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Physiology

Change of apparent stoichiometry of proximal-tubule Na^+–HCO_3^- cotransport upon experimental reversal of its orientation

(amphibian kidney/Ba^{2+}/intracellular pH/ion-selective microelectrodes)

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ABSTRACT **Electrogenic cotransport of Na^+ with HCO_3^- has been reported in numerous tissues. It has always been shown with a net transfer of negative charge, but in some situations achieves net outward transport of both species with a stoichiometry of at least three HCO_3^- ions per Na^+ ion (3:1), and in other situations achieves net inward transport of both species and has a stoichiometry of at most two HCO_3^- ions per Na^+ ion (2:1). This suggests either that there may be more than one protein responsible for Na^+–HCO_3^- cotransport in different tissues or that if there is a single protein, its stoichiometry may differ depending on the orientation of net transport. The present study, using conventional or double-barreled ion-selective microelectrodes to follow basolateral membrane potential and intracellular pH or Na^+ activity in *Necturus* proximal convoluted tubule *in vivo*, shows that the orientation of the basolateral Na^+–HCO_3^- cotransporter can be reversed upon switching from a perfusate simulating normal acid–base conditions to one that imposes peritubular isohydric hypercapnia. Moreover, accompanying the reversal of orientation is a change of apparent stoichiometry from 3:1 to 2:1. Given that the observed change of orientation and accompanying change of apparent stoichiometry occur within seconds and in the same preparation, these results suggest that a single transport protein is responsible for both types of behavior.**

The cotransport of Na^+ and HCO_3^-, first demonstrated in the proximal convoluted tubule (PCT) of the salamander (1), has now been reported in numerous epithelial and nonepithelial tissues (for a recent review, see ref. 2). In most cases this transporter has been shown to be Cl^--independent, inhibited by stilbene derivatives, and rheogenic, with a net transfer of negative charge (HCO_3^- or related species). Depending on the tissue under consideration, the Na^+–HCO_3^- cotransport may be outward-directed (extrusion of base-equivalents from the cell) or inward-directed.

Interestingly, the stoichiometry of this cotransporter appears to be $3HCO_3^-$:$1Na^+$ in the kidney (3, 4) where the net transport is outward, whereas it appears to be 2:1 in other tissues, both epithelial (5) and nonepithelial (6), where net transport is inward-directed. In certain preparations, the stoichiometry has been reported to be 1:1 (7) or has not been clearly established (8–10). These results suggest either that there may be more than one Na^+–HCO_3^- transport protein or that there is a single protein whose stoichiometry may change.

In the *Necturus* PCT, Lopes *et al.* (11) clearly established the presence of an outward-directed Na^+–HCO_3^- cotransport with a stoichiometry of at least $3HCO_3^-$:$1Na^+$. In the present study, we have applied a thermodynamic approach to estimate the stoichiometry of Na^+–HCO_3^- cotransport in *Necturus* PCT under normal acid–base conditions (outward-directed transport) and under experimental conditions that reverse the direction of net transport. Our results suggest that a change of apparent stoichiometry accompanies the change of orientation of the transporter, though we cannot say whether there is a cause–effect relationship between stoichiometry and orientation.

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METHODS

Animal Preparation and Peritubular Perfusion. Salamanders (*Necturus maculosus*) were anesthetized by immersion in a solution of tricaine methanesulfonate (700 μg/ml), and anesthesia was maintained during the experiment by bathing the gills with a 1:5 dilution of the same solution. After the peritoneal cavity was opened, the right kidney was superfused with 87 mM NaCl/3 mM KCl/1 mM $MgCl_2$/1.8 mM $CaCl_2$/10 mM Tes, pH 7.5.

A peritubular vessel was perfused by using single or double micropipettes to which the perfusion solutions were delivered by gravity feed via polyethylene catheters. The catheters and micropipettes were hermetically sheathed in a larger, CO_2-impermeable tube within which a gas mixture of CO_2, O_2, and N_2 was constantly perfused.

Two experimental acid–base conditions were used—either physiological acid–base conditions or imposed isohydric hypercapnia. To simulate physiological conditions, the peritubular perfusion solution was 91.8 mM NaCl/3 mM KCl/1 mM $MgCl_2$/1.8 mM $CaCl_2$/8.2 mM $NaHCO_3$, equilibrated to pH 7.6 by bubbling with 1% CO_2/20% O_2/79% N_2. In the experiments using ion-selective microelectrodes to measure intracellular pH (pH_i) or intracellular Na^+ activity [$(Na^+)_i$], the perfusion solution was 81 mM sodium gluconate/10.8 mM NaCl/3 mM KCl/1 mM $MgCl_2$/5.4 mM $CaCl_2$/8.2 mM $NaHCO_3$, equilibrated with the same gas mixture. (Due to chelation of calcium by gluconate, it was necessary to increase the concentration of $CaCl_2$ in order for free Ca^{2+} to be at the same concentration in both solutions.) The low Cl^- concentration of this solution was chosen to match that of the solution used for isohydric hypercapnia.

To impose isohydric hypercapnia, the peritubular perfusion solution was 18 mM NaCl/3 mM KCl/1 mM $MgCl_2$/1.8 mM $CaCl_2$/82 mM $NaHCO_3$, equilibrated to pH 7.6 by bubbling with 10% CO_2/20% O_2/70% N_2.

In a separate set of experiments, we eliminated the luminal compartment by injecting colored castor oil through the glomerulus. We then sectioned Bowman's capsule wide-open and sectioned the glomerular capillaries. In this way, the proximal tubular epithelium was bathed only by the peritubular perfusate.

Abbreviations: PCT, proximal convoluted tubule; pH_i, intracellular pH; $(Na^+)_i$ and $[Na^+]_i$, intracellular Na^+ activity and concentration, respectively; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid; Ψ_m, basolateral membrane potential; E_K, K^+ equilibrium potential.

Under both of these experimental conditions, we studied the effects of 2 mM $BaCl_2$ (Sigma) and 1 mM 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS) (BDH).

Electrophysiology. Basolateral membrane potential, Ψ_m, was measured with conventional microelectrodes filled with 1 M KCl. For simultaneous measurements of Ψ_m and pH_i or $(Na^+)_i$, we constructed double-barreled ion-selective microelectrodes by our customary methods (12). To improve response time of the double electrodes, their points were carefully sharpened with a microbeveler (De Marco Engineering, Geneva). The slope, S, of the double-barreled ion-selective microelectrodes was 48–54 mV per decade for the Na^+ electrodes and 50–58 mV per decade for the pH electrodes. The insensitivity of these microelectrodes to the partial pressure of CO_2 (P_{CO_2}) was verified by using them to measure the pH in solutions buffered to the same pH with either CO_2/HCO_3^- or Tes.

$(Na^+)_i$ was calculated according to $(Na^+)_i = (Na^+)_{ref}\, 10^{(\Psi_{Na}-\Psi_m)/S}$, where $(Na^+)_{ref}$ is the Na^+ activity in the superfusion solution and Ψ_{Na} is the measured electrochemical potential difference for Na^+.

pH_i was calculated according to $pH_i = pH_{ref} - (\Psi_H - \Psi_m)/S$, where pH_{ref} is the pH in the superfusion solution and Ψ_H is the measured electrochemical potential difference for H^+. Intracellular concentration of HCO_3^- ($[HCO_3^-]_i$) was calculated from individual pH_i measurements by using the Henderson–Hasselbalch equation with $pK_a = 6.17$ and $\alpha_{CO_2} = 0.043$ (at 22°C), which are the appropriate values for cold-blooded animals (13, 14). We assumed that intracellular P_{CO_2} was identical to that of the peritubular perfusate. This assumption implies that the luminal P_{CO_2} near the impaled cells was not significantly different from that in the peritubular perfusate. The validity of this assumption was tested by doing a series of experiments in which the lumen was blocked by oil (see above).

Unless explicitly stated otherwise, all results reported below refer to experiments in which the lumen was in the free-flow condition.

Activity coefficient was taken to be equal to 0.75 for both intracellular and extracellular ions. Concentrations are indicated with square brackets, [ ], and activities with parentheses, ( ).

Thermodynamic Estimate of Na^+–HCO_3^- Stoichiometry. By studying the effects of Ba^{2+} and SITS on Ψ_m, we determined the position of the equilibrium potential for the Na^+–HCO_3^- cotransporter with respect to Ψ_m.

Ba^{2+} is a classic inhibitor of K^+ conductance (15). Its effect on the value of Ψ_m therefore indicates the position of the K^+ equilibrium potential, E_K, with respect to Ψ_m; that is, a Ba^{2+}-induced depolarization indicates that E_K is more negative than Ψ_m, whereas a Ba^{2+}-induced hyperpolarization indicates that E_K is more positive than Ψ_m (16). In both cases, the responses also indicate that there must be a significant membrane conductance to some other ion whose equilibrium potential is situated on the other side of Ψ_m.

SITS was used here to inhibit the Na^+–HCO_3^- cotransport, though it is known that its effects are not specific to only this transporter (17, 18). We considered, as have other authors, that a SITS-induced hyperpolarization reflects inhibition of a transporter carrying net negative charge outward (11) and that a SITS-induced depolarization reflects inhibition of a transporter carrying net negative charge inward (5).

The equilibrium potential $E_{Na^+-HCO_3^-}$ is given by the equation

$$E_{Na^+-HCO_3^-} = \frac{RT}{(N-1)\mathcal{F}} \ln \frac{(Na^+)_i (HCO_3^-)_i^N}{(Na^+)_o (HCO_3^-)_o^N}, \quad [1]$$

Table 1. Ψ_m under normal physiological acid–base conditions (1% CO_2, pH 7.6): Effects of 2 mM Ba^{2+} and 1 mM SITS

	Ψ_m, mV				
		1% CO_2			
Exp.	Blood	Control	Ba^{2+}	SITS	*n*
1	−62.9 ± 3.0	−65.6 ± 2.7*	−52.6 ± 2.8†	—	9
2	−67.6 ± 3.1	−67.1 ± 3.0*	—	−73.3 ± 3.3†	7

*Not statistically significant compared with blood.
†Statistically significant, $P < 0.001$, compared with the preceding condition.

where R is the universal gas constant, $\mathcal{F}$ is the faraday (≈96, 510 C), $T = 22$°C, the suffix o refers to the extracellular bath, and N is the stoichiometric ratio of HCO_3^- to Na^+.

Using this equation and the measured $(Na^+)_i$ and $(HCO_3^-)_i$ (from pH_i), we estimated upper or lower integer bounds for N from the inequalities $E_{Na^+-HCO_3^-} > \Psi_m$, for net outward-directed transport, and $E_{Na^+-HCO_3^-} < \Psi_m$, for net inward-directed transport.

Results are given as means ± standard errors. Significance of the results was assessed by using Student's t test for paired samples.

RESULTS

Effects of Ba^{2+} and SITS on Ψ_m. In the first series of experiments, the effects of Ba^{2+} and SITS on Ψ_m were studied with conventional intracellular microelectrodes. This was done under two distinct experimental conditions (see *Methods*)—normal acid-base conditions or imposed isohydric hypercapnia.

Upon perfusion with a solution approaching normal physiological acid–base conditions (i.e., peritubular perfusion with a solution at a P_{CO_2} of 1%, pH 7.6), Ψ_m had a value similar to that measured spontaneously (i.e., blood perfusion). Introduction of Ba^{2+} depolarized Ψ_m, with a return to control values after removal of Ba^{2+} from the perfusate. Introduction of SITS, on the other hand, hyperpolarized Ψ_m, with a partial or complete return to control values after its removal from the perfusate (Table 1).

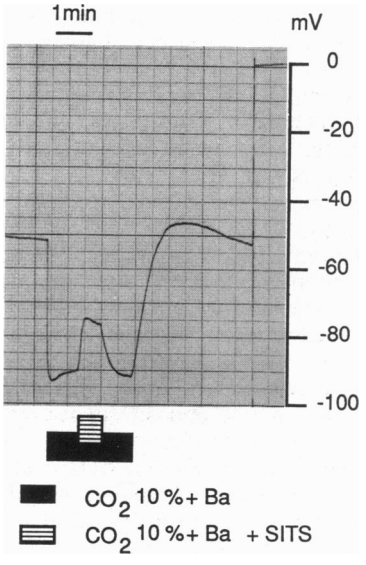

FIG. 1. Effect of 1 mM SITS on Ψ_m (mV) in the presence of 2 mM Ba^{2+} during imposed isohydric hypercapnia. Original tracing, obtained with a conventional intracellular microelectrode, is shown. Timing of perfusion with experimental solutions is indicated by the horizontal bars below the graph. Before and after these experimental solutions, perfusion was with blood from normal circulation.

Table 2. Ψ_m under imposed isohydric hypercapnia (10% CO_2, pH 7.6): Individual and combined effects of 2 mM Ba^{2+} and 1 mM SITS

	Ψ_m, mV					
		10% CO_2				
Exp.	Blood	Control	Ba^{2+}	SITS	Ba^{2+} + SITS	*n*
1	−64.9 ± 2.1	−88.0 ± 3.1*	−90.9 ± 3.8†	—	—	14
2	−66.4 ± 2.9	−88.0 ± 1.9*	—	−80.2 ± 1.8*	—	11
3	−56.9 ± 3.5	—	−100.4 ± 4.3*	—	−84.1 ± 5.1*	7

*Statistically significant, $P < 0.001$, compared with preceding condition.
†Statistically significant, $P < 0.05$, compared with preceding condition.

Under imposed isohydric hypercapnia (i.e., peritubular perfusion with a solution at P_{CO_2} of 10%, pH 7.6), Ψ_m was considerably more negative than under perfusion with circulating blood. Under these conditions, introduction of Ba^{2+} (n = 14) led sometimes to a 2- to 3-mV depolarization (3/14), sometimes had no effect on Ψ_m (2/14), and most often led to a 2- to 10-mV hyperpolarization (9/14); on the average, Ba^{2+} caused a small but significant hyperpolarization of 2.9 ± 1.2 mV ($P < 0.05$). Introduction of SITS to the perfusate always led to depolarization of Ψ_m followed by partial or complete return to baseline after removal of SITS. In the presence of Ba^{2+}, introduction of SITS provoked a larger depolarization (Fig. 1 and Table 2).

These results suggest that SITS inhibits an anionic current that is outward-directed under P_{CO_2} of 1% and inward-directed under P_{CO_2} of 10%. In addition, the hyperpolarization usually induced by Ba^{2+} under P_{CO_2} of 10% indicates that under these conditions Ψ_m is close to E_K and that it is most often more negative than E_K.

To evaluate the relative importance of HCO_3^- and K^+ for Ψ_m, we did a series of experiments (n = 12) in which we switched from a solution at a P_{CO_2} of 10%, pH 7.6, supplemented with 2 mM Ba^{2+} to a solution which also contained 2 mM Ba^{2+} at the same pH but which was nominally CO_2/HCO_3^--free (Tes-buffered). Removal of CO_2 and HCO_3^- in the presence of Ba^{2+} depolarized Ψ_m toward zero (Fig. 2).

Effects of SITS on pH_i. In the second series of experiments, we verified the hypothesis that SITS inhibits a current due to HCO_3^- (or related species). We studied the effects of SITS on Ψ_m and pH_i by using double-barreled pH-selective microelectrodes under the same two experimental conditions.

Under normal physiological acid–base conditions, SITS hyperpolarized Ψ_m by 5.9 ± 0.7 mV (n = 8, $P < 0.001$) and increased pH_i from 7.36 ± 0.04 to 7.42 ± 0.04 (n = 8, $P <$ 0.001). Fig. 3 *Left* shows a representative record from one of these experiments.

Under imposed isohydric hypercapnia, SITS depolarized Ψ_m by 5.8 ± 0.5 mV (n = 9, $P < 0.001$) and decreased pH_i from 7.09 ± 0.03 to 7.02 ± 0.03 (n = 9, $P < 0.001$). Fig. 3 *Right* shows a representative record from one of these experiments.

Taken together, these results support the hypothesis that SITS inhibits a current due to HCO_3^- or related species. Since the electrochemical driving force for HCO_3^- across the basolateral membrane is outward-directed and of similar magnitude in both experimental conditions, the fact that the effect of SITS on Ψ_m and pH_i is opposite under our two experimental conditions rules out the possibility that the effect may be the result of inhibition of a pure HCO_3^- conductance. The action of SITS is, however, consistent with inhibition of a Na^+–HCO_3^- symport whose direction reverses, as we have argued above.

Thermodynamic Estimate of Na^+–HCO_3^- Stoichiometry. In the last series of experiments, $(Na^+)_i$ and pH_i (used to calculate $[HCO_3^-]_i$) were measured with double-barreled ion-selective microelectrodes. An experimental problem may arise, since it is possible that the $[CO_2]$ in tubule cells could be lower than the $[CO_2]$ in blood at the time of the experiments. Two experimental approaches could be used to circumvent this uncertainty. First, tubules *and* blood could be perfused with solutions of identical P_{CO_2}; alternatively, a tubule oil block could be used to prevent luminal P_{CO_2} from affecting cell $[CO_2]$ and pH. We have elected the second approach for technical reasons, and these values, as well as those in unblocked tubules, are shown in Table 3. The values of $(Na^+)_i$ and $(HCO_3^-)_i$ measured during perfusion with P_{CO_2} of 1% or 10% were used in Eq. 1 along with $(Na^+)_o$ = 75 mM and $(HCO_3^-)_o$ = 6.15 mM (for P_{CO_2} of 1%) or $(HCO_3^-)_o$ = 61.5 mM (for P_{CO_2} of 10%). With these activities, $E_{Na^+-HCO_3^-}$ was calculated for both N = 2 and N = 3.

Under normal acid–base conditions, the calculation gave $E_{Na^+-HCO_3^-}$ = −84.0 mV for N = 2 or −48.3 mV for N = 3. Since Ψ_m under these conditions was −68.3 ± 3.3 mV (n = 13), a stoichiometry of 2:1 would imply inward-directed net driving force for the Na^+–HCO_3^- cotransporter, and a stoichiometry of 3:1 would imply an outward-directed net driving force. The observed SITS-induced hyperpolarization implies that $E_{Na^+-HCO_3^-}$ was less negative than Ψ_m, which, together with the observed cell alkalinization, argues for net outward transport with a stoichiometry of (at least) 3:1.

Under imposed isohydric hypercapnia, the calculation gave $E_{Na^+-HCO_3^-}$ = −112.1 mV for N = 2 or −72.1 mV for N

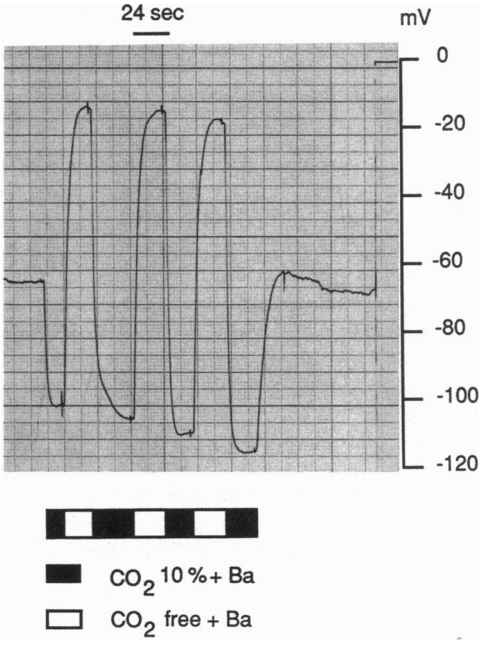

FIG. 2. Response of Ψ_m (mV) to removal of CO_2/HCO_3^- (shift from P_{CO_2} of 10%, 82 mM HCO_3^-, pH 7.6, to a nominally CO_2/HCO_3^--free, Tes-buffered, pH 7.6 solution) in the presence of 2 mM Ba^{2+}. Original tracing, obtained with a conventional intracellular microelectrode, is shown. Timing of perfusion with experimental solutions is indicated by the horizontal bars below the graph. Before and after these experimental solutions, perfusion was with blood from normal circulation.

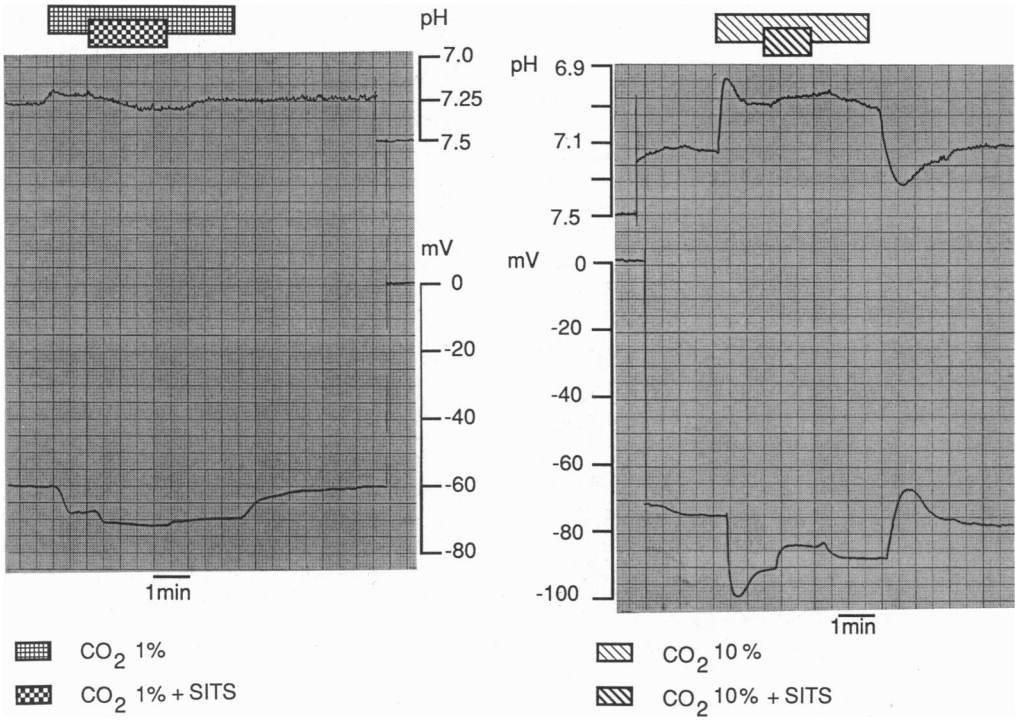

FIG. 3. Responses of pH_i (upper traces) and Ψ_m (mV) (lower traces) to 1 mM SITS under conditions of physiological acid–base conditions (1% CO_2) (*Left*) or acute peritubular isohydric hypercapnia (10% CO_2) (*Right*). Original tracings, obtained with a double-barreled pH-selective microelectrode, are shown. Note that the pH trace precedes the voltage trace by 18 sec, due to placement of pens on the chart recorder. Timing of perfusion indicated by the horizontal bars corresponds to the pH trace. See *Methods* for complete description of the two perfusion solutions.

= 3. The measured Ψ_m under these conditions was -88.9 ± 2.1 mV ($n = 18$). Thus, as for the case considered above, a stoichiometry of 2:1 would imply an inward-directed net driving force for the Na^+–HCO_3^- cotransporter, and a stoichiometry of 3:1 would imply an outward-directed net driving force. Under these conditions, however, SITS led to depolarization and cell acidification, meaning that $E_{Na^+-HCO_3^-}$ was more negative than Ψ_m and leading to the conclusion of net inward transport with a stoichiometry of (at most) 2:1.

DISCUSSION

Electrogenic coupled transport of Na^+ and HCO_3^- has been demonstrated by many authors (3, 5, 6, 11, 19, 20) on the basis of verification of the "predictions of the [general] model for electrogenic Na^+–HCO_3^- cotransport" [as defined by Boron and Boulpaep (2)]. The demonstration rests on the observation of a membrane depolarization associated with intracellular acidification during the reduction, at constant Pco_2, of $[HCO_3^-]_o$ and/or $[Na^+]_o$, but it does not presume the direction of net cotransport, which may be inward-directed (5) or outward-directed (1). Analysis of the effects of stilbene derivatives permits determination of the direction of the net transport: in frog retinal pigmented epithelium, the application of 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) depolarizes Ψ_m and decreases pH_i (inhibition of net inward transport) (5), whereas in the *Necturus* PCT, application of SITS hyperpolarizes Ψ_m and increases pH_i (inhibition of net outward transport) (11). As in these earlier reports, we have studied the effects of SITS on Ψ_m and pH_i.

Our results are consistent with an outward-directed Na^+–HCO_3^- symport under normal acid–base conditions. The thermodynamic approach we used here to conclude that the Na^+–HCO_3^- cotransporter has a stoichiometry of $3HCO_3^-$:$1Na^+$ under these conditions is similar to that used by Lopes *et al.* (11), except that they were obliged to calculate the stoichiometry by using values of $(Na^+)_i$ measured by other authors (21, 22), whereas we were able to measure $(Na^+)_i$ and $(HCO_3^-)_i$ in the same preparation.

During acute isohydric hypercapnia, SITS depolarized Ψ_m and decreased pH_i by about 0.07 pH unit, suggesting that SITS blocked an inward-directed Na^+–HCO_3^- cotransport. The membrane depolarization slightly preceded the fall in pH_i (as shown in Fig. 3) and persisted in the presence of Ba^{2+}, which indicates that it was not due to a secondary pH effect on a pH-sensitive K^+ conductance (23). In fact, in the presence of Ba^{2+}, the depolarization induced by SITS was actually amplified, implying that in the presence of Ba^{2+} either the Na^+–HCO_3^- transporter represents a larger fraction

Table 3. Paired measurements of Ψ_m and $(Na^+)_i$, or Ψ_m and pH_i, in three experimental series: blood vs. 1% CO_2, blood vs. 10% CO_2, and blood vs. 10% CO_2 under luminal oil block

Exp.	Condition	Ψ_m, mV	$(Na^+)_i$, mM	*n*	Ψ_m, mV	pH_i	$[HCO_3^-]_i$, mM	*n*
1	Blood	−62.5 ± 2.9	7.94 ± 0.97	7	−61.7 ± 5.7	7.35 ± 0.04	—	6
	1% CO_2	−67.7 ± 3.4	7.70 ± 0.93		−69.1 ± 6.4	7.34 ± 0.04	4.91 ± 0.49	
2	Blood	−60.4 ± 2.9	7.99 ± 0.90	9	−56.2 ± 2.9	7.36 ± 0.05	—	9
	10% CO_2	−88.5 ± 2.1	11.44 ± 1.21		−89.3 ± 3.8	6.99 ± 0.06	23.15 ± 2.90	
3	Under luminal oil block:							
	Blood				−62.1 ± 3.1	7.32 ± 0.04	—	10
	10% CO_2				−91.2 ± 1.9	7.03 ± 0.04	24.45 ± 1.94	

$[HCO_3^-]_i$ was calculated from pH_i in individual experiments.

of basolateral membrane conductance, or the basolateral membrane resistance is increased, or both.

The Ba^{2+}-induced hyperpolarization observed under conditions of isohydric hypercapnia is an argument in favor of an inward-directed Na^+–HCO_3^- symport, because it means that Ψ_m was more negative than E_K (16), which is only possible if there is another major conductance whose equilibrium potential is more negative than Ψ_m—namely, the SITS-sensitive Na^+–HCO_3^- cotransport with a stoichiometry of ≤ 2. The hyperpolarization effect of Ba^{2+} was unexpected, but has been observed before (24), and suggests that Ψ_m is largely determined by inward-directed Na^+–HCO_3^- cotransport. Results presented in Fig. 2 confirm that under acute isohydric hypercapnia, HCO_3^- ions are a major determinant of Ψ_m. In the BSC1 monkey kidney cell line, an inward-directed Na^+–HCO_3^- symport is also apparently responsible for the negativity of Ψ_m (25).

Although our results do argue in favor of an apparent stoichiometry change of the Na^+–HCO_3^- cotransport in *Necturus* proximal tubule, they do not allow a decision as to the exact nature of the transported species. In the kidney, where the Na^+–HCO_3^- cotransport assures transepithelial reabsorption of base-equivalents, a stoichiometry of 3:1 has been reported (3), corresponding to three distinct sites: one for free Na^+, one for CO_3^{2-}, and one for HCO_3^-, the last one being near saturation at physiological concentrations (4). In BSC1 cells, the Na^+–HCO_3^- symport is inward-directed with a net transfer of one negative charge, corresponding to transport of the pair $NaCO_3^-$ (one site) (26), whereas in retinal pigmented cells, which also present inward-directed transport with a stoichiometry of 2:1 (5), the transport of $NaCO_3^-$ is excluded, as is that of a free Na^+ and a CO_3^{2-} (27), which leads to the suggestion of a transporter with three sites ($1Na^+$:$2HCO_3^-$).

Our results would also be consistent with a conformational change of the transport protein, resulting in a change of the affinity of a CO_3^{2-} site in favor of HCO_3^-, which would also have the effect of a change of apparent stoichiometry.* A similar conclusion was also reached recently in a study of the stoichiometry of Na^+–HCO_3^- cotransport in rabbit proximal tubules (28).

Such a conformational change, leading to altered substrate requirement, has been suggested for the furosemide-sensitive cotransport of the medullary thick ascending limb; it has been suggested that a change of stoichiometry from Na^+:Cl^- to Na^+:K^+:$2Cl^-$ may be mediated by phosphorylation of the transport protein itself or of a regulating protein (29). Phosphorylation probably also plays an important role in the regulation of renal Na^+–HCO_3^- cotransport, since its activation parallels that of the apical Na^+/H^+ antiporter (30) and since it has recently been shown that its activity is regulated by cAMP- and Ca^{2+}-dependent protein kinases (31). Another common feature between Na^+–HCO_3^- cotransport and the Na^+/H^+ antiporter is the presence of an internal proton-sensitive modifier site in both proteins (32, 33). In our experiments, we noticed that pH_i fell by about 0.3 pH unit during imposition of isohydric hypercapnia, compared with normal physiologic conditions. The modulation by pH_i of the transporters that regulate pH_i (a sort of feedback effect) may be associated with protein phosphorylation, as has been shown to be the case for the Na^+/H^+ exchanger isoform NHE_1 (34) or for the Na^+-dependent Cl^-/HCO_3^- exchanger (35). At present, the influence of pH_i (or of other intracellular parameters such as $[Ca^{2+}]_i$) and/or altered protein phosphorylation of the transporter or of a regulator protein on the apparent stoichiometry of Na^+–HCO_3^- cotransport remains to be clarified.

*It can be shown, by assuming equilibrium of the chemical reactions, that if the P_{CO_2} is identical on both sides of the membrane, then $([HCO_3^-]_i/[HCO_3^-]_o)^2 = ([CO_3^{2-}]_i/[CO_3^{2-}]_o)$. It is then straightforward to show, for example, that the equilibrium potential for a $1Na$:$1CO_3^{2-}$ cotransporter is equal to that of a $1Na^+$:$2HCO_3^-$ cotransporter.

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