE II

[HCO_3^-]_o Dependence of akNBC and rkNBC[‡]

NBC	$K_m(HCO_3^-)$	Relative v^*_{max}	α^*	n	Method
	mM		*mM^{-1}*		
akNBC	10.6 ± 1.2	1.22 ± 0.06	0.00226	33	Membrane voltage
rkNBC	10.8 ± 4.4	1.25 ± 0.21	0.00177	45	Membrane voltage
rkNBC	6.5 ± 0.7	0.97 ± 0.03	0.00577	58	Membrane current

[‡]The parameter values were obtained using Eqs. 5 and 6.

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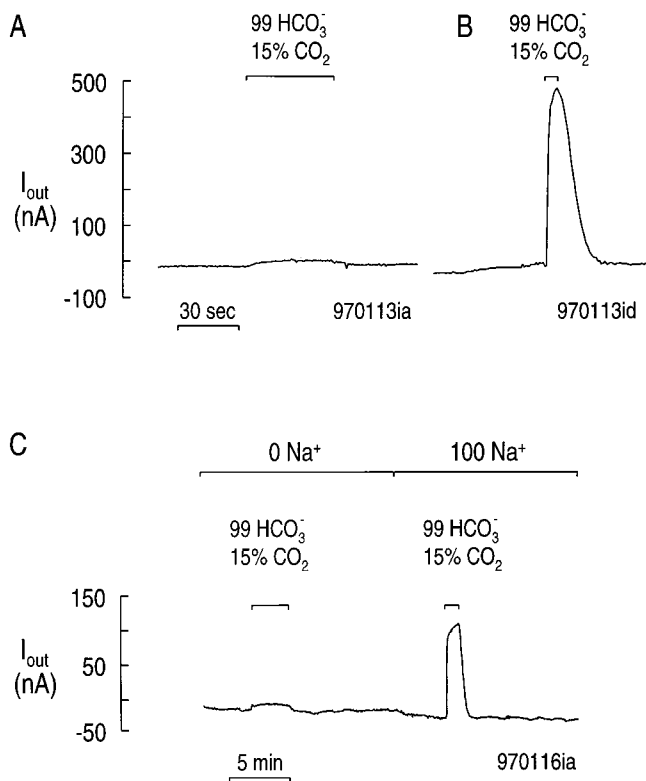Figure 4. Dependence of HCO_3^--evoked currents on the expression rkNBC and the presence of Na^+. (A) H_2O-injected, control oocyte. (B) Oocyte expressing rkNBC. (C) Effect of removing Na^+ in an oocyte expressing rkNBC. In each case, we pulsed the oocyte with a pH 7.5 solution containing 99 mM HCO_3^-/15% CO_2. V_{hold} = -60 mV, 22°C.

Na^+. Restoring the extracellular Na^+ substantially increased the current elicited by adding 99 mM HCO_3^-/15% CO_2. Thus, the current elicited by HCO_3^- depends on the expression of rkNBC and requires Na^+. Previous work has shown that pretreating oocytes with 200 μM DIDS blocks the activity of rkNBC (Romero et al., 1996, 1997b).

Effect on membrane current of adding graded levels of CO_2/HCO_3^- at a constant pH_o of 7.5. We used the peak amplitude of the current induced by exposing oocytes to HCO_3^-/CO_2 as a measure of the inward, electrogenic transport of Na^+ and HCO_3^- via rkNBC. Otherwise, the protocol we used was the same as in Fig. 2. A typical experiment is shown in Fig. 5. We computed a normalized ΔI by dividing the ΔI of the test pulse to the mean ΔI of the two bracketing standard pulses ([HCO_3^-]$_o$ = 33 mM). The normalized ΔI data are summarized in Fig. 6 (●). The curve represents the result of a nonlinear least-squares fit of Eq. 5. The apparent $K_m(HCO_3^-)$ was 6.5 mM (Table II). This $K_m(HCO_3^-)$ value for rkNBC in ΔI experiments is substantially less than for the same clone in ΔV_m experiments.

Effect of Sulfate and Sulfite/Bisulfite on the [HCO_3^-]$_o$ Dependence of the rkNBC Current

To test the hypothesis (see introduction) that NBC can transport $SO_3^=$ or HSO_3^-, we first examined the effect of $SO_3^=$/HSO_3^- on membrane currents carried by rkNBC expressed in *Xenopus* oocytes. As a control, we examined the effects of another divalent anion, sulfate ($SO_4^=$).

Effect of $SO_4^=$ and $SO_3^=$ on rkNBC current evoked by 33 mM HCO_3^-. Fig. 7 shows a voltage-clamp experiment in which we examined the effect of $SO_4^=$ and $SO_3^=$/HSO_3^- on the peak current produced by 33 mM HCO_3^- in an oocyte expressing rkNBC. The changes in current evoked by 33 mM HCO_3^- were virtually identical regardless of whether the dominant background anion was 66 mM gluconate, 33 mM $SO_4^=$ or 33 mM total $SO_3^=$/HSO_3^- (i.e., 26.4 mM $SO_3^=$ + 6.6 mM HSO_3^-). In a total of six similar experiments, the ratio[2] of the current in $SO_4^=$ to the bracketing-paired currents in gluconate was 0.982. Similarly, in seven experiments, the ratio[3] of the current in $SO_3^=$/HSO_3^- to the bracketing-paired currents in gluconate was 0.999. The difference between these mean ratios is not statistically significant ($P = 0.13$, one tail t test). Thus, under the conditions of our experiments, the current carried by rkNBC in 33 mM HCO_3^- is virtually identical in the presence of $SO_4^=$ or $SO_3^=$/HSO_3^-. It is therefore likely that $SO_3^=$/HSO_3^- per se has no effect on the NBC current.

Effect of $SO_4^=$ and $SO_3^=$ on the extracellular HCO_3^- dependence of the rkNBC current. Fig. 8 summarizes the results of typical voltage-clamp experiments in which we examined the [HCO_3^-]$_o$ dependence of the rkNBC current using the same protocol as in Fig. 5, but with either $SO_4^=$ (Fig. 8 A) or $SO_3^=$/HSO_3^- (B) as the dominant anion. Because, in these experiments, [gluconate]$_o$ was only 33 mM when [HCO_3^-]$_o$ was 0 mM, we could only investigate the HCO_3^- dependence of NBC in the [HCO_3^-]$_o$ range of 0–33 mM.

Fig. 8 C summarizes the data as well as the curve fits. Because the $SO_4^=$ and $SO_3^=$/HSO_3^- data in the range of 0–33 mM HCO_3^- did not permit an accurate determination of a slope of the linear component (i.e., α^*), we assumed that α^* was the same as that obtained in the fit of the gluconate data in Fig. 6 ([HCO_3^-]: 0–99 mM). The results of these curve fits (Eq. 7) are summarized in Table III, and show that the apparent K_m and v_{max} values are virtually identical, regardless of whether HCO_3^- was varied in the presence of gluconate, $SO_4^=$ or $SO_3^=$/HSO_3^-.

Effect of Sulfite/Bisulfite on DIDS-sensitive pH_i Change in Oocytes Expressing rkNBC

Is it possible that $SO_3^=$/HSO_3^- could ride rkNBC and yet not produce a change in current? If NBC could nei-

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[2]The log-normal mean was 0.982 +0.028/−0.027.
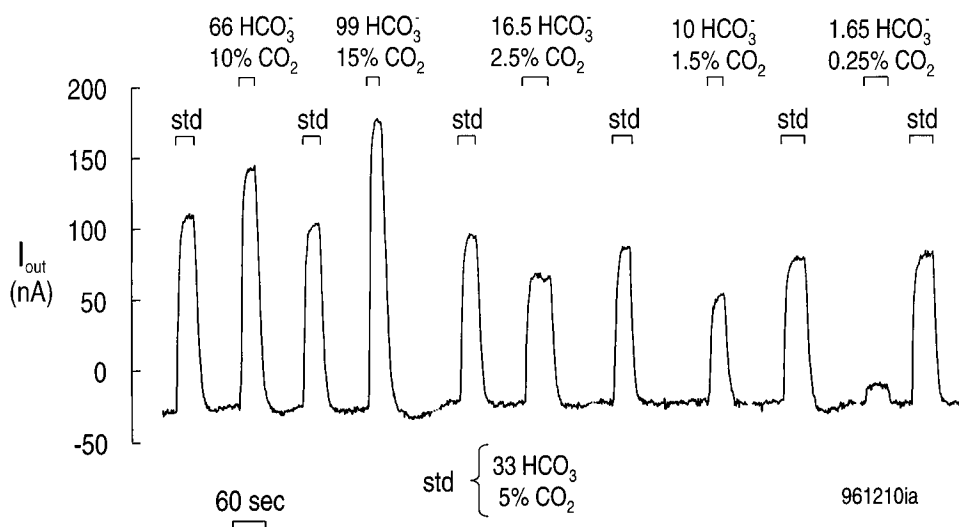
[3]The log-normal mean was 0.999 +0.025/−0.025.

Figure 5. Membrane current of rkNBC-expressing *Xenopus laevis* oocyte during superfusion of solutions with different levels of HCO_3^-/CO_2. The protocol for changing the extracellular solutions was the same as in Fig. 2. The standard (std) solution contained 33 mM HCO_3^-/5% CO_2 (Table I, Solution 2). Typical of eight experiments. $V_{hold} = -60$ mV, 22°C.

ther distinguish $SO_3^=$ from $CO_3^=$, nor HSO_3^- from HCO_3^-, then introducing $SO_3^=/HSO_3^-$ would have no effect on the current carried by NBC if the transporter were already near v_{max}. However, because the pK values governing the reactions $SO_3^= + H^+ \leftrightarrow HSO_3^-$ and $HSO_3^- + H^+ \leftrightarrow H_2SO_3$ are so much lower than for the corresponding reactions involving $CO_3^=$, HCO_3^-, and H_2CO_3, the pH_i changes for NBC carrying $SO_3^=/HSO_3^-$ would be much slower than for NBC carrying $CO_3^=/HCO_3^-$ (see discussion). We therefore examined the possibility that $SO_3^=/HSO_3^-$ would slow the pH_i produced by NBC in the presence of CO_2/HCO_3^-.

Our assay was to expose an rkNBC-expressing oocytes to an extracellular solution buffered with 3.3 mM HCO_3^-/0.5% CO_2. As shown in Fig. 9 A, an experiment conducted in the absence of $SO_3^=/HSO_3^-$, applying HCO_3^-/CO_2 causes a rapid but small pH_i decrease (a–b), followed by a pH_i increase (b–c). After ~30 min,

when pH_i was recovering at a constant rate in the HCO_3^-/CO_2 solution, we applied 1 mM DIDS for ~15 min. This DIDS blocked the NBC-mediated alkalinization, unmasking a slow acidification (c–d). We took the difference between the alkalinization rate in the absence of DIDS (b–c) and the presence of DIDS (c–d) as an index of the net base influx mediated by rkNBC. In a total of five similar experiments, the DIDS-dependent alkalinization rate was $0.98 \pm 0.27 \times 10^{-4}$ pH U/s, with a mean initial pH_i value of 7.34 ± 0.04.

The experiment in Fig. 9 B is the same as in A, except that the oocyte was exposed to 26.4 mM $SO_3^=$/6.6 mM HSO_3^- during the application of the 3.3 mM HCO_3^-/ 0.5% CO_2 solution. In a total of six such experiments, the mean net base influx was $0.88 \pm 0.38 \times 10^{-4}$ pH U/s, which is not significantly different from the value in the absence of $SO_3^=/HSO_3^-$ ($P = 0.28$, an unpaired one tail t test). The mean initial pH_i in the $SO_3^=/HSO_3^-$
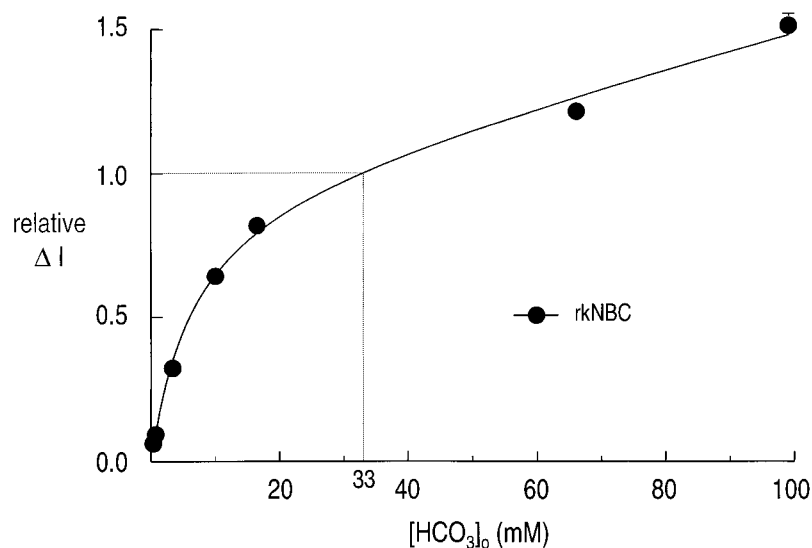

Figure 6. $[HCO_3^-]_o$ dependence of rkNBC current. The solid curve represents the result of a nonlinear least-squares curve fit of Eq. 5 to the data (●) similar to those shown in Fig. 5. Each symbol represents the mean of five to eight data points obtained in separate experiments. The vertical bar represents the SEM; the bars are omitted when they are smaller than the size of the symbol. The kinetic parameters are summarized in the last line of Table II.
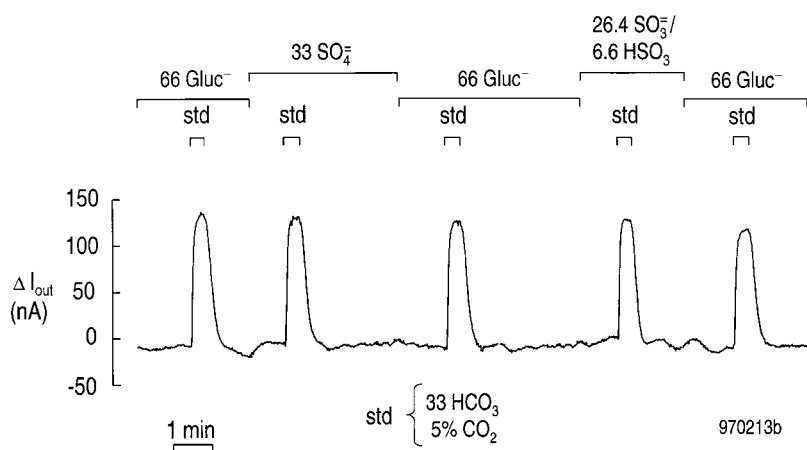
Figure 7. Effect of $SO_4^=$ and $SO_3^=/HSO_3^-$ on the current carried by rkNBC. The oocyte was exposed five times to a solution containing 33 mM HCO_3^-/5% CO_2. For the first, third, and fifth pulses, we switched from a HEPES solution (Table I, Solution 1) to a solution containing 33 mM HCO_3^-/5% CO_2 solution (Table I, Solution 2). For the second HCO_3^-/CO_2 pulse, we switched from a HEPES solution containing 33 mM $SO_4^=$ (Table I, Solution 3) to a 33 mM HCO_3^-/5% CO_2 that also contained 33 mM $SO_4^=$ (Table I, Solution 4). For the fourth HCO_3^- /CO_2 pulse, we switched from a HEPES-containing 33 mM $SO_3^=/HSO_3^-$ (Table I, Solution 5) to a 33-mM HCO_3^-/5% CO_2 solution that also contained 33 mM $SO_3^=/HSO_3^-$ (Table I, Solution 6). Typical of six experiments. $V_{hold} = -60$ mV, 22°C.

experiments was 7.39 ± 0.06, which also is not significantly different from the value in the absence of $SO_3^=/HSO_3^-$ ($P = 0.20$, unpaired two tail t test).

Effect of Altering $[CO_3^=]_o$ and pH_o on the Current Carried by rkNBC

Because the data introduced above make it unlikely that rkNBC, as expressed in *Xenopus* oocyte, interacts with HSO_3^- or $SO_4^=$, we asked whether rkNBC transports $CO_3^=$. Our approach was to hold $[HCO_3^-]_o$ constant at 33 mM while raising $[CO_2]$ from 0.1% (pH_o 9.2, $[CO_3^=]_o = \sim3,500$ μM) to 100% (pH_o 6.2, $[CO_3^=]_o = \sim3.5$ μM). Our protocol was similar to that in Fig. 5, with two pulses of our standard solution (Table I, Solution 2, $[CO_3^=]_o = \sim70$ μM) bracketing each test pulse. Because $CaCO_3$ precipitated from the pH 9.2 solution, which nominally contains $\sim3,500$ μM $CO_3^=$, we replaced all Ca^{2+} with Mg^{2+}. Control experiments showed that this switch has no effect on the NBC current.[4]

Fig. 10 A summarizes our results, expressed as normalized ΔI data as a function of $[CO_3^=]_o$. The dashed curve, which represents the best fit of a normalized Michaelis-Menten equation (total residual variance = 0.0341), systematically passes above or below points, depending where they lie along the curve. The solid curve, which represents the best fit of the normalized Michaelis-Menten equation plus a linear component[5]

[total residual variance (trv) = 0.0088], also systematically misfits the data. On the other hand, when we plot the same data as a function of pH_o (Fig. 10 B), whether the best-fit pH titration curve (pK = 7.50 ± 0.05) passes above or below a point does not depend systematically on the position of the point. In addition, the total residual variance of this fit (trv = 0.0055) is comparable with that in Fig. 6 (trv = 0.0043).

DISCUSSION

Why Is the NBC Current–$[HCO_3^-]_o$ Relationship Not Sigmoidal?

As expressed in the *Xenopus* oocyte, rkNBC is electrogenic. This observation is consistent with a Na^+:HCO_3^- stoichiometry of 1:2, or perhaps 1:3, as has been observed in membrane vesicles prepared from rabbit kidney (Soleimani et al., 1987). Recent voltage-clamp experiments suggest that rkNBC, at least as expressed in oocytes, has a stoichiometry of 1:2 (Heyer et al., 1999; Sciortino and Romero, 1999). Thus, one would not be surprised if the relationship between NBC current and $[HCO_3^-]_o$ were more complex than a simple right-rectangular hyperbola, for example. In fact, we found that the current–$[HCO_3^-]_o$ relationship is well described by the sum of a hyperbola and a line. Why did we not observe a sigmoidal current–$[HCO_3^-]_o$ relationship?

First, it is possible that, as expressed in *Xenopus* oocytes, rkNBC has but a single HCO_3^--related substrate. If rkNBC carried a single $CO_3^=$ (equivalent to two HCO_3^-), then the Na^+:HCO_3^- stoichiometry would be 1:2, and thus one would expect the current–$[HCO_3^-]_o$ relationship to be a right-rectangular hyperbola. Thus, our data are consistent with the hypothesis that rkNBC binds a single HCO_3^- related species, $CO_3^=$.

Second, if rkNBC carried two HCO_3^- ions (for a stoichiometry of 1:2) or one HCO_3^- and one $CO_3^=$ (for a stoichiometry of 1:3), then the current–$[HCO_3^-]_o$ relationship might show a foot at low $[HCO_3^-]_o$, but only if
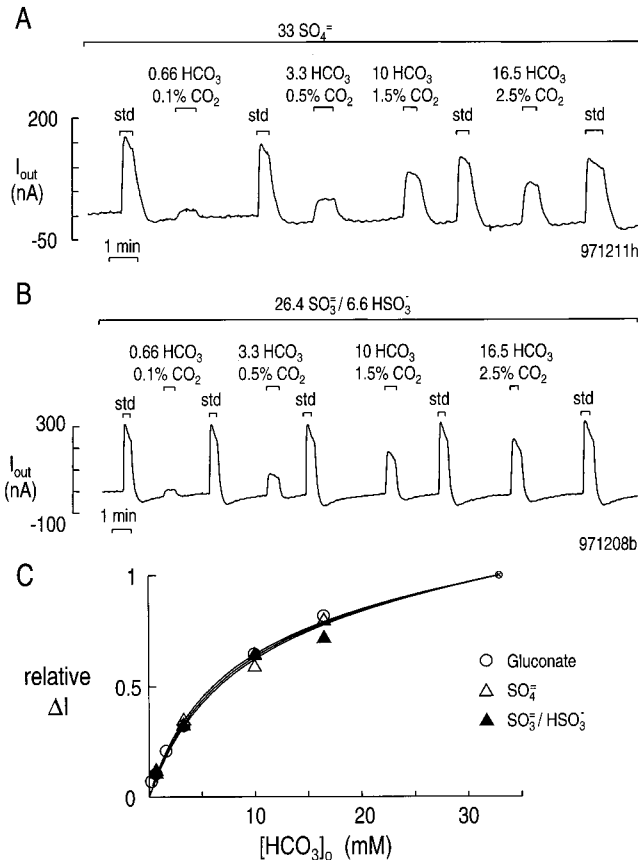
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[4]We compared NBC currents in two pH 7.8 solutions ($[CO_3^=]_o = \sim138$ mM); one was a Ca^{2+}-containing solution in which we observed no precipitation, and the other was a solution in which Mg^{2+} replaced Ca^{2+}. The ratio of NBC current in the pH 7.8, nominally Ca^{2+}-free solution to the currents in the bracketing standard pH 7.5 solution had a log-normal mean of $1.097 +0.056/-0.054$ ($n = 3$). The comparable ratio for the pH 7.8 Ca^{2+}-containing solution had a log-normal mean of $1.079 +0.047/-0.047$ ($n = 4$). The difference between these mean ratios is not statistically significant ($P = 0.318$, one tail t test).

[5]Eq. 5, but with $[CO_3^=]$ replacing $[HCO_3^-]$. The standard $[CO_3^=]$ was 70 μM.

Figure 8. Effect of $SO_4^=$ and $SO_3^=/HSO_3^-$ on the $[HCO_3^-]_o$ dependence of the current carried by rkNBC. (A) Experiments conducted in 33 mM $SO_4^=$. The experimental protocol was the same as in Fig. 5, except that all solutions contained 33 mM $SO_4^=$. Typical of eight experiments. $V_{hold} = -60$ mV, 22°C, pH 7.5. (B) Experiments conducted in 26.4 mM $SO_3^=/6.6$ mM HSO_3^-. The protocol was the same as in A. Typical of 10 experiments. (C) Effect of $SO_4^=$ and $SO_3^=/HSO_3^-$ on $[HCO_3^-]_o$ dependency of rkNBC. One of the solid curves is the same as that in Fig. 6, and represents the fit of Eq. 5 to the data obtained in the absence of $SO_4^=$ and $SO_3^=/HSO_3^-$ (O). The other two solid curves represent the fits of Eq. 7 to the data obtained in $SO_4^=$ (△), as in A, and the data obtained in $SO_3^=/HSO_3^-$ (▲), as in B. Each symbol represents the mean of 6–17 data points, obtained in separate experiments. The bars representing SEM are omitted because they are smaller than the size of the symbol.

the K_m values for the two binding sites were sufficiently similar and high. For example, if rkNBC carried two HCO_3^- ions, and the K_m values for one binding site was 6.5 mM (as observed), but the K_m for the other was only 0.1 mM, then we would not have been able to detect a foot,[6] given the precision of our data. Thus, our results are consistent with the hypothesis that rkNBC binds two HCO_3^--related species, but that we cannot detect a foot due to a low K_m value.

Third, if rkNBC carried three HCO_3^- ions (for a sto-

ichiometry of 1:3), then the current–$[HCO_3^-]_o$ relationship might show a foot, but, again, only if the K_m values for all three were sufficiently similar and high. For example, if the K_m values were 6.5, 6.5, and 0.1 mM, or 6.5, 0.1, and 0.1 mM, we would not have been able to detect a foot.[7] Thus, our data are consistent with the hypothesis that rkNBC binds three HCO_3^-, but that we cannot detect a foot due to a low K_m value.

Fourth, it is possible that a systematic error in the way we monitored rkNBC activity may have masked a foot. For example, when we expose a cell to HCO_3^-/CO_2, rkNBC transports Na^+ and HCO_3^- into the cell, and the passive entry of CO_2 leads to the production of HCO_3^- and H^+. We attempted to minimize such effects by making our measurements very soon after exposing the cell to HCO_3^-/CO_2. Nevertheless, any buildup of intracellular Na^+, HCO_3^-, and/or H^+ that might have occurred in the vicinity of rkNBC would have slowed the cotransporter; the effect would have been greater at higher HCO_3^-/CO_2 levels.

The "Linear Component"

The present study represents the first kinetic experiments on a member of the newly cloned NBC family. As suggested above, we would not have been surprised had the current–$[HCO_3^-]_o$ relationship been sigmoidal. Instead, the shape of the relationship appears to be the sum of a hyperbola and a line. We could not adequately fit the current versus $[HCO_3^-]_o$ data using any of several rapid-equilibrium models[8] for random or ordered binding of $HCO_3^-/CO_3^=$ to the cotransporter. We therefore suggest that some additional process, which is a first-order function of $[HCO_3^-]_o$, contributes to the current, especially at $[HCO_3^-]_o$ values above 33 mM. This linear component is not present in water-injected oocytes. As shown in Fig. 4 A, the transition from HEPES to 99 mM HCO_3^- caused a slow and small (~6 nA) outward current in control oocytes. In contrast, as shown in Fig. 4 B, the same maneuver caused a rapid and large (~490 nA) outward current in rkNBC-expressing oocytes.

The linear component also requires Na^+. As shown in Fig. 4 C, a transition from Na^+-free HEPES to Na^+-free 99 mM HCO_3^- caused only slow and small (~9 nA) outward current in rkNBC-expressing *Xenopus* oocytes. However, in the presence of Na^+, the transition from HEPES to 99 mM HCO_3^- produced a much larger current (~140 nA). Because virtually the entire HCO_3^--

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[6]We modeled a rapid-equilibrium, random bireactant system (Segel, 1993) with dissociation constants of 6.5 and 0.1 mM, and interaction factors of $\alpha = \beta = 1$.

[7]We modeled a rapid-equilibrium, random terreactant system (Segel, 1993) with dissociation constants of 6.5, 6.5 (or 0.1), and 0.1 mM, and interaction factors of $\alpha = \beta = 1$.

[8]We explored the following random-binding models for HCO_3^--related species: 2 HCO_3^-, 3 HCO_3^-, 1 $HCO_3^- + 1 CO_3^=$. We also tested the following ordered-binding models: 2 HCO_3^-, 3 HCO_3^-, HCO_3^- then $CO_3^=$, $CO_3^=$ then HCO_3^-.

Effect of $SO_4^=$ and $SO_3^=/HSO_3^-$ on Kinetic Parameters of rkNBC[‡]

Major extracellular anion	$K_m(HCO_3^-)$	Relative v_{max}	$\alpha*$	n
	mM		*mM^{-1}*	
66 mM gluconate$^-$	6.5 ± 0.7	0.97 ± 0.03	0.00577	58
33 mM SO$_4^=$	7.1 ± 0.5	0.98	0.00577 (fixed)	24
26.4 mM SO$_3^=$/6.6mM HSO$_3^-$	7.6 ± 0.6	0.99	0.00577 (fixed)	48

[‡]The parameter values for 66 mM gluconate were obtained using Eqs. 5 and 6, whereas those for $SO_4^=$ and $SO_3^=/HSO_3^-$ were obtained using Eqs. 7 and 8.

induced current at 99 mM HCO_3^- requires both rkNBC and Na^+, it is very likely that the linear component is carried by rkNBC or a closely related protein. What are the possible sources of the linear component of the current?

First, expression of rkNBC might induce the expression of a previously silent, endogenous NBC-like protein with a low affinity for HCO_3^-. As described in several reports, the expression of exogenous membrane proteins induces various endogenous channels in *Xenopus* oocytes (Attali et al., 1993, 1995; Shimbo et al., 1995; Tzounopoulos et al., 1995; Buyse et al., 1997).

Second, the linear component could represent a parallel HCO_3^--conductance pathway that is part of rkNBC. The glutamate transporters (Fairman et al., 1995) have an intrinsic Cl^- conductance, and the electroneutral Na/HCO_3 cotransporter has an intrinsic conductance to Na^+ (Choi, I., C. Aalkjaer, E.L. Boulpaep, and W.F. Boron, personal communication).

Third, it is possible that increases in $[CO_2]$ and/or $[HCO_3^-]$ cause the $Na^+:HCO_3^-$ stoichiometry of rkNBC to shift from, say, 1:2 to 1:3. If the turnover of rkNBC were governed by a classical kinetic model, then the shift in stoichiometry would lead to greater currents at greater values of $[HCO_3^-]_o$.

Fourth, it is possible that changes in the concentrations of gluconate and $CO_3^=$, both of which chelate Ca^{2+}, led to changes in free $[Ca^{2+}]_o$ that affected NBC. However, in the series of experiments summarized in Fig. 10, we showed that replacing all Ca^{2+} with Mg^{2+} has no effect on the current carried by NBC.

Effect of Extracellular $HSO_3^-/SO_3^=$ on rkNBC

We found that $SO_3^=/HSO_3^-$ affected neither the currents (Fig. 7) nor the pH_i changes (Fig. 9) produced by rkNBC as it functions as a heterologously expressed protein in *Xenopus* oocytes. To assess these results, we examined a series of models (Fig. 11, A–G and A′–G′) for how $SO_3^=/HSO_3^-$ might interact with NBC, and predicted the effects of these interactions on the currents and pH_i changes produced by NBC. In A and A′, we assume that neither $SO_3^=$ nor HSO_3^- is capable of interacting with NBC, so that $SO_3^=/HSO_3^-$ should have no effect on either NBC-mediated currents or pH_i changes, as we in fact observed.

In Fig. 11, B–D and B′– D′, NBC transports $SO_3^=$ and/or HSO_3^-. Although it is conceivable that $SO_3^=/HSO_3^-$ might not affect the currents that NBC carries (depending on the concentrations and K_m values for $SO_3^=$, HSO_3^-, $CO_3^=$, and HCO_3^-), the pH_i changes would be appreciably slower for three reasons. (a) The pK of the reaction $SO_3^= + H^+ \leftrightarrow HSO_3^-$ is ~6.9, compared with ~10 for the equilibrium $CO_3^= + H^+ \leftrightarrow HCO_3^-$. (b) The pK of the reaction $HSO_3^- + H^+ \leftrightarrow H_2SO_3$ is ~1.6, compared with ~3.4 for the equilibrium $HCO_3^- + H^+ \leftrightarrow H_2CO_3$. (c) $[H_2SO_3]_i$ is so low that the net efflux of H_2SO_3 is expected to be negligible. In contrast, H_2CO_3 forms CO_2, which is present at relatively high concentrations and can rapidly exit the cell. The net effect is that incoming $SO_3^=$ and/or HSO_3^- will neutralize fewer H^+ than incoming $CO_3^=$ and/or HCO_3^-. Because we

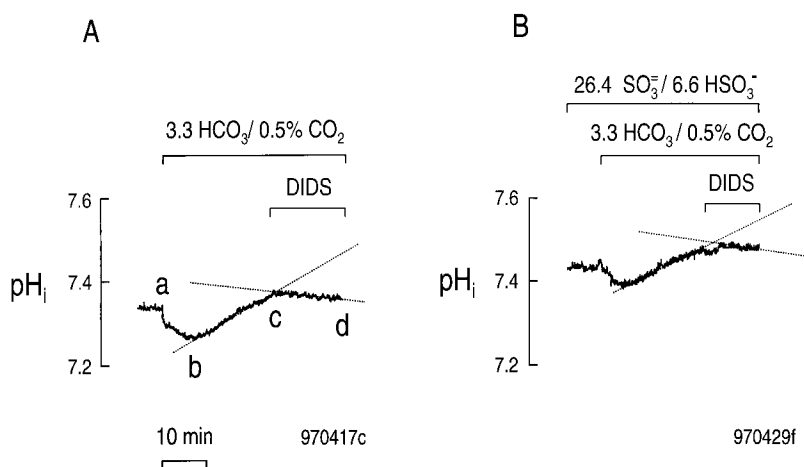**Figure 9.** Effect of $SO_3^=/HSO_3^-$ on the DIDS-sensitive recovery of pH_i from a CO_2-induced acid load. (A) Absence of $SO_3^=/HSO_3^-$. During the indicated time, the solution bathing an oocyte expressing rkNBC was switched from standard HEPES (Table I, Solution 1) to a solution containing 3.3 mM HCO_3^-/0.5% CO_2. During the pH_i recovery from the CO_2-induced acid load, we blocked rkNBC by applying 1 mM DIDS. (B) Presence of 26.4 mM $SO_3^=$/6.6 mM HSO_3^-. The protocol was the same as in A, except that all solutions contained 26.4 mM $SO_3^=$/6.6 mM HSO_3^-.

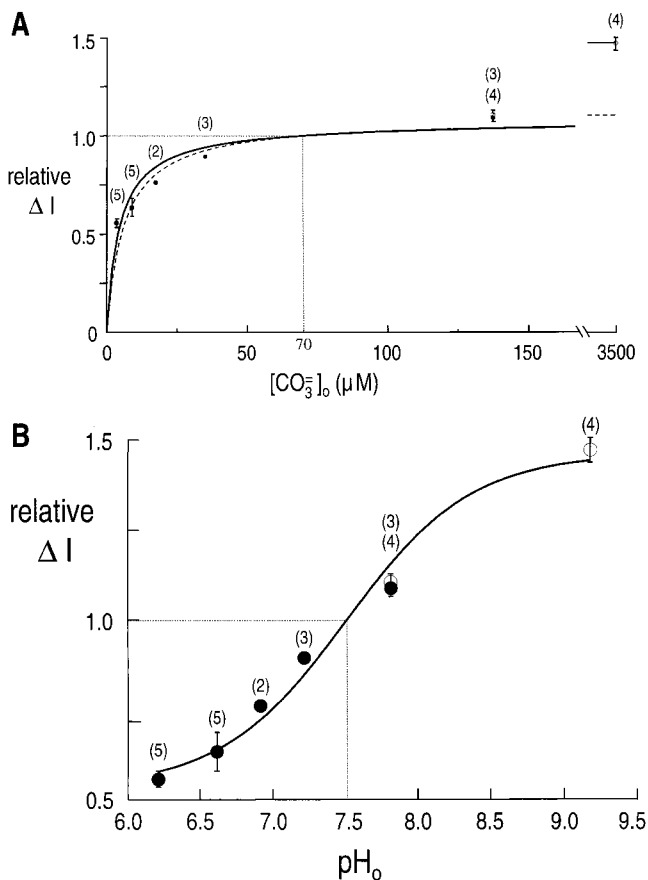**Figure 10.** Effect of varying $[CO_3^=]_o$ and pH_o on the rkNBC current. (A) Relative rkNBC current as a function of $[CO_3^=]_o$. ● represent data obtained in the presence of Ca^{2+}, and ○, with Mg^{2+} replacing Ca^{2+}. The dashed curve is the result of a nonlinear least-squares fit of the data by a normalized Michaelis-Menten equation. The best-fit value for $K_m(CO_3^=)$ was 6.1 ± 1.5 μM, and for I_{max}, 1.09. The solid curve represents the best fit of the data by a normalized Michaelis-Menten equation plus a linear component.[5] The best-fit value for $K_m(CO_3^=)$ was 4.5 ± 0.6 μM, for I_{max} was 1.05, and for α was 0.000122 $μM^{-1}$. (B) Relative rkNBC current as a function of pH_o. The solid curve is the result of a nonlinear least-squares fit of the data by a normalized pH titration curve (Boron and Knakal, 1992). The best-fit value for pK was 7.50 ± 0.05. The number of determinations is given in parentheses. The vertical bars indicate SEM values; they are omitted where the length of the bar is smaller than the size of the symbol.

found that $SO_3^=/HSO_3^-$ had no effect on NBC-mediated pH_i changes, Fig. 11, B–D and B′–D′, must be incorrect (i.e., NBC cannot transport $SO_3^=$ and/or HSO_3^- under the conditions of our experiments).

In Fig. 11, E–G and E′–G′, $SO_3^=$ and/or HSO_3^- are competitive inhibitors for the transport of $CO_3^=$ and/or HCO_3^-, respectively. In these cases, adding $SO_3^=/HSO_3^-$ should decrease both the NBC current and pH_i changes. Inasmuch as we found that $SO_3^=/HSO_3^-$ had no effect on either, E–G and E′–G′ must be incorrect (i.e., neither $SO_3^=$ nor HSO_3^- can competitively inhibit NBC under the conditions of our experiments).

Thus, we conclude that neither $SO_3^=$ nor HSO_3^- interacts with rkNBC under the conditions of our experiments. This conclusion is in agreement with an observation of Jentsch et al. (1986) on bovine corneal endothelial cells. However, Soleimani and Aronson (1989), in studies on renal basolateral membrane vesicles, reported that $SO_3^=/HSO_3^-$, when applied in the presence of HCO_3^-, stimulates ^{22}Na uptake mediated by the Na/HCO_3 cotransporter. Based on this and other data, those authors concluded that NBC transports Na^+, $CO_3^=$, and HCO_3^- in a stoichiometry of 1:1:1, and that $SO_3^=$ can substitute for $CO_3^=$ at the $CO_3^=$ binding site. This line of reasoning was the first, and probably the strongest, evidence that NBC can transport $CO_3^=$.

We could reconcile our data and those of Jentsch et al. (1986) with the data of Soleimani and Aronson (1989) by proposing that (a) oocytes and corneal endothelial cells have a "factor" that prevents the $SO_3^=/HSO_3^-$–rkNBC interaction, or (b) oocytes and corneal endothelial cells lack a factor required for the $SO_3^=/HSO_3^-$–rkNBC interaction. We think that the latter is more likely. The missing factor could be an enzyme(s) that catalyzes a posttranslational modification of NBC (e.g., phosphorylation) that is essential for the NBC–$SO_3^=/HSO_3^-$ interaction, or the missing factor could be an additional NBC subunit that confers sensitivity to $SO_3^=/HSO_3^-$. Alternatively, the missing factor triggered by $SO_3^=/HSO_3^-$ could be part of a purely regulatory pathway that modulates NBC (i.e., not an intrinsic part of NBC). Thus, although the rkNBC protein expressed in *Xenopus* oocytes can carry out all other known functions of the renal NBC, rkNBC by itself cannot interact with $SO_3^=$ or HSO_3^-.

Effect of Altering $[CO_3^=]_o$ and pH_o on rkNBC

Fig. 10 shows the effect on the current carried by rkNBC of simultaneously varying $[CO_3^=]_o$ and pH_o. The best-fit Michaelis-Menten curve, with or without a linear component, fails to adequately fit the data, expressed in terms of $[CO_3^=]_o$ (Fig. 10 A). On the other hand, the best-fit pH titration curve nicely fits the data, expressed in terms of pH_o, over the entire range of pH_o values. One possible explanation for these results is that rkNBC transports $CO_3^=$ with a K_m of ~6 μM, but that an idiosyncratic pH_o sensitivity is responsible for the poor fits at the extreme $[CO_3^=]_o$ values. However, the most straightforward explanation for these data is that rkNBC is not sensitive to $[CO_3^=]_o$ in the range 3.5–3,500 μM, but has a single titratable site that inhibits NBC when protonated. For example, this site could be an HCO_3^--binding site that has a lower affinity for its substrate when protonated. Note that we cannot rule out the possibility that rkNBC transports $CO_3^=$ with an extremely high affinity (i.e., a $K_m \ll 3.5$ μM).
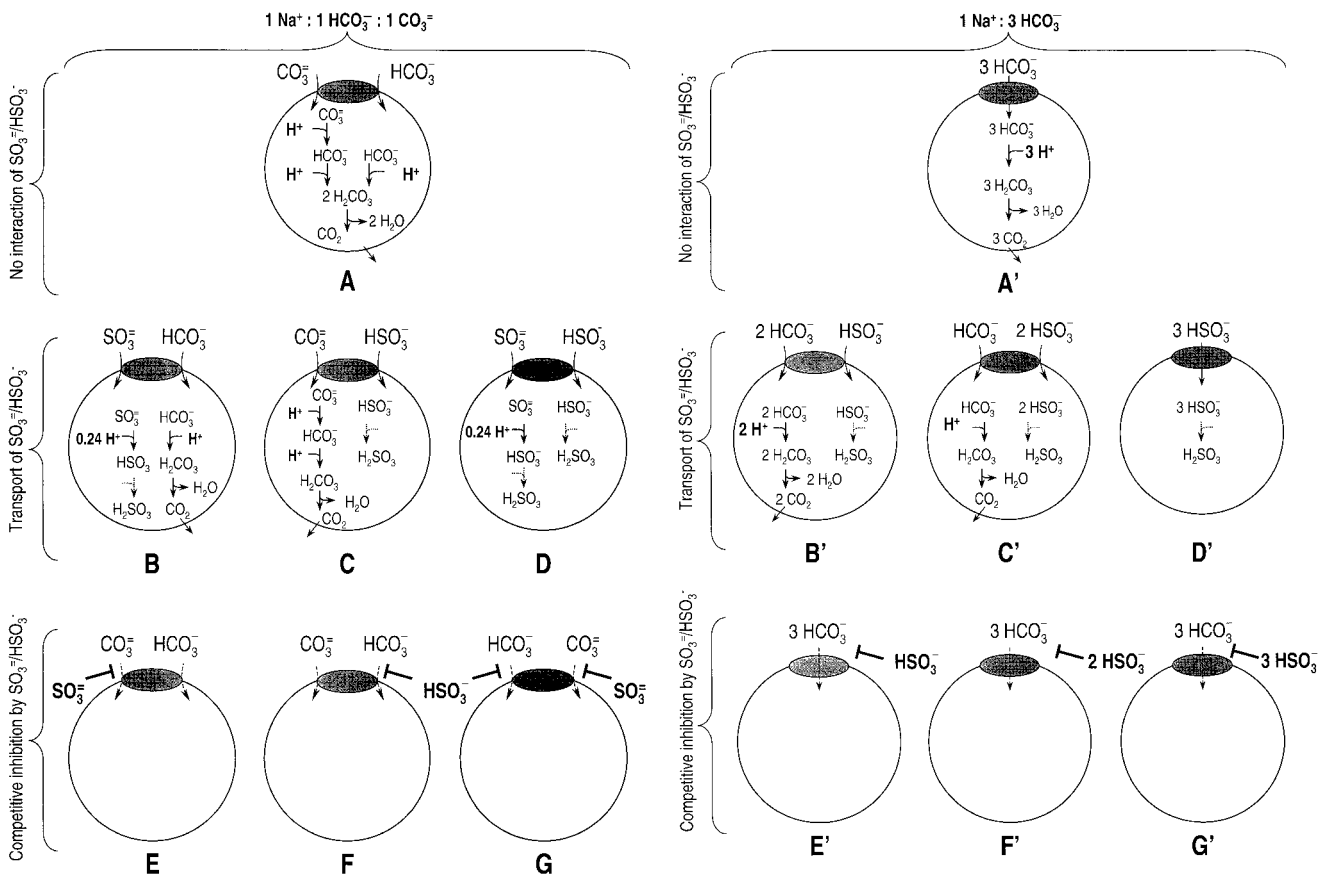
Figure 11. Predicted effects of $SO_3^=$ and/or HSO_3^- on the pH_i changes mediated by NBC. A–G refer to a general scheme in which NBC transports one Na^+, one $CO_3^=$, and one HCO_3^-. (A) Neither $SO_3^=$ nor HSO_3^- interact with cotransporter. The entering $CO_3^=$ can neutralize two H^+, and the entering HCO_3^- can neutralize an additional H^+, for a total of three H^+ neutralized. (B) $SO_3^=$ replaces $CO_3^=$. To the extent that $SO_3^=$ replaces $CO_3^=$, only 1.24 H^+ are neutralized, and thus the expected rate of pH_i increase will be 41% of that in A. (C) HSO_3^- replaces HCO_3^-. Only two H^+ are neutralized, and thus the expected rate of pH_i increase will be only 67% of that in A. (D) $SO_3^=$ and HSO_3^- replace, respectively, $CO_3^=$ and HCO_3^-. Only total 0.24 H^+ ions are neutralized, and thus the expected rate of pH_i increase will be only 8% of that in A. (E) $SO_3^=$ acts as a competitive inhibitor of $CO_3^=$. (F) HSO_3^- acts as a competitive inhibitor of HCO_3^-. (G) $SO_3^=$ and HSO_3^- both are competitive inhibitors. A′–G′ refer to a general scheme in which NBC transports one Na^+ and three HCO_3^-. (A′) Neither $SO_3^=$ nor HSO_3^- interact with cotransporter. A total of three H^+ are neutralized. (B′) HSO_3^- replaces one HCO_3^-. Only two H^+ are neutralized, and thus the expected rate of pH_i increase will be 67% of that in A′. (C′) Two HSO_3^- replace two HCO_3^-. Only one H^+ is neutralized, and thus the expected rate of pH_i increase will be only 33% of that in A′. (D′) Three HSO_3^- replace three HCO_3^-. No H^+ ions are neutralized, and thus the expected rate of pH_i increase will be 0% of that in A′. In E′–G′, HSO_3^- acts as a competitive inhibitor at one, two, and three HCO_3^--binding sites, respectively.

REFERENCES

Abuladze, N., I. Lee, D. Newman, J. Hwang, K. Boorer, A. Pushkin, and I. Kurtz. 1998a. Molecular cloning, chromosomal localiza-tion, tissue distribution, and functional expression of the human pancreatic sodium bicarbonate cotransporter. *J. Biol. Chem.* 273: 17689–17695.

Abuladze, N., I. Lee, D. Newman, J. Hwang, A. Pushkin, and I. Kurtz. 1998b. Axial heterogeneity of sodium-bicarbonate cotransporter expression in the rabbit proximal tubule. *Am. J. Physiol. Renal Physiol.* 274:F628–F633.

Akiba, T., R.J. Alpern, J. Eveloff, J. Calamina, and D.G. Warnock. 1986. Electrogenic sodium/bicarbonate cotransport in rabbit renal cortical basolateral membrane vesicles. *J. Clin. Invest.* 78: 1472–1478.

Alpern, R.J. 1985. Mechanism of basolateral membrane $H^+/OH^-/$ HCO_3^- transport in the rat proximal convoluted tubule. A sodium-coupled electrogenic process. *J. Gen. Physiol.* 86:613–636.

Attali, B., E. Guillemare, F. Lesage, E. Honore, G. Romey, M. Lazdunski, and J. Barhanin. 1993. The protein IsK is a dual activator of K^+ and Cl^- channels. *Nature.* 365:850–852.

Attali, B., H. Latter, N. Rachamim, and H. Garty. 1995. A cortico-steroid-induced gene expressing an "IsK-like" K^+ channel activity in *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA.* 92:6092–6096.

Boron, W.F., and E.L. Boulpaep. 1983. Intracellular pH regulation in the renal proximal tubule of the salamander: basolateral HCO_3^- transport. *J. Gen. Physiol.* 81:53–94.

Boron, W.F., and E.L. Boulpaep. 1989. The electrogenic Na/HCO_3 cotransporter. *Kidney Int.* 36:392–402.

Boron, W.F., M.A. Hediger, E.L. Boulpaep, and M.F. Romero. 1997. The renal electrogenic $Na^+:HCO_3^-$ cotransporter. *J. Exp. Biol.* 200:263–268.

Boron, W.F., and R.C. Knakal. 1992. Na^+-dependent Cl-HCO_3 exchange in the squid axon. Dependence on extracellular pH. *J. Gen. Physiol.* 99:817–837.

Burnham, C.E., H. Amlal, Z. Wang, G.E. Shull, and M. Soleimani. 1997. Cloning and functional expression of a human kidney $Na^+:HCO_3^-$ cotransporter. *J. Biol. Chem.* 272:19111–19114.

Buyse, G., T. Voets, J. Tytgat, C. De Greef, G. Droogmans, B. Nilius, and J. Eggermont. 1997. Expression of human pICln and ClC-6 in *Xenopus* oocytes induces an identical endogenous chloride conductance. *J. Biol. Chem.* 272:3615–3621.

Choi, I., M.F. Romero, N. Khandoudi, A. Bril, and W.F. Boron. 1999. Cloning and characterization of a human electrogenic Na^+-HCO_3^- cotransporter isoform (hhNBC). *Am. J. Physiol. Cell Physiol.* 276:C576–C584.

Fairman, W.A., R.J. Vandenberg, J.L. Arriza, M.P. Kavanaugh, and S.G. Amara. 1995. An excitatory amino-acid transporter with properties of a ligand-gated chloride channel. *Nature.* 375:599–603.

Gross, E., and U. Hopfer. 1998. Voltage and cosubstrate dependence of the Na-HCO_3 cotransporter kinetics in renal proximal tubule cells. *Biophys. J.* 75:810–824.

Heyer, M., S. Müller-Berger, M.F. Romero, W.F. Boron, and E. Frömter. 1999. Stoichiometry of the rat kidney Na^+-HCO_3^- cotransporter expressed in *Xenopus laevis* oocytes. *Pflügers Arch.* 438:322–329.

Jensen, L., B.M. Schmitt, D. Brown, U.V. Berger, M.A. Hediger, W.F. Boron, and S. Breton. 1999. Localization of sodium bicarbonate co-transporter (NBC) protein and mRNA in rat epididymis. *Biol. Reprod.* 60:573–579.

Jentsch, T.J., B.S. Schill, P. Schwartz, H. Matthes, S.K. Keller, and J. Wiederholt. 1985. Kidney epithelial cells of monkey origin (BSC-1) express a sodium bicarbonate cotransport. *J. Biol. Chem.* 260:15554–15560.

Jentsch, T.J., P. Schwartz, B.S. Schill, B. Langner, A.P. Lepple, S.K. Keller, and M. Wiederholt. 1986. Kinetic properties of the sodium bicarbonate (carbonate) symport in monkey kidney epithelial cells (BSC-1). *J. Biol. Chem.* 261:10673–10679.

Marino, C.R., V. Jeanes, W.F. Boron, and B.M. Schmitt. 1999. Expression and distribution of the Na^+-HCO_3^- cotransporter in human pancreas. *Am. J. Physiol. Gastrointest. Liver Physiol.* 277:G487–G494.

Nakhoul, N.L., B.A. Davis, M.F. Romero, and W.F. Boron. 1998. Effect of expressing the water channel aquaporin-1 on the CO_2 permeability of *Xenopus* oocytes. *Am. J. Physiol. Cell Physiol.* 274:C543–C548.

Romero, M.F., P. Fong, U.V. Berger, M.A. Hediger, and W.F. Boron. 1998. Cloning and functional expression of rNBC, an electrogenic Na^+-HCO_3^- cotransporter from rat kidney. *Am. J. Physiol. Renal Physiol.* 274:F425–F432.

Romero, M.F., M.A. Hediger, E.L. Boulpaep, and W.F. Boron. 1996. Cloning and functional expression of the rat renal electrogenic Na/HCO_3 cotransporter (rNBC). *J. Am. Soc. Nephrol.* 7:1259. (Abstr.)

Romero, M.F., M.A. Hediger, E.L. Boulpaep, and W.F. Boron. 1997a. Expression cloning and characterization of a renal electrogenic Na^+/HCO_3^- cotransporter. *Nature.* 387:409–413.

Romero, M.F., M.A. Hediger, P. Fong, and W.F. Boron. 1997b. Expression of the rat renal electrogenic Na/HCO_3 cotransporter (rkNBC). *FASEB J.* 11:25. (Abstr.)

Schmitt, B.M., D. Biemesderfer, M.F. Romero, E.L. Boulpaep, and W.F. Boron. 1999. Immunolocalization of the electrogenic Na^+/HCO_3^- cotransporter in mammalian and amphibian kidney. *Am. J. Physiol. Renal Physiol.* 276:F27–F36.

Sciortino, C.M., and M.F. Romero. 1999. Cation and voltage dependence of rat kidney electrogenic Na^+-HCO_3^- cotransporter, rkNBC, expressed in oocytes. *Am. J. Physiol. Renal Physiol.* 277:F611–F623.

Segel, I.H. 1993. Enzyme Kinetics. John Wiley & Sons, Inc. New York, NY. 957 pp.

Shimbo, K., D.L. Brassard, R.A. Lamb, and L.H. Pinto. 1995. Viral and cellular small integral membrane proteins can modify ion channels endogenous to *Xenopus* oocytes. *Biophys. J.* 69:1819–1829.

Siebens, A.W., and W.F. Boron. 1987. Effect of electroneutral luminal and basolateral lactate transport on intracellular pH in salamander proximal tubules. *J. Gen. Physiol.* 90:799–831.

Soleimani, M., and P.S. Aronson. 1989. Ionic mechanism of sodium bicarbonate cotransport in rabbit renal basolateral membrane vesicles. *J. Biol. Chem.* 264:18302–18308.

Soleimani, M., S.M. Grassl, and P.S. Aronson. 1987. Stoichiometry of Na^+-HCO_3^- cotransport in basolateral membrane vesicles isolated from rabbit renal cortex. *J. Clin. Invest.* 79:1276–1280.

Stim, J., A.A. Bernardo, F.T. Kear, Y.Y. Qiu, and J.A.L. Arruda. 1994. Renal cortical basolateral Na^+/HCO_3^- cotransporter: II. Detection of conformational changes with fluorescein isothiocyanate labeling. *J. Membr. Biol.* 140:39–46.

Tzounopoulos, T., J. Maylie, and J.P. Adelman. 1995. Induction of endogenous channels by high levels of heterologous membrane proteins in *Xenopus* oocytes. *Biophys. J.* 69:904–908.

Yoshitomi, K., B.C. Burckhardt, and E. Frömter. 1985. Rheogenic sodium-bicarbonate cotransport in the peritubular cell membrane of rat renal proximal tubule. *Pflügers Arch.* 405:360–366.