Extracellular HCO$_3^-$ Dependence of Electrogenic Na/ HCO$_3^-$ Cotransporters Cloned from Salamander and Rat Kidney

Irina I. Grichtchenko, Michael F. Romero, and Walter F. Boron

From the Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut 06520

**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 ($\Delta V_m$) 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 ($\Delta I$) elicited by rkNBC. Briefly exposing an NBC-expressing oocyte to HCO$_3^-$/CO$_2$ (0.33–99 mM HCO$_3^-$, pH$_i$ 7.5) elicited an immediate, DIDS (4,4-diisothiocyanatostilbene-2,2-disulfonic acid)-sensitive and Na$^+$-dependent hyperpolarization (or outward current). In $\Delta V_m$ 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$_3^-$ nor 33 mM SO$_3^-$/HSO$_3^-$ substantially affects the apparent $K_m$ for HCO$_3^-$ of rkNBC. 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$_i$, 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$_i$. 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) from Ambystoma kidney (Romero et al., 1997a), closely related cDNAs have been cloned from human kidney (Burnham et al., 1999), rat kidney (Romero et al., 1998), and human pancreas 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
pressed in that NBC, as expressed by itself in oocytes, transports pH we suggest that increased pH stimulates NBC with a pK of 7.5, but that NBC does not interact with CO.

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, #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 (15140-121; 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 ug/ul 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\(_3^2\), or SO\(_3^2\)/HSO\(_3^\) our HEPES-buffered HCO\(_3^\)/CO\(_2\) 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\(^-\)] 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\(_3^2\) 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\(_3^2\) and 33 mM mannitol. In the HCO\(_3^\)/CO\(_2\)-containing solutions, we kept [SO\(_3^2\)] fixed at 33 mM, and substituted HCO\(_3^\) for gluconate. Because the solutions contained a maximal [gluconate], 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\(_3^2\) and HSO\(_3^\) (pK 6.9). Thus, a pH 7.5 solution containing 33 mM “total SO\(_3^2\)” actually contains 26.4 mM SO\(_3^2\) and 6.6 mM HSO\(_3^\). Similar to the situation for the SO\(_3^2\) solutions, we replaced 66 of the 99 mM gluconate in the HEPES-buffered solution with 33 mM total SO\(_3^2\)/HSO\(_3^\) and 33 mM mannitol (Solution 5 in Table I). In the HCO\(_3^\)/CO\(_2\)-containing solutions, we kept [total SO\(_3^2\)/HSO\(_3^\)] fixed at 33 mM, but substituted HCO\(_3^\) for gluconate. In all solutions, pH was 7.5.

To determine the pH\(_o\) or [CO\(_3^\)] 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\(_3^\)] varied from ~3.5 \mu M to ~3.5 mM. Our standard 33 mM HCO\(_3^\)/5% CO\(_2\) (Solution 2 in Table I) contained ~70 \mu M 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\(_o\) measurements. In some experiments, we used the change in membrane potential (\(\Delta V_m\)) 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.
TABLE I
Composition of Standard Solutions

| Component       | 1 standard HEPES | 2 standard HCO₃ | 3 standard SO₄ + HEPES | 4 standard SO₄ + HCO₃ | 5 standard SO₃ + HEPES | 6 standard SO₃ + HCO₃ |
|-----------------|-----------------|-----------------|-----------------------|----------------------|-----------------------|----------------------|
| Na⁺             | 104 mM          | 104 mM          | 104 mM                | 104 mM               | 97.4 mM               | 97.4 mM              |
| K⁺              | 2 mM            | 2 mM            | 2 mM                  | 2 mM                 | 2 mM                  | 2 mM                 |
| Mg²⁺            | 1 mM            | 1 mM            | 1 mM                  | 1 mM                 | 1 mM                  | 1 mM                 |
| Ca²⁺            | 1.8 mM          | 1.8 mM          | 1.8 mM                | 1.8 mM               | 1.8 mM                | 1.8 mM               |
| Total cations, meq | 111.6 meq     | 111.6 meq       | 111.6 meq             | 111.6 meq            | 105 meq               | 105 meq              |
| Cl⁻             | 7.6 mM          | 7.6 mM          | 7.6 mM                | 7.6 mM               | 7.6 mM                | 7.6 mM               |
| Gluconate⁻      | 99 mM           | 66 mM           | 33 mM                 | 0 mM                 | 33 mM                 | 0 mM                 |
| HCO₃⁻           | 0 mM            | 0 mM            | 33 mM                 | 33 mM                | 0 mM                  | 0 mM                 |
| SO₄²⁻           | 0 mM            | 0 mM            | 0 mM                  | 0 mM                 | 26.4 mM               | 26.4 mM              |
| HSO₃⁻           | 0 mM            | 0 mM            | 0 mM                  | 0 mM                 | 6.6 mM                | 6.6 mM               |
| HEPES⁻          | 5 mM            | 5 mM            | 5 mM                  | 5 mM                 | 5 mM                  | 5 mM                 |
| Total anions, meq | 111.6 meq     | 111.6 meq       | 111.6 meq             | 111.6 meq            | 105 meq               | 105 meq              |
| Mannitol        | 0 mM            | 0 mM            | 33 mM                 | 33 mM                | 33 mM                 | 33 mM                |
| HEPES (neutral) | 5 mM            | 5 mM            | 5 mM                  | 5 mM                 | 5 mM                  | 5 mM                 |
| pH              | 7.5             | 7.5             | 7.5                   | 7.5                  | 7.5                   | 7.5                  |

‡ Solutions containing 33 mM HCO₃ were equilibrated with 5% CO₂.

1.16 mm i.d. x 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 measurements. In some experiments, we used the rate of pH increase (dPH/dt) as an index of the flux of HCO₃⁻ into oocytes expressing rKnBC. We corrected for bath junction potentials. 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 (Vhold) was −60 mV. The currents were filtered at 20 Hz (four-pole Bessel filter).

Data Acquisition

The pH, Vm, and Iout data were recorded digitally on an 80486-based personal computer. The analogue-to-digital converter (ADC-30; Contec Microelectronics U.S.A., Inc.) sampled the Vm and pH 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 change (dPH/dt) by fitting a line to pH versus time data using a linear least-squares method. All average dPH/dt, ∆Vm, and ∆I data are reported as mean ± 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 ∆Vm (or ∆I) data obtained in the absence of SO₄²⁻ or SO₃⁻/HSO₃⁻ (i.e., when gluconate and HCO₃⁻ were the major anions), we normalized absolute values of ∆Vm (or ∆I) obtained under “test” conditions to bracketing values of ∆Vm (or ∆I) obtained under “standard” conditions of 33 mM HCO₃⁻. As noted in the discussion, the simplest equation that adequately fitted our data was a model having a Michaelis-Menten dependence on [HCO₃⁻]o, plus a linear component:

\[ v = \frac{[HCO_3^-]}{[HCO_3^-] + K_m} v_{max} + \alpha [HCO_3^-], \]  

where \( v \) is an absolute value of the velocity of the reaction (i.e., ∆Vm or ∆I) at each value of [HCO₃⁻], \( v_{max} \) is the maximum velocity, and \( \alpha \) is a constant. We can rearrange Eq. 1 to obtain \( \alpha \) as follows (Eq. 2):

\[ \alpha = \frac{v}{[HCO_3^-]} - \frac{v_{max}}{[HCO_3^-] + K_m}. \]

Under our standard conditions of [HCO₃⁻] = [HCO₃⁻]std = 33 mM, \( \alpha \) becomes:

\[ \alpha = \frac{v_{std}}{[HCO_3^-]_{std}} - \frac{v_{max}}{[HCO_3^-]_{std} + K_m}. \]

Substituting Eq. 3 into Eq. 1 yields:
used a nonlinear least-squares curve fitting approach to obtain
normalized data is:

\[ \text{normalized data} = \frac{\text{data}}{\text{data in the absence of}} \]

and sustained fall in pH, as well as a slowly developing
depolarization. These changes in pH and V_m are fully
reversible.

**RESULTS**

[HCO₃]₀ Dependence of akNBC, Based on Changes in V_m

Effect of adding 33 mM HCO₃/5% CO₂ on pH_i and V_m.

Fig. 1A illustrates the results of an experiment on a wa-
ter-injected (i.e., control) oocyte. As described previ-
ously (Romero et al., 1997a), switching the extracellu-
lar solution from one buffered with HEPES to one buf-
fered with HCO₃/CO₂, at a constant pH_i, 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. 1B illustrates the results of a similar ex-
periment, but performed on an oocyte injected 3 d ear-
er with cRNA encoding rkNBC. Although there is a
modest recovery of pH_i from the initial CO₂-induced
acidification, the major difference between this exper-
iment and the one in Fig. 1A is that applying HCO₃/CO₂
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
hyperpolarization are both consistent with the elec-
rogenic influx of Na⁺ and HCO₃. 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 HCO₃/CO₂ at a
constant pH_i of 7.5. The expression level of the akNBC
cloned, as judged in voltage-clamp experiments (not
shown), was not sufficiently high to allow us to measure
NCNs accurately at low values of [HCO₃]₀. To obtain a first approximation of the [HCO₃]₀ depen-
dence of akNBC, we monitored changes in V_m while
briefly applying extracellular solutions containing vari-
cious levels of HCO₃/CO₂. Fig. 2 shows a typical exper-
iment. We began with the oocyte in our standard gluco-
late-HEPES solution (Table I, Solution 1). We then
switched to our standard gluanate-HEPES solution (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 HCO₃/CO₂
solutions, each having a pH_i of 7.50. During the rest of
the experiment, we bracketed each test HCO₃/CO₂
pulse with a standard HCO₃/CO₂ pulse. To compensate
for differences in the expression level of akNBC in individual
ocytex, 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.

Curvefitting. As noted in the discussion, we at-
tempted to fit the normalized akNBC data (Fig. 3, ■)
with a variety of “single-enzyme” rapid-equilibrium ki-
netic models. Visually, none of these fits was fully satis-

---

\[ v = \frac{[\text{HCO}_3]}{[\text{HCO}_3]} \cdot v_{\text{max}} \frac{1}{[\text{HCO}_3] + K_m} - \frac{1}{[\text{HCO}_3] + K_m} \]

+ \frac{[\text{HCO}_3]}{[\text{HCO}_3]} \cdot v_{\text{std}}. \quad (4)

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

\[ v^* = \frac{v}{v_{\text{std}}} = \frac{[\text{HCO}_3]}{[\text{HCO}_3]} \cdot v_{\text{max}} \]

\[ = \frac{1}{[\text{HCO}_3] + K_m} - \frac{1}{[\text{HCO}_3] + K_m} + \frac{[\text{HCO}_3]}{[\text{HCO}_3]} \cdot v_{\text{std}}, \quad (5) \]

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

\[ \alpha^* = \frac{\alpha}{v_{\text{std}}} = \frac{\text{v}_{\text{max}}}{[\text{HCO}_3] + K_m}. \quad (6) \]

In analyzing normalized ΔI data obtained in the presence of sulfate or sulfite, ([HCO₃] between 0 and 33 mM), we fitted the data with a normalized version of a function similar to Eq. 1, ex-
cept that we assumed that α* was fixed to the same value ob-
tained from the curve fit of the data obtained in the absence of sulfate or sulfite (see Table III). In this case, the equation for normal-
ized data is:

\[ v^* = \frac{v}{v_{\text{std}}} = \frac{[\text{HCO}_3] + K_m}{[\text{HCO}_3] + K_m} \cdot \frac{[\text{HCO}_3]}{[\text{HCO}_3] + K_m}, \]

\[ = (1 - \alpha^*)[\text{HCO}_3] + \alpha^*[HCO_3]. \quad (7) \]

where α* = 0.00577 mM⁻¹. After obtaining K_m by curve fitting,
we obtained the value of v_{\text{max}} using Eq. 8:

\[ v_{\text{max}} = \{1 - \alpha^*\}[HCO_3] + \frac{[HCO_3]}{[HCO_3] + K_m}. \quad (8) \]
factory. We also fitted our normalized $\Delta V_m$ 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., $z_{231}$ mM) was far higher than the highest $[HCO_3^-]$ 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 $D V_m$ 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.

$[HCO_3^-]_0$ 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. 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).

$[HCO_3^-]_0$ Dependence of rkNBC, Studied in Voltaged-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 $[HCO_3^-]_0$ dependence of rkNBC.

Effect of 99 mM $HCO_3^-$ on membrane current. Fig. 4 A shows that briefly exposing a control (i.e., H$_2$O-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 $\sim 500$ 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**

| $[HCO_3^-]_0$ Dependence of akNBC and rkNBC‡ |
|---|---|---|---|---|
| NBC | $K_m'(HCO_3^-)$ | Relative $v_{max}^*$ | $\alpha^*$ | $n$ | Method |
| 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.
Na⁺. Restoring the extracellular Na⁺ substantially increased the current elicited by adding 99 mM HCO₃⁻/15% CO₂. Thus, the current elicited by HCO₃⁻ 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 CO₂/HCO₃⁻ at a constant pH₀ of 7.5. We used the peak amplitude of the current induced by exposing oocytes to HCO₃⁻/CO₂ as a measure of the inward, electrogenic transport of Na⁺ and HCO₃⁻ 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 (ΔI_data are summarized in Fig. 6 (●). The curve represents the result of a nonlinear least-squares fit of Eq. 5. The apparent Kₘ(HCO₃⁻) was 6.5 mM (Table II). This Kₘ(HCO₃⁻) value for rKNBC in ΔI experiments is substantially less than for the same clone in ΔVₘ experiments.

Fig. 4 Dependence of HCO₃⁻-evoked currents on the expression rKNBC and the presence of Na⁺. (A) H₂O-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 HCO₃⁻/15% CO₂. Vₘ₀ = −60 mV, 22°C.

Effect of Sulfate and Sulfite/Bisulfite on the [HCO₃⁻]₀ Dependence of the rKNBC Current

To test the hypothesis (see introduction) that NBC can transport SO₄²⁻ or HSO₄⁻, we first examined the effect of SO₄²⁻/HSO₄⁻ on membrane currents carried by rKNBC expressed in Xenopus oocytes. As a control, we examined the effects of another divalent anion, sulfate (SO₄²⁻).

Effect of SO₄²⁻ and SO₄²⁻ on rKNBC current evoked by 33 mM HCO₃⁻. Fig. 7 shows a voltage-clamp experiment in which we examined the effect of SO₄²⁻ and SO₄²⁻/HSO₄⁻ on the peak current produced by 33 mM HCO₃⁻ in an oocyte expressing rKNBC. The changes in current evoked by 33 mM HCO₃⁻ were virtually identical regardless of whether the dominant background anion was 66 mM gluconate, 33 mM SO₄²⁻ or 33 mM total SO₄²⁻/HSO₄⁻ (i.e., 26.4 mM SO₄²⁻ + 6.6 mM HSO₄⁻). In a total of six similar experiments, the ratio² of the current in SO₄²⁻ to the bracketing-paired currents in gluconate was 0.982. Similarly, in seven experiments, the ratio² of the current in SO₄²⁻/HSO₄⁻ 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 HCO₃⁻ is virtually identical in the presence of SO₄²⁻..

Fig. 8 C summarizes the data as well as the curve fits. Because the SO₄²⁻ and SO₄²⁻/HSO₄⁻ data in the range of 0–33 mM HCO₃⁻ 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 (ΔI [HCO₃⁻]: 0–99 mM). The results of these curve fits (Eq. 7) are summarized in Table III, and show that the apparent Kₘ and Vₘ values are virtually identical, regardless of whether HCO₃⁻ was varied in the presence of gluconate, SO₄²⁻ or SO₄²⁻/HSO₄⁻.

Effect of Sulfite/Bisulfite on DIDS-sensitive pH change in Oocytes Expressing rKNBC

Is it possible that SO₄²⁻/HSO₄⁻ could ride rKNBC and yet not produce a change in current? If NBC could nei-

<sup>2</sup>The log-normal mean was 0.982 ± 0.028/–0.027.
<sup>3</sup>The log-normal mean was 0.999 ± 0.025/–0.025.
ther distinguish SO\textsubscript{3} from CO\textsubscript{3}, nor HSO\textsubscript{3} from HCO\textsubscript{3}, then introducing SO\textsubscript{3}/HSO\textsubscript{3} would have no effect on the current carried by NBC if the transporter were already near \(v_{\text{max}}\). However, because the pK values governing the reactions SO\textsubscript{3} + H\textsuperscript{+} \rightleftharpoons HSO\textsubscript{3} and HSO\textsubscript{3} + H\textsuperscript{+} \rightleftharpoons H\textsubscript{2}SO\textsubscript{3} are so much lower than for the corresponding reactions involving CO\textsubscript{3}, HCO\textsubscript{3}, and H\textsubscript{2}CO\textsubscript{3}, the pH\textsubscript{i} changes for NBC carrying SO\textsubscript{3}/HSO\textsubscript{3} would be much slower than for NBC carrying CO\textsubscript{3}/HCO\textsubscript{3} (see discussion). We therefore examined the possibility that SO\textsubscript{3}/HSO\textsubscript{3} would slow the pH\textsubscript{i} produced by NBC in the presence of CO\textsubscript{2}/HCO\textsubscript{3}.

Our assay was to expose an rkNBC-expressing oocytes to an extracellular solution buffered with 3.3 mM HCO\textsubscript{3}/0.5% CO\textsubscript{2}. As shown in Fig. 9 A, an experiment conducted in the absence of SO\textsubscript{3}/HSO\textsubscript{3}, applying HCO\textsubscript{3}/CO\textsubscript{2} causes a rapid but small pH\textsubscript{i} decrease (a–b), followed by a pH\textsubscript{i} increase (b–c). After \(\sim\)30 min, when pH\textsubscript{i} was recovering at a constant rate in the HCO\textsubscript{3}/CO\textsubscript{2} solution, we applied 1 mM DIDS for \(\sim\)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\textsubscript{i} value of 7.34 \(\pm\) 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\textsubscript{3}/6.6 mM HSO\textsubscript{3} during the application of the 3.3 mM HCO\textsubscript{3}/0.5% CO\textsubscript{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\textsubscript{3}/HSO\textsubscript{3} (\(P = 0.28\), an unpaired one tail t test). The mean initial pH\textsubscript{i} in the SO\textsubscript{3}/HSO\textsubscript{3}
Effect of Altering \( [\text{CO}\_3]_o \) and \( \text{pH}_o \) on the Current Carried by rkNBC

Because the data introduced above make it unlikely that rkNBC, as expressed in *Xenopus* oocyte, interacts with \( \text{HSO}_3^- \) or \( \text{SO}_4^{2-} \), we asked whether rkNBC transports \( \text{CO}_3^- \). Our approach was to hold \( [\text{HCO}_3^-]_o \) constant at 33 mM while raising \( [\text{CO}_2] \) from 0.1% \( (\text{pH}_o 9.2, [\text{CO}_3^-]_o \sim 3,500 \mu M) \) to 100% \( (\text{pH}_o 6.2, [\text{CO}_3^-]_o \sim 3.5 \mu M) \). Our protocol was similar to that in Fig. 5, with two pulses of our standard solution (Table I, Solution 2, \( [\text{CO}_3^-]_o \sim 70 \mu M \)) bracketing each test pulse. Because \( \text{CaCO}_3 \) precipitated from the \( \text{pH} 9.2 \) solution, which nominally contains \( \sim 3,500 \mu M \) \( \text{CO}_3^- \), we replaced all \( \text{Ca}^{2+} \) with \( \text{Mg}^{2+} \). Control experiments showed that this switch has no effect on the NBC current.4

Fig. 10 A summarizes our results, expressed as normalized \( \Delta I \) data as a function of \( [\text{CO}_3^-]_o \). 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.

Why Is the NBC Current–[\text{HCO}_3^-]_o Relationship Not Sigmoidal?

As expressed in the *Xenopus* oocyte, rkNBC is electrogenic. This observation is consistent with a \( \text{Na}^+:\text{HCO}_3^- \) 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; Sciotino and Romero, 1999). Thus, one would not be surprised if the relationship between NBC current and \( [\text{HCO}_3^-]_o \) were more complex than a simple right-rectangular hyperbola, for example. In fact, we found that the current–[\text{HCO}_3^-]_o relationship is well described by the sum of a hyperbola and a line. Why did we not observe a sigmoidal current–[\text{HCO}_3^-]_o relationship?

First, it is possible that, as expressed in *Xenopus* oocytes, rkNBC has but a single \( \text{HCO}_3^- \)-related substrate. If rkNBC carried a single \( \text{CO}_3^- \) (equivalent to two \( \text{HCO}_3^- \)), then the \( \text{Na}^+:\text{HCO}_3^- \) stoichiometry would be 1:2, and thus one would expect the current–[\text{HCO}_3^-]_o relationship to be a right-rectangular hyperbola. Thus, our data are consistent with the hypothesis that rkNBC binds a single \( \text{HCO}_3^- \) related species, \( \text{CO}_3^- \).

Second, if rkNBC carried two \( \text{HCO}_3^- \) ions (for a stoichiometry of 1:2) or one \( \text{HCO}_3^- \) and one \( \text{CO}_3^- \) (for a stoichiometry of 1:3), then the current–[\text{HCO}_3^-]_o relationship might show a foot at low \( [\text{HCO}_3^-]_o \), but only if [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 \( \text{pH}_o \) (Fig. 10 B), whether the best-fit pH titration curve \((pK = 7.50 \pm 0.05)\) 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 \((\text{trv} = 0.0055)\) is comparable with that in Fig. 6 \((\text{trv} = 0.0043)\).

DISCUSSION

Experiments was 7.39 ± 0.06, which also is not significantly different from the value in the absence of \( \text{SO}_3^- / \text{HSO}_3^- \) \((P = 0.20\), unpaired two tail \( t \) test).

**Figure 7.** Effect of \( \text{SO}_3^- / \text{HSO}_3^- \) on the current carried by rkNBC. The oocyte was exposed five times to a solution containing 33 mM \( \text{HCO}_3^- /5\% \text{ CO}_2 \). For the first, third, and fifth pulses, we switched from a HEPES solution (Table I, Solution 1) to a solution containing 33 mM \( \text{HCO}_3^- /5\% \text{ CO}_2 \) solution (Table I, Solution 2). For the second \( \text{HCO}_3^- /\text{CO}_2 \) pulse, we switched from a HEPES solution containing 33 mM \( \text{SO}_3^- /\text{HSO}_3^- \) (Table I, Solution 3) to a 33 mM \( \text{HCO}_3^- /5\% \text{ CO}_2 \) that also contained 33 mM \( \text{SO}_3^- /\text{HSO}_3^- \) (Table I, Solution 4). For the fourth \( \text{HCO}_3^- /\text{CO}_2 \) pulse, we switched from a HEPES-containing 33 mM \( \text{SO}_3^- /\text{HSO}_3^- \) (Table I, Solution 5) to a 33-mM \( \text{HCO}_3^- /5\% \text{ CO}_2 \) solution that also contained 33 mM \( \text{SO}_3^- /\text{HSO}_3^- \) (Table I, Solution 6). Typical of six experiments. \( V_{\text{hold}} = -60 \text{ mV}, 22^\circ \text{C} \).

| \text{HCO}_3^- | \% \text{ CO}_2 | \text{SO}_3^- | \text{HSO}_3^- |
|---|---|---|---|
| 33 | 5 | - | - |
| 6.6 | 3 | - | - |

4We compared NBC currents in two \( \text{pH} 7.8 \) solutions \([\text{CO}_3^-]_o \sim 138 \text{ mM} \); one was a \( \text{Ca}^{2+} \)-containing solution in which we observed no precipitation, and the other was a solution in which \( \text{Mg}^{2+} \) replaced \( \text{Ca}^{2+} \). The ratio of NBC current in the \( \text{pH} 7.8 \), nominally \( \text{Ca}^{2+} \)-free solution to the currents in the bracketing standard \( \text{pH} 7.5 \) solution had a log-normal mean of 1.097 ± 0.056/−0.054 \((n = 3)\). The comparable ratio for the \( \text{pH} 7.8 \) \( \text{Ca}^{2+} \)-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\( [\text{CO}_3^-]_o \) replacing [\( \text{HCO}_3^- \)]. The standard \([\text{CO}_3^-]_o \) was 70 \( \mu M \).
the K\textsubscript{m} values for the two binding sites were sufficiently similar and high. For example, if rkNBC carried two HCO\textsubscript{3}\textsuperscript{-} ions, and the K\textsubscript{m} values for one binding site was 6.5 mM (as observed), but the K\textsubscript{m} for the other was only 0.1 mM, then we would not have been able to detect a foot, given the precision of our data. Thus, our results are consistent with the hypothesis that rkNBC binds two HCO\textsubscript{3}\textsuperscript{-}-related species, but that we cannot detect a foot due to a low K\textsubscript{m} value.

Third, if rkNBC carried three HCO\textsubscript{3}\textsuperscript{-} ions (for a stoichiometry of 1:3), then the current-[HCO\textsubscript{3}\textsuperscript{3}]\textsubscript{o} relationship might show a foot, but, again, only if the K\textsubscript{m} values for all three were sufficiently similar and high. For example, if the K\textsubscript{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. Thus, our data are consistent with the hypothesis that rkNBC binds three HCO\textsubscript{3}\textsuperscript{-}, but that we cannot detect a foot due to a low K\textsubscript{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\textsubscript{3}/CO\textsubscript{2}, rkNBC transports Na\textsuperscript{+} and HCO\textsubscript{3}\textsuperscript{-} into the cell, and the passive entry of CO\textsubscript{2} leads to the production of HCO\textsubscript{3}\textsuperscript{-} and H\textsuperscript{+}. We attempted to minimize such effects by making our measurements very soon after exposing the cell to HCO\textsubscript{3}/CO\textsubscript{2}. Nevertheless, any buildup of intracellular Na\textsuperscript{+}, HCO\textsubscript{3}\textsuperscript{-}, and/or H\textsuperscript{+} that might have occurred in the vicinity of rkNBC would have slowed the cotransporter; the effect would have been greater at higher HCO\textsubscript{3}/CO\textsubscript{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\textsubscript{3}\textsuperscript{3}]\textsubscript{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\textsubscript{3}\textsuperscript{3}]\textsubscript{o} data using any of several rapid-equilibrium models for random or ordered binding of HCO\textsubscript{3}/CO\textsubscript{2} to the cotransporter. We therefore suggest that some additional process, which is a first-order function of [HCO\textsubscript{3}\textsuperscript{3}]\textsubscript{o}, contributes to the current, especially at [HCO\textsubscript{3}\textsuperscript{3}]\textsubscript{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\textsubscript{3}\textsuperscript{-} 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\textsuperscript{+}. As shown in Fig. 4 C, a transition from Na\textsuperscript{+}-free HEPES to Na\textsuperscript{+}-free 99 mM HCO\textsubscript{3}\textsuperscript{-} caused only slow and small (~9 nA) outward current in rkNBC-expressing X. laevis oocytes. However, in the presence of Na\textsuperscript{+}, the transition from HEPES to 99 mM HCO\textsubscript{3}\textsuperscript{-} produced a much larger current (~140 nA). Because virtually the entire HCO\textsubscript{3}\textsuperscript{-}...
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/H CO$_3^-$ 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 [H CO$_3^-$] cause the Na$^+$/H CO$_3^-$ 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 [H CO$_3^-$].

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.

Table III

| Major extracellular anion | $K_m$(H CO$_3^-$) | Relative $v_{max}$ | $\alpha^*$ | n  |
|--------------------------|------------------|-------------------|-----------|----|
| 66 mM gluconate$^*$      | 6.5 ± 0.7        | 0.97 ± 0.03       | 0.00577   | 58 |
| 33 mM SO$_3^-$           | 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$_3^-$ and SO$_3^-$ /HSO$_3^-$ were obtained using Eqs. 7 and 8.

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 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 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 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 changes would be appreciably slower for three reasons. (a) The pK of the reaction SO$_3^-$ + H$^+$ « HSO$_3^-$ is ~6.9, compared with ~10 for the equilibrium CO$_3^-$ + H$^+$ « HCO$_3^-$ (b) The pK of the reaction HSO$_3^-$ + H$^+$ « H$_2$SO$_3$ is ~1.6, compared with ~3.4 for the equilibrium HCO$_3^-$ + H$^+$ « H$_2$CO$_3$. (c) [H$_2$SO$_3$] is so low that the net efflux of H$_2$SO$_3$ is expected to be negligible. In contrast, H$_2$CO$_3$ 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
found that SO\textsubscript{3}/HSO\textsubscript{3} had no effect on NBC-mediated pH\textsubscript{i} changes. In Fig. 11, E-G and E’-G’, SO\textsubscript{3} and/or HSO\textsubscript{3} are competitive inhibitors for the transport of CO\textsubscript{2} and/or HCO\textsubscript{3} under the conditions of our experiments.

In Fig. 11, E-G and E’-G’, SO\textsubscript{3} and/or HSO\textsubscript{3} are competitive inhibitors for the transport of CO\textsubscript{2} and/or HCO\textsubscript{3}, respectively. In these cases, adding SO\textsubscript{3}/HSO\textsubscript{3} should decrease both the NBC current and pH\textsubscript{i} changes. Inasmuch as we found that SO\textsubscript{3}/HSO\textsubscript{3} had no effect on either, E-G and E’-G’ must be incorrect (i.e., neither SO\textsubscript{3} nor HSO\textsubscript{3} can competitively inhibit NBC under the conditions of our experiments).

Thus, we conclude that neither SO\textsubscript{3} nor HSO\textsubscript{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\textsubscript{3}/HSO\textsubscript{3}, when applied in the presence of HCO\textsubscript{3}, stimulates \(^{22}\text{Na}\) uptake mediated by the Na/HCO\textsubscript{3} cotransporter. Based on this and other data, those authors concluded that NBC transports Na\textsuperscript{+}, CO\textsubscript{3}, and HCO\textsubscript{3} in a stoichiometry of 1:1:1, and that SO\textsubscript{3} can substitute for CO\textsubscript{3} at the CO\textsubscript{3} binding site. This line of reasoning was the first, and probably the strongest, evidence that NBC can transport CO\textsubscript{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\textsubscript{3}/HSO\textsubscript{3}-rkNBC interaction, or (b) oocytes and corneal endothelial cells lack a factor required for the SO\textsubscript{3}/HSO\textsubscript{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\textsubscript{3}/HSO\textsubscript{3} interaction, or the missing factor could be an additional NBC subunit that confers sensitivity to SO\textsubscript{3}/HSO\textsubscript{3}. Alternatively, the missing factor triggered by SO\textsubscript{3}/HSO\textsubscript{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 X\textsuperscript{enopus} oocytes can carry out all other known functions of the renal NBC, rkNBC by itself cannot interact with SO\textsubscript{3} or HSO\textsubscript{3}.

Effect of Altering [CO\textsubscript{3}]\textsubscript{o} and pH\textsubscript{o} on rkNBC

Fig. 10 shows the effect on the current carried by rkNBC of simultaneously varying [CO\textsubscript{3}]\textsubscript{o} and pH\textsubscript{o}. The best-fit Michaelis-Menten curve, with or without a linear component, fails to adequately fit the data, expressed in terms of [CO\textsubscript{3}]\textsubscript{o} (Fig. 10 A). On the other hand, the best-fit pH titration curve nicely fits the data, expressed in terms of pH\textsubscript{i}, over the entire range of pH\textsubscript{o} values. One possible explanation for these results is that rkNBC transports CO\textsubscript{3} with a K\textsubscript{m} of ~6 \textmu M, but that an idiosyncratic pH\textsubscript{o} sensitivity is responsible for the poor fits at the extreme [CO\textsubscript{3}]\textsubscript{o} values. However, the most straightforward explanation for these data is that rkNBC is not sensitive to [CO\textsubscript{3}]\textsubscript{o} in the range 3.5–3.500 \textmu M, but has a single titratable site that inhibits NBC when protonated. For example, this site could be an HCO\textsubscript{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\textsubscript{3} with an extremely high affinity (i.e., a K\textsubscript{m} << 3.5 \textmu M).
We thank Drs. C.L. Slayman, B.M. Schmitt, and M.O. Bevensee for helpful discussions. We are also indebted to Mr. D. Wong for developing the software.

This work was supported by grants from the National Institutes of Health to W.F. Boron (DK30344). I.I. Grichtchenko was supported by a fellowship from the American Heart Association (AHA), Connecticut Affiliate. M.F. Romero was supported by a Scientist Development Grant from the AHA.

Submitted: 17 August 1999
Revised: 22 February 2000
Accepted: 23 February 2000

REFERENCES

Abuladze, N., I. Lee, D. Newman, J. Hwang, K. Boorer, A. Pushkin, and I. Kurtz. 1998a. Molecular cloning, chromosomal localization, 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−/ HCO3− 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. Lazardinski, and J. Barhanin. 1993. The protein Isk is a dual activator of K+ and Cl− channels. Nature 365:850–852.

Figure 11. Predicted effects of SO3− and/or HSO3− on the pHi changes mediated by NBC. A–G refer to a general scheme in which NBC transports one Na+, one CO3−, and one HCO3−. (A) Neither SO3− nor HSO3− interact with cotransporter. The entering CO3− can neutralize two H+ and the entering HCO3− can neutralize an additional H+, for a total of three H+ neutralized. (B) SO3− replaces CO3−. To the extent that SO3− replaces CO3−, only 1.24 H+ are neutralized, and thus the expected rate of pHi increase will be 41% of that in A. (C) HSO3− replaces HCO3−. Only two H+ are neutralized, and thus the expected rate of pHi increase will be only 67% of that in A. (D) SO3− and HSO3− replace, respectively, CO3− and HCO3−. Only total 0.24 H+ ions are neutralized, and thus the expected rate of pHi increase will be only 8% of that in A. (E) SO3− acts as a competitive inhibitor of CO3−. (F) HSO3− acts as a competitive inhibitor of HCO3−. (G) SO3− and HSO3− both are competitive inhibitors. A′–G′ refer to a general scheme in which NBC transports one Na+ and three HCO3−. (A′) Neither SO3− nor HSO3− interact with cotransporter. A total of three H+ are neutralized. (B′) HSO3− replaces one HCO3−. Only two H+ are neutralized, and thus the expected rate of pHi increase will be 67% of that in A′. (C′) Two HSO3− replace two HCO3−. Only one H+ is neutralized, and thus the expected rate of pHi increase will be only 33% of that in A′. (D′) Three HSO3− replace three HCO3−. No H+ ions are neutralized, and thus the expected rate of pHi increase will be 0% of that in A′. In E′–G′, HSO3− acts as a competitive inhibitor at one, two, and three HCO3−-binding sites, respectively.
Attali, B., H. Latter, N. Rachamim, and H. Garty. 1995. A corticosteroid-induced gene expressing an "Isk-like" K\(^+\) channel activity in \textit{Xenopus} oocytes. \textit{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. \textit{J. Gen. Physiol.} 81:53–94.

Boron, W.F., and E.L. Boulpaep. 1989. The electrogenic Na/\(\text{HCO}_3\) 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. \textit{J. Exp. Biol.} 200:263–268.

Boron, W.F., and R.C. Knakal. 1992. Na\(^+\)-dependent \(\text{Cl-HCO}_3\) exchange in the squid axon. Dependence on extracellular pH. \textit{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. \textit{J. Biol. Chem.} 272:19111–19114.

Buyse, G., T. Voets, J. Tytgat, C. De Grefe, G. Droogmans, B. Nilius, and J. Eggemont. 1997. Expression of human pClC-6 and CIC-6 in \textit{Xenopus} oocytes induces an identical endogenous chloride conductance. \textit{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). \textit{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. 1989. Voltage and cosubstrate dependence of the Na-HCO\(_3^\) cotransporter kinetics in renal proximal tubule cells. \textit{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 \textit{Xenopus laevis} oocytes. \textit{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. \textit{Biol. Reprod.} 60:571–579.

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 cotransporter. \textit{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). \textit{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. \textit{Am. J. Physiol. Gastrointest. Liver Physiol.} 277:G487–G494.

Nakhoul, N.L., B.A. Davies, M.F. Romero, and W.F. Boron. 1998. Effect of expressing the water channel aquaporin-1 on the CO\(_2\) permeability of \textit{Xenopus} oocytes. \textit{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. \textit{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. \textit{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. \textit{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 (rNBC). \textit{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. \textit{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, rNBC, expressed in oocytes. \textit{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 \textit{Xenopus} oocytes. \textit{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. \textit{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. \textit{J. Biol. Chem.} 264:18302–18308.

Soleimani, M., S.M. Grass, and P.S. Aronson. 1987. Stoichiometry of Na\(^+\)/HCO\(_3^\) cotransport in basolateral membrane vesicles isolated from rabbit renal cortex. \textit{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. \textit{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 \textit{Xenopus} oocytes. \textit{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. \textit{Pflügers Arch.} 405:360–366.