Basolateral Membrane Na/Base Cotransport Is Dependent on CO₂/HCO₃ in the Proximal Convoluted Tubule

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ABSTRACT The mechanism of basolateral membrane base transport was examined in the in vitro microperfused rabbit proximal convoluted tubule (PCT) in the absence and presence of ambient CO₂/HCO₃ by means of the microfluorometric measurement of cell pH. The buffer capacity of the cells measured using rapid NH₃ washout was 42.8 ± 5.6 mmol-litter⁻¹-pH unit⁻¹ in the absence and 84.6 ± 7.3 mmol-litter⁻¹-pH unit⁻¹ in the presence of CO₂/HCO₃. In the presence of CO₂/HCO₃, lowering peritubular pH from 7.4 to 6.8 acidified the cell by 0.30 pH units and lowering peritubular Na from 147 to 0 mM acidified the cell by 0.25 pH units. Both effects were inhibited by peritubular 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonate (SITS). In the absence of exogenous CO₂/HCO₃, lowering peritubular pH from 7.4 to 6.8 acidified the cell by 0.25 pH units and lowering peritubular Na from 147 to 0 mM decreased cell pH by 0.20 pH units. Lowering bath pH from 7.4 to 6.8 induced a proton flux of 643 ± 51 pmol-mm⁻¹-min⁻¹ in the presence of exogenous CO₂/HCO₃ and 223 ± 27 pmol-mm⁻¹-min⁻¹ in its absence. Lowering bath Na from 147 to 0 mM induced proton fluxes of 596 ± 77 pmol-mm⁻¹-min⁻¹ in the presence of exogenous CO₂/HCO₃ and 147 ± 13 pmol-mm⁻¹-min⁻¹ in its absence. The cell acidification induced by lowering bath pH or bath Na in the absence of CO₂/HCO₃ was inhibited by peritubular SITS or by acetazolamide, whereas peritubular amiloride had no effect. In the absence of exogenous CO₂/HCO₃, cyanide blocked the cell acidification induced by bath Na removal, but was without effect in the presence of exogenous CO₂/HCO₃. We reached the following conclusions. (a) The basolateral Na/base cotransporter in the rabbit PCT has an absolute requirement for CO₂/HCO₃. (b) In spite of this CO₂ dependence, in the absence of exogenous CO₂/HCO₃, metabolically produced CO₂/HCO₃ is sufficient to keep the transporter running at 30% of its control rate in the presence of ambient CO₂/HCO₃. (c) There is no apparent amiloride-sensitive Na/H antiporter on the basolateral membrane of the rabbit PCT.
INTRODUCTION

The mammalian proximal convoluted tubule (PCT) reabsorbs 80% of the filtered bicarbonate. Most of this bicarbonate reabsorption depends on two Na-dependent mechanisms, one on each side of the proximal tubule cell: an amiloride-sensitive Na/H antiporter on the apical membrane, and a recently described, stilbene-inhibitable, electrogenic Na/base cotransporter on the basolateral membrane. This latter transport system, first described in the salamander (Boron and Boulpaep, 1983), has now been found in the rat (Alpern, 1985; Yoshitomi et al., 1985) and rabbit PCT (Sasaki et al., 1985; Biagi and Sothell, 1986), in basolateral membrane vesicles from the rabbit renal cortex (Akiba et al., 1986a; Grassl and Aronson, 1986), in bovine corneal endothelial cells (Jentsch et al., 1984), and in a kidney epithelial cell line from the monkey (BSC-1; Jentsch et al., 1985). It has not been resolved whether the mechanism of Na/base exit is Na/HCO₃ or Na/OH cotransport (or, equivalently, Na/H antiport). Because it is not possible to eliminate CO₂/HCO₃ in vivo, in vitro studies are needed to differentiate among these possibilities.

A basolateral membrane amiloride-sensitive Na/H antiporter has been described in the salamander PCT (Boron and Boulpaep, 1983), but could not be found in rabbit cortical basolateral membrane vesicles (Ives et al., 1983) and in in vitro perfused S₃ segments of the rabbit PCT (Nakhoul and Boron, 1985). In rabbit basolateral membrane vesicles, ₂⁻Na uptake was stimulated by pH gradients in the absence of CO₂/HCO₃, but at a much slower rate than in its presence (5–20%), which raises the possibility that the Na/base cotransporter can transport HCO₃ and/or OH⁻ (Grassl and Aronson, 1986).

The purposes of our studies were therefore (a) to examine the mechanisms of basolateral Na-coupled base exit in the rabbit PCT, (b) to determine the CO₂/HCO₃ dependence of the base exit step, and (c) to confirm the presence or absence of a basolateral membrane Na/H antiporter in the rabbit PCT. To examine these questions, we have adapted the technique of measuring intracellular pH using the pH-sensitive dye (2',7')-bis-(carboxyethyl)-(5,6)-carboxyfluorescein (BCECF) to the in vitro microperfused rabbit PCT. The results demonstrate that the basolateral membrane of the rabbit PCT contains an Na/HCO₃ cotransporter with an absolute requirement for CO₂/HCO₃. In spite of this CO₂ dependence, the cotransporter is able to run at approximately one-third of its control rate in the absence of exogenous CO₂ (utilizing metabolic CO₂). There is no detectable amiloride-sensitive Na/H antiporter on this membrane.

Portions of this work have been presented previously and have appeared in abstract form (Krapf et al., 1987).

METHODS

In this study, the technique of in vitro microperfusion of isolated rabbit PCT, as previously described (Burg et al., 1966), was used. Kidneys from New Zealand white rabbits, killed by decapitation, were quickly removed and cut into thin (~1 mm) coronal slices. Cortical PCT (S₁ and S₂ segments) were dissected in the cooled (4°C) solution of the respective experiment (Table I). Late PCT, as identified by their attachment to straight tubules, were not used. The tubules were transferred to a bath chamber with a volume of ~150 μl. The bath fluid was continuously exchanged at ~10 ml/min by hydrostatic pressure.
With this setup, a complete bath fluid exchange could be achieved within ~1 s. This was confirmed when solutions were changed from a control solution to one containing a fluorescent dye (BCECF salt; see below). The bath pH was continuously monitored by placing a flexible commercial pH electrode (MI 21960, Microelectrodes, Inc., Londonderry, NH) into the bath. The bath solutions were preheated to 38°C and equilibrated with appropriate gases (see Table I). Another water bath, placed just before the bath chamber, permitted adjustment of the bath temperature to a constant 38 ± 0.5°C.

The perfusion solutions used in this study are listed in Table I. CO₂/HCO₃⁻-free solutions were bubbled with 100% O₂ passed through a 3-N KOH CO₂ trap. The protocol that excluded exogenous CO₂/HCO₃⁻ was always performed first, when the effects of the absence or presence of exogenous CO₂/HCO₃⁻ were compared to ensure the absence of CO₂/HCO₃⁻. SITS was obtained from ICN Pharmaceuticals (Cleveland, OH). Amiloride, nigericin, and acetazolamide were purchased from Sigma Chemical Co. (St. Louis, MO).

After the tubules were allowed to equilibrate at 38°C for 15 min, they were loaded with the acetoxymethyl derivative of BCECF (BCECF-AM; Molecular Probes, Inc., Eugene, OR). This compound does not fluoresce and is lipid soluble. It therefore diffuses rapidly into the cells, where cytoplasmic esterases cleave off the acetoxymethyl group, forming the fluorescent BCECF, which leaves the cells only slowly owing to its anionic charges. The tubules were loaded for 5–8 min. Since the intracellular cleavage of ester bonds constitutes an acid load to the cell, the first fluorescence measurements were made no earlier than 5 min after loading the tubules. During the performance of these studies, we found that the tubules could be loaded from the lumen (dye concentration, 100 μM) as well as from the bath (dye concentration, 4 μM). Loading from the bath yielded, on average, a higher signal-to-background ratio than loading from the lumen. The intracellular calibration curve of the dye (see below) was similar in tubules loaded from the lumen (n = 8) and from the bath (n = 4).

**Cell pH Measurement**

Measurements were made with an inverted fluorescent microscope (Fluovert, E. Leitz, Inc., Rockleigh, NJ) using a 25× objective. An adjustable measuring diaphragm was placed over the tubule and opened to ~40–70 μm². Within this range, no difference in the reliability of the data was observed. The average tubule length exposed to the bath fluid was 300–400 μm. Background fluorescence was measured before loading the tubule with the dye. After this measurement, the measuring diaphragm was left in the same place for the entire experiment. The signal-to-background ratio at the end of the experiments varied from ~25 to 200 at 500 nm excitation and from ~15 to 120 at 450 nm excitation (see analysis below).

**Analysis**

BCECF has a peak excitation at 504 nm that is pH sensitive, and an isosbestic point at 436 nm, where fluorescence is independent of pH. Peak emission is at 526 nm (Alpern, 1985). Fluorescence was measured, as previously described (Alpern, 1985), alternately at 500 and 450 nm excitation and at an emission wavelength of 530 nm (interference filters, Corion Corp., Holliston, MA). After correcting all measurements for background, the mean of two 500-nm excitation measurements was divided by the 450-nm excitation measurement between them, thereby yielding the fluorescence excitation ratio (F₅₀₀/F₄₅₀). For each determination, the measurements were performed twice and their mean was used to estimate cell pH. The use of the ratio provides a measurement that is unaffected by changes in the dye concentration (Thomas et al., 1979). After a solution change, the steady state cell pH values were determined when the 500-nm excitation fluorescence had stabilized.
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All concentrations are in millimoles per liter.
Dye Calibration

In order to correlate the fluorescence excitation ratios with cell pH, the dye was calibrated intracellularly using the method of Thomas et al. (1979). Tubules were perfused with well-buffered solutions (25 mM HEPES, 33 mM phosphate) containing 7 μM nigericin (a K/H antiporter) and 66 mM K. Since there are no data on the intracellular K activity for the rabbit PCT, we used the values as reported for the proximal straight tubule (PST) of the rabbit (48 mM; activity coefficient, 0.73; Biagi et al., 1981). In the above setting, cell pH is predicted to approximately equal extracellular pH. In order to test whether a difference in the K concentration would affect the calibration, three tubules were calibrated at pH 7.3 and external K concentrations of 66 or 132 mM. The ratios obtained were similar in the same tubules at these two external K concentrations. (The lack of an effect of a twofold increase in external K on the calibration is probably due to the high K conductance in PCT cells, which, together with the K/H antiporter, would be expected to equilibrate internal and external K, even if they were initially unequal.) Before exposure to nigericin, the tubules were loaded with BCECF and were then perfused in the lumen and the bath with the above solutions at different pH values. A typical tracing is shown in Fig. 1. The results of this calibration in a total of 12 tubules are shown in Fig. 2. As noted by others (Alpern, 1985; Chaillet and Boron, 1985), the effect of cell pH on the intracellular excitation ratios is shifted toward higher pH values as compared with the results of the extracellular calibration.

Acidification Rate

To measure \( \frac{d[H_+]}{dt} \), fluorescence was followed at 500 nm while a fluid exchange was performed, and recorded on a chart recorder (LS 52, Linseis, Inc., Princeton Junction, NJ). The slope of a line drawn tangent to the initial deflection \( (dF_{500}/dt) \) defined the initial rate of change in 500 nm fluorescence. Because fluorescence with 450 nm excitation is pH insensitive (Alpern, 1985), it can be considered constant. By measuring fluorescence at 450 nm before and after the fluid exchange, the actual value of the 450-nm excitation
at the time of the initial deflection of 500 nm could be interpolated. The rate of change in the fluorescence ratio was then calculated using the formula:

$$\frac{d(F_{500}/F_{450})}{dt} = \frac{(dF_{500}/dt)}{F_{450}}$$

(1)

Because the slope of the line (intracellular calibration) relating fluorescence ratio to pH in Fig. 2 is 1.13 pH unit⁻¹:

$$\frac{d\text{pH}}{dt} = \frac{(dF_{500}/dt)}{F_{450} \times 1.13}$$

(2)

**Buffer Capacity**

The buffer capacity ($B$) was determined using the technique of rapid NH₃ washout (Roos and Boron, 1981). Tubules were perfused at pH 7.4 in the control period. The bath solution was then changed to a similar solution with 20 mM NH₃/NH₄⁺ added (solutions 9-12, Table I). The NH₃ in these solutions rapidly enters the cells and combines with intracellular protons to form NH₄⁺. When external NH₃/NH₄⁺ are rapidly removed,

intracellular NH₄ dissociates into NH₃ and protons. Because of its high permeability (6 × 10⁻² cm/s in the rabbit PCT; Hamm et al., 1985), NH₃ rapidly diffuses out of the cell, while the protons are left behind and constitute the intracellular acid load. Since for each NH₄⁺ molecule dissociated, one intracellular proton is produced, the acid load per liter cell is $\Delta[NH_4^+]$. The buffer capacity (in millimoles per liter times pH units) is given by the formula

$$B = \frac{\Delta[NH_4^+]}{\Delta\text{pH}_i},$$

(3)

where $[NH_4^+]$ is the intracellular NH₄⁺ concentration just before removal of external NH₃/NH₄. This is calculated as

$$[NH_4^+] = [NH_3] \times 10^{pK_a-pH_i},$$

(4)

where $[NH_3]$ is the intracellular NH₃ concentration (assumed to equal the extracellular NH₃ concentration). pH$_i$ was calculated from the fluorescence excitation ratios described above. A pK$_a$ of 9.4 was used.

**Calculation of Proton Fluxes**

The proton fluxes ($J_{H^+}$ in picomoles per liter times millimeters times minutes) induced by the maneuvers in the different protocols were calculated using the formula

FIGURE 2. Dye calibration by fluorescence microscopy. The results of the extracellular and intracellular calibrations (n = 12 tubules) are shown. The intracellular calibration curve is shifted upward by ~0.24 pH units at pH 7.4.
\[ J_H = \frac{dpH}{dt} \cdot V \cdot \text{mm}^{-1} \cdot B, \]

where \( \frac{dpH}{dt} \) is the initial rate of cell acidification (in pH units per minute), \( V \cdot \text{mm}^{-1} \) is the approximate cellular volume of the tubules per millimeter, and \( B \) is the buffer capacity (in millimoles per liter times pH units). For an outer tubular diameter of 60 \( \mu \text{m} \) and an inner diameter of 25 \( \mu \text{m} \), \( V = 23.4 \times 10^{-10} \text{ liter} \cdot \text{mm}^{-1} \). Reported \( J_H \) values represent the means of the \( J_H \) values for the acidification induced by the experimental solution and the alkalinization induced by the control solution in the recovery period. In the steady state, cell pH is constant and there are proton fluxes from bath to cell and from cell to lumen. The proton flux \( (J_H) \) referred to here is actually the change in the proton flux induced by the experimental maneuver.

**Statistics**

All studies were paired, comparing two protocols within the same tubule. After the first protocol, the tubules were left to equilibrate in the control solution of the second protocol for 5 min. The data were analyzed using the paired t test. The calibration data were fitted using linear regression. Results are reported as means \( \pm \) standard error.

**RESULTS**

**Determination of Buffer Capacity**

Buffer capacity was determined in the absence and presence of \( \text{CO}_2/\text{HCO}_3^- \). An accurate determination of the buffer capacity requires that acid-extrusion processes be blocked. We attempted to meet this requirement by perfusing the tubules with 1 mM amiloride in the lumen (to block the apical Na/H antiporter) and 1 mM SITS in the bath (to block the Na/base cotransporter and Cl/base exchanger). To prevent competition of Na ions to the amiloride-binding site on the antiporter (Kinsella and Aronson, 1981), the tubules were perfused symmetrically with 50 mM Na (solutions 9–12, Table I). That the acid extrusion processes were effectively blocked in this setting is illustrated by the fact that cell pH defense against the acid load induced by NH3 washout was very slow (e.g., 0.05–0.08 pH units \( \cdot \text{min}^{-1} \) in the absence of exogenous \( \text{CO}_2/\text{HCO}_3^- \)). As illustrated by Table II, in the absence of exogenous \( \text{CO}_2/\text{HCO}_3^- \), the buffer capacity of the cells was 42.8 \( \pm \) 5.6 mmol liter \( \cdot \text{pH unit}^{-1} \) \( (B_i) \), and in its presence, the buffer capacity was 84.6 \( \pm \) 7.3 mmol liter \( \cdot \text{pH unit}^{-1} \) \( (B_f, n = 10) \). The mean resting cell pH was 7.26 \( \pm \) 0.02 in the presence and 7.24 \( \pm \) 0.03 in the absence of \( \text{CO}_2/\text{HCO}_3^- \) (NS).

**Na/Base Cotransport in Rabbit PCT**

To determine whether an Na/base cotransporter is present in the rabbit PCT, we examined the effects of changes in peritubular pH and Na concentration on cell pH in the absence and presence of 1 mM bath SITS. As shown in Fig. 3, lowering the bath HCO\(_3^-\) concentration from 25 to 5 mM (solutions 1 and 2, Table I) decreased cell pH by 0.30 \( \pm \) 0.02 pH units in the absence of SITS as compared with 0.09 \( \pm \) 0.02 in the presence of SITS \( (p < 0.001, n = 6) \). The proton flux, \( J_H \), was 592 \( \pm \) 71 pmol \( \cdot \text{mm}^{-1} \cdot \text{min}^{-1} \) in the control period and was inhibited 94\% to 37 \( \pm \) 14 pmol \( \cdot \text{mm}^{-1} \cdot \text{min}^{-1} \) by bath SITS. When peritubular Na was lowered from 147 to 0 mM (Fig. 4; solutions 5 and 6, Table I), the cells acidified by a mean of 0.25 \( \pm \) 0.03 pH units. In the presence of SITS, the pH
TABLE II

Intracellular Buffer Capacity of Rabbit PCT
in the Presence and Absence of CO₂/HCO₃⁻

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Comparison of the total buffer capacity (Bᵢ) with the buffer capacity in the absence of exogenous CO₂/HCO₃⁻ (Bᵢ). The difference between these two values is the buffer capacity of CO₂/HCO₃⁻ (B₂CO₂ measured). Calculated B₂CO₂ represents the buffer capacity calculated from the estimated intracellular HCO₃⁻ concentration (see text).

decrease was reduced to 0.03 ± 0.02 (p < 0.001, n = 5). The JₙH was 332 ± 51 pmol·mm⁻¹·min⁻¹ in the control period and was reduced to 10 ± 5 pmol·mm⁻¹·min⁻¹ by SITS.

These studies show the presence of a stilbene-inhibitable, Na-coupled base exit mechanism on the basolateral membrane of the rabbit PCT and confirm studies that found this transporter in the salamander (Boron and Boulpaep, 1983), rat (Alpern, 1985; Yoshitomi et al., 1985), and rabbit (Biagi, 1985; Sasaki et al., 1985; Biagi and Sothell, 1986) PCT.

CO₂/HCO₃⁻ Dependence of Na⁺/Base Cotransport

Since this Na-coupled transporter could possibly transport either HCO₃⁻ and/or H⁺/OH⁻ coupled to Na, the next studies were designed to determine the CO₂ requirements of this transport system. Tubules were symmetrically perfused at pH 7.4 and the bath fluid was changed to pH 6.8, first in the absence (solutions 3 and 4, Table I) and then in the presence of exogenous CO₂/HCO₃⁻ (solutions 1 and 2, Table I). As illustrated in Fig. 5 (left), lowering bath pH in the absence of CO₂/HCO₃⁻ decreased cell pH from 7.38 ± 0.04 to 7.14 ± 0.04 (p < 0.001, n = 10). Cell pH returned to 7.40 ± 0.04 (p < 0.001) when bath pH was returned to 7.4. In the presence of CO₂/HCO₃⁻ (Fig. 5, right), lowering bath pH acidified the cells from 7.35 ± 0.04 to 6.97 ± 0.04 (p < 0.001), with a recovery.
to 7.36 ± 0.05 (p < 0.001). The mean decrease of cell pH was 0.25 ± 0.04 in the absence of exogenous CO2/HCO3 and 0.38 ± 0.04 in its presence (p < 0.005). The associated fH values were 223 ± 27 pmol·mm⁻¹·min⁻¹ in the absence of CO2/HCO3 and 643 ± 51 pmol·mm⁻¹·min⁻¹ in its presence (p < 0.001). Thus, the fH in response to a given change in bath pH is reduced by only 65% upon removal of ambient CO2/HCO3.

The effect of lowering the bath Na concentration from 147 to 0 mM in the absence and presence of CO2/HCO3 (solutions 5–8, Table I) is shown in Fig. 6. In the absence of CO2/HCO3, cell pH decreased from 7.25 ± 0.03 to 7.08 ± 0.02 (p < 0.001) and recovered to 7.21 ± 0.04 (p < 0.001, n = 7). In the presence of CO2/HCO3, cell pH decreased from 7.28 ± 0.03 to 6.93 ± 0.05 (p < 0.001) and returned to 7.30 ± 0.05 (p < 0.001). The mean change of cell pH was 0.17 ± 0.03 in the absence of CO2/HCO3 and 0.35 ± 0.07 in its presence (p < 0.005), and fH values were 147 ± 13 and 596 ± 77 pmol·mm⁻¹·min⁻¹, respectively (p < 0.005). Again, a significant fH value in response to lowering the bath Na concentration was observed in the absence of ambient CO2/HCO3.

<table>
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<tr>
<td>ΔpH</td>
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<td>JH</td>
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<td>643 ± 51</td>
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</table>

Figure 4. Effect of bath Na removal in the presence of exogenous CO2/HCO3 on cell pH and fH. Bath Na was replaced by choline in the presence and absence of 1 mM bath SITS. * p < 0.001, n = 5.

Figure 5. Effect of lowering bath pH in the absence and presence of exogenous CO2/HCO3 on cell pH and fH. Bath pH was lowered from 7.4 to 6.8. * p < 0.001, n = 10.
Thus, these studies show the persistence of Na/base cotransport in the absence of CO₂/HCO₃⁻. In theory, the above results could be explained by the presence of an Na/OH or an Na/HCO₃ cotransporter and/or an Na/H antiporter. To differentiate among these mechanisms, we studied the effects of SITS (an inhibitor of Na/OH and Na/HCO₃ cotransporters) and amiloride (an inhibitor of Na/H antiporters) in the absence of exogenous CO₂/HCO₃⁻.

**Effect of SITS**

The effects of 1 mM peritubular SITS on the cell pH response to changes in bath pH or bath Na in the absence of ambient CO₂/HCO₃⁻ were examined. A typical tracing is shown in Fig. 7. SITS markedly inhibited the response of cell pH to changing bath pH in the absence of exogenous CO₂/HCO₃⁻. Fig. 8 shows results from six tubules. In the absence of SITS, lowering bath pH from 7.4 to 6.8 caused a decrease in cell pH from 7.52 ± 0.04 to 7.23 ± 0.03 (p < 0.001)
and a return to 7.53 ± 0.05 in the recovery period (p < 0.001). In the presence of 1 mM bath SITS, cell pH decreased from 7.44 ± 0.05 to 7.26 ± 0.05 (p < 0.005) and recovered to 7.43 ± 0.08 (p < 0.005). The mean changes in cell pH and $J_{H}$ were 0.30 ± 0.04 pH units and 338 ± 36 pmol·mm$^{-1}$·min$^{-1}$, respectively, in the absence of SITS and 0.17 ± 0.05 (p < 0.005) pH units and 62 ± 23 pmol·mm$^{-1}$·min$^{-1}$ (p < 0.001) in the presence of SITS.

Fig. 9 demonstrates that SITS decreased the mean change in cell pH induced by removing bath Na from 0.16 ± 0.03 to 0.05 ± 0.02 pH units (p < 0.001, n = 8) and inhibited $J_{H}$ from 165 to 33 to 13 ± 7 pmol·mm$^{-1}$·min$^{-1}$ (p < 0.001). In the period without SITS, cell pH decreased from 7.47 ± 0.04 to 7.30 ± 0.04 (p < 0.001) and returned to 7.45 ± 0.04 in the recovery period (p < 0.025). In the presence of 1 mM bath SITS, cell pH changed from 7.34 ± 0.03 to 7.31 ± 0.06 when bath Na was removed (NS) and was unchanged (7.31 ± 0.06) upon readdition of Na.
These data show that cell acidification induced by lowering bath pH or Na in the absence of exogenous CO₂/HCO₃⁻ is SITS sensitive and is inhibited by >80% by this agent. In these protocols, SITS caused a slight decrease of cell pH (see Figs. 8 and 9). Since time controls did not show any acidification, this effect seems to be SITS related, although its mechanism is presently unclear.

**Effect of Amiloride**

Fig. 10 shows that 1 mM bath amiloride did not inhibit the cell acidification induced by removing bath Na in the absence of exogenous CO₂/HCO₃⁻. Cell acidification was 0.18 ± 0.03 pH units in the absence of amiloride and 0.19 ± 0.04 pH units in the presence of amiloride (NS, n = 11). The corresponding $J_{H}$ values were 131 ± 23 and 118 ± 21 pmol·mm⁻¹·min⁻¹ (NS). In terms of absolute pH values, Na removal caused a decrease of cell pH from 7.33 ± 0.06 to 7.13 ± 0.07 ($p < 0.001$), with a recovery to 7.30 ± 0.05 ($p < 0.001$) in the absence of amiloride. In the presence of amiloride, cell pH decreased from 7.33 ± 0.06 to 7.11 ± 0.06 ($p < 0.005$) and returned to 7.28 ± 0.06 ($p < 0.005$).

The absence of an amiloride effect in these studies could be due to the high Na concentration used, since amiloride competes with Na on the Na/H antiporter (Kinsella and Aronson, 1981). When the same protocol was repeated in six additional tubules, but with Na decreased from 50 to 0 mM, there was still no detectable inhibition of cell acidification. The mean changes in cell pH were 0.11 ± 0.01 without and 0.14 ± 0.03 with 1 mM bath amiloride (NS). The $J_{H}$ was 148 ± 12 pmol·mm⁻¹·min⁻¹ and was unaltered by amiloride (152 ± 16 pmol·mm⁻¹·min⁻¹; NS).

Thus, these studies rule out an important role for an amiloride-sensitive Na/H antiporter in the Na-coupled basolateral base exit mechanism.

**Figure 10.** Effect of amiloride on cell pH changes and $J_{H}$ induced by bath Na removal in the absence of exogenous CO₂/HCO₃⁻. Na was removed from the bath in the absence and presence of 1 mM bath amiloride. * $p < 0.001$, ** $p < 0.005$, $n = 11$. 

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<tr>
<td>ΔpH</td>
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<td>0.19 ± 0.04 (NS)</td>
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</table>

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Effect of Acetazolamide and Cyanide

The significant rate of basolateral Na-coupled base exit in the absence of ambient CO$_2$/HCO$_3^-$, although it is slower than in the presence of CO$_2$/HCO$_3^-$, raises the possibility that the cotransport system might operate as an Na/OH cotransporter. Alternatively, there might be sufficient metabolic CO$_2$ produced in the cell to drive an Na/HCO$_3^-$ cotransporter in the absence of external CO$_2$/HCO$_3^-$.

The effect of acetazolamide on the cell acidification induced by lowering bath pH or bath Na might aid in distinguishing between these two possibilities. Acetazolamide would be expected to inhibit the rate of Na/HCO$_3^-$ cotransport, because carbonic anhydrase would be required to hydroxylate CO$_2$ to HCO$_3^-$. In contrast, acetazolamide would not be expected to inhibit the rate of Na/OH cotransport, unless carbonic anhydrase were an integral part of the Na/OH symporter protein. Such a function for carbonic anhydrase has not been previously described (Dobyan and Bulger, 1982; Silverman and Vincent, 1983).

The effect of 0.1 mM acetazolamide added to bath and lumen when bath pH was lowered is shown in Fig. 11. When bath pH was lowered from 7.4 to 6.8 in the absence of exogenous CO$_2$/HCO$_3^-$, the cell pH decreased by 0.27 ± 0.04 in the absence of acetazolamide, but only by 0.15 ± 0.05 (p < 0.001, n = 7) in the presence of acetazolamide. Acetazolamide substantially inhibited $J_H$ from 212 ± 39 to 15 ± 3 pmol·mm$^{-1}$·min$^{-1}$ (p < 0.001). When bath Na was removed (Fig. 12), acetazolamide prevented (100% inhibition) cell acidification. The mean cell pH change was 0.12 ± 0.02 in the absence and 0.01 ± 0.02 in the presence of acetazolamide (p < 0.001, n = 8). $J_H$ was inhibited from 125 ± 13 to 1 ± 3 pmol·mm$^{-1}$·min$^{-1}$ (p < 0.001).

As discussed above, acetazolamide sensitivity of the cell pH change is compa-
Figure 12. Effect of acetazolamide on cell pH changes and $J_H$ induced by removing bath Na in the absence of exogenous CO$_2$/HCO$_3^-$. Bath Na was changed from 147 to 0 mM in the absence and presence of 0.1 mM acetazolamide in the luminal and bath perfusates. * $p < 0.001$, $n = 8$.

Figure 13. Effect of acetazolamide on cell pH changes and $J_H$ induced by removing luminal Na in the absence of exogenous CO$_2$/HCO$_3^-$. Glucose and alanine were eliminated from the luminal perfusate and 1 mM SITS was added to the bath. Luminal Na was changed from 141 to 0 mM in the absence and presence of 0.1 mM acetazolamide in luminal and bath perfusates. * $p < 0.001$, $n = 4$. 

***
Dependence of Na/Base Cotransport on CO₂/HCO₃

Figure 14. Effect of 2 mM bath KCN on cell pH changes and \( J_H \) induced by removing bath Na in the absence of exogenous CO₂/HCO₃. * \( p < 0.001 \), ** \( p < 0.005 \), \( n = 6 \).

\[ 141 \pm 12 \] \( J_H \)
\[ 3 \pm 5 \]
\[ 0.16 \pm 0.02 \] \( \Delta \text{pH}_i \)
\[ \pm 0.02 \pm 0.02 \]

\[ \pm 34 \text{ pmol} \cdot \text{mm}^{-1} \cdot \text{min}^{-1} \] without acetazolamide and \( 147 \pm 41 \text{ pmol} \cdot \text{mm}^{-1} \cdot \text{min}^{-1} \) with acetazolamide (NS).

Thus, the acetazolamide sensitivity suggests that in the absence of exogenous CO₂/HCO₃, HCO₃ provided by metabolism is the transported base species. If this is the case, metabolic inhibitors would be expected to inhibit the transporter by restriction of substrate availability. On the other hand, they should have no effect in the presence of exogenous CO₂/HCO₃. The effect of 2 mM bath KCN (2 mM KCN replaced 2 mM KCl in solutions 7 and 8, Table 1) when bath Na was removed in the absence of exogenous CO₂/HCO₃ is shown in Fig. 14. Na removal lowered cell pH by 0.16 ± 0.02 pH units in the absence of KCN. In its presence, however, cell acidification was completely abolished (mean cell pH change plus 0.02 ± 0.02 pH units; \( p < 0.001 \), \( n = 6 \)). KCN inhibited \( J_H \) from 141 ± 12 to 3 ± 5 pmol·mm⁻¹·min⁻¹ (\( p < 0.001 \)). Cyanide inhibition of the transporter could be due to an ATP dependence rather than a CO₂ dependence. To rule this out, additional experiments were performed on four tubules (Fig. 15) in the presence of exogenous CO₂/HCO₃ (2 mM KCN replaced 2 mM KCl in solutions 5 and 6, Table 1). In this setting, KCN had no significant effect on

\[ 412 \pm 55 \] \( J_H \)
\[ 434 \pm 61 \]
\[ 0.21 \pm 0.03 \] \( \Delta \text{pH}_i \)
\[ 0.26 \pm 0.04 \]

\[ 0 \text{ mM} \]

Figure 15. Effect of 2 mM bath KCN on cell pH changes and \( J_H \) induced by removing bath Na in the presence of exogenous CO₂/HCO₃. * \( p < 0.001 \), ** \( p < 0.025 \), \( n = 4 \).
the cell acidification induced by bath Na removal: the cells acidified by 0.21 ± 0.03 pH units in the absence and by 0.26 ± 0.04 in the presence of KCN. \( J_H \) was 412 ± 55 pmol·mm⁻¹·min⁻¹ in the absence of KCN and 434 ± 61 pmol·mm⁻¹·min⁻¹ (NS) in its presence.

The fact that restriction of substrate (HCO₃⁻) availability by acetazolamide and KCN inhibits cell pH changes induced by bath Na removal also provides evidence that the transporter is Na coupled. We could not prove this formally when we attempted to show an inhibitory effect of symmetrical Na removal on cell pH change induced by lowering bath pH from 7.4 to 6.8, because Na removal caused a massive, stable, and fully reversible cell acidification from 7.28 ± 0.04 to 6.84 ± 0.05 (\( p < 0.001 \), \( n = 5 \); solutions 3 and 8, Table I). The decrease in the intracellular base concentration associated with this degree of cell acidification would by itself decrease the driving force across the transporter.

**Discussion**

Since the main purposes of these studies were to determine the CO₂/HCO₃⁻ dependence of the Na/base cotransporter and to analyze the nature of the base exit, we used the technique of in vitro microperfusion of rabbit PCT, which permits vigorous exclusion of exogenous CO₂/HCO₃⁻. Microcalorimetric analysis of bath fluid samples confirmed that the total CO₂ concentration was not different from zero. In addition, the special setup for these studies permitted rapid, serial fluid exchanges for the bath and the luminal perfusates independently. Therefore, we were able to assess the kinetic properties of the Na-coupled base transporter under a variety of experimental conditions. Cell pH was measured using fluorescence ratios of BCECF at two different wavelengths (500 and 450 nm), as previously described (Alpern, 1985).

In order to correlate the cell pH to the fluorescent ratios, the dye was calibrated using nigericin (a K/H antiporter) and a high external K concentration that approximated intracellular K activity (Thomas et al., 1979). In this setting, external pH approximately equals cell pH. The intracellular calibration curve was shifted to higher pH values when compared with the extracellular calibration, as also noted for the rat (Alpern, 1985) and the salamander PCT (dimethyl-6-carboxyfluorescein; Chaillet and Boron, 1985). In this study, the shift was equivalent to 0.24 pH units at pH 7.4 (Fig. 2). When tubules were perfused symmetrically with HCO₃⁻-containing solutions (solution 1, Table I), the cell pH was 7.29 ± 0.02 (\( n = 42 \)). This value is comparable to cell pH determinations with microelectrodes in the rabbit PST (Sasaki et al., 1985), but is lower than microfluorometrically obtained cell pH values in the rabbit PST (pH 7.46; Nakhoul and Boron, 1985). However, in the latter study, the pH data were obtained in the nominal absence of CO₂ and in the presence of monocarboxylate. We observed some intertubular variability in the resting cell pH, which probably relates to the fact that the tubules were not calibrated individually. However, as all our studies were paired, comparing a control with an experimental procedure, and since the interpretation of our results depends on the changes of the cell pH rather than their absolute values, this variability is probably of minor importance.
Determination of Buffer Capacity

Buffer capacity was measured in the presence and absence of exogenous CO₂/HCO₃⁻ and was 84.6 ± 7.3 mmol·liter⁻¹·pH unit⁻¹ in the presence and 42.8 ± 5.6 mmol·liter⁻¹·pH unit⁻¹ in the absence of CO₂/HCO₃⁻. The difference between total buffer capacity and the buffer capacity in the absence of exogenous CO₂/HCO₃⁻ is equal to the buffer capacity of CO₂/HCO₃⁻ (B_CO₂). According to the formula

$$B_{CO_2} = 2.3 \times [HCO_3^-]$$

(Roos and Boron, 1981), the CO₂/HCO₃⁻ buffer capacity can be directly calculated. Since the mean pH in this set of studies was 7.26, [HCO₃⁻] would be 17 mM and B_CO₂, would therefore be 39 mmol·liter⁻¹·pH unit⁻¹. As can be seen, this value is in excellent agreement with our measured difference of 42.2 mmol·liter⁻¹·pH unit⁻¹ (see Table II). The value for the total buffer capacity is comparable to that in the rat PCT of 102.7 ± 11.2 mmol·liter⁻¹, when cells were exposed to a pCO₂ of 40 kPa or 0.5 kPa, respectively (Yoshitomi and Froemter, 1984), and the value of 65.3 ± 8.7 mmol·liter⁻¹ when pCO₂ was varied from 5 to 10 kPa only (Yoshitomi et al., 1985).

Persistence of a Stilbene-inhibitable, Na-coupled Base Exit in the Absence of Exogenous CO₂/HCO₃⁻

In the presence of exogenous CO₂/HCO₃⁻, our studies confirmed the presence of a stilbene-sensitive, Na-coupled base exit in the rabbit PCT (Figs. 3 and 4). The observed mean cell pH change of 0.30 pH units when bath bicarbonate was lowered from 25 to 5 mM is comparable to findings in the rabbit PST (microelectrode study; Sasaki et al., 1985) and the rat PCT (fluorescent studies; Alpern, 1985; Alpern and Chambers, 1986), where mean changes of ~0.31 and 0.23–0.30 pH units were recorded, respectively. Similarly, Na removal from the bath caused a mean acidification of 0.25 pH units, again quite close to values in the rabbit PST (0.21; Sasaki et al., 1985) and the rat PCT (0.20, where Na was not totally removed, but lowered to 25 mM; Alpern, 1985).

When the effects of lowering peritubular Na or pH were compared in the absence and presence of CO₂/HCO₃⁻ in the same tubule (Figs. 5 and 6), it could be demonstrated that a significant acidification persisted in the total absence of exogenous CO₂/HCO₃⁻. The persisting proton fluxes were ~25% of the observed fluxes in the presence of CO₂/HCO₃⁻ when bath Na was removed and ~35% when bath pH was lowered. The facts that (a) SITS inhibited these effects (Figs. 7–9) and (b) amiloride had no effect (Fig. 10) both support the existence of a stilbene-sensitive, Na-coupled base exit in the absence of exogenous CO₂/HCO₃⁻. The failure to find an amiloride-sensitive Na/H antiporter on the basolateral membrane agrees with studies in vesicles from the rabbit and rat renal cortex (Ives et al., 1983; Sabolic and Burckhardt, 1983) and with studies in the isolated rabbit PST (Nakhoul and Boron, 1985).
Nature of the Na-coupled Base Exit in the Rabbit PCT

In the absence of exogenous CO2/HCO3, an Na-coupled base exit could occur by two basic transport mechanisms: (a) an Na/OH cotransporter (equivalent to an amiloride-insensitive Na/H antiporter), or (b) an Na/HCO3 cotransporter.

The presence of an amiloride-insensitive Na/H antiporter has recently been described in endosomes from the rabbit renal cortex (Gurich and Warnock, 1986), but there are no data to suggest its presence in the basolateral membrane of the PCT; in addition, it would not be expected to be SITS sensitive.

The finding that acetazolamide inhibited the cell pH changes in this study could be consistent with an Na/OH cotransporter: (a) if cytoplasmic carbonic anhydrase is needed to minimize a cellular diffusion barrier to base equivalents (Gutknecht et al., 1977), (b) if basolateral carbonic anhydrase is an integral functional component of the transporter, or (c) if the transporter shares a common structural domain with carbonic anhydrase, rendering it susceptible to acetazolamide.

If carbonic anhydrase facilitated diffusion of mobile buffers from the bulk phase in the cytoplasm to the membrane transporter, acetazolamide also should have inhibited the apical Na/H antiporter in the absence of exogenous CO2/HCO3. As such an inhibition was not found, carbonic anhydrase does not seem to have an important role in facilitating cytoplasmic buffer diffusion.

Because CO2-dependent 22Na uptake in rabbit cortical basolateral membrane vesicles occurs in the presence of acetazolamide (Akiba et al., 1986; Soleimani and Aronson, 1987), a direct influence of acetazolamide on the Na/base cotransporter seems improbable. The results of these studies and the effect of acetazolamide on the rate of the Na-coupled base exit observed in our study would argue against an Na/OH and favor an Na/HCO3 cotransport mechanism.

Even more convincing is the dependence of the basolateral membrane transporter in the absence of exogenous CO2/HCO3 on metabolism. In the absence of exogenous CO2/HCO3, inhibition of metabolism by bath cyanide completely inhibited basolateral membrane Na/HCO3 cotransport. To prove that this was not due to a direct effect on the transporter, we showed that cyanide was without effect in the presence of exogenous CO2/HCO3. Thus, sensitivity to acetazolamide and cyanide, in the absence of exogenous CO2/HCO3, proves that the transporter is dependent on CO2/HCO3, a dependence that is probably due to the fact that HCO3 is the transported base species.

The observations that, in the absence of exogenous CO2/HCO3, (a) changes in bath Na concentration were able to affect changes in transport rate and (b) changes in bath pH were reversible suggest that there is a significant local CO2/HCO3 concentration next to the outside of the basolateral membrane. This could be due to the effect of unstirred layers. Based on calculations of metabolic CO2 production, the intracellular HCO3 concentration can be estimated to be <0.37 mM, while the local extracellular concentration of HCO3 would be even lower.
In the presence of exogenous CO$_2$/HCO$_3^-$, the intracellular HCO$_3^-$ concentration at a pH of 7.3 is $\sim$19 mM. However, in the absence of exogenous CO$_2$/HCO$_3^-$, the transporter is able to run at $\sim$30% of its control rate in the presence of ambient CO$_2$/HCO$_3^-$ This observation suggests that the transporter is sensitive to very low concentrations of bicarbonate. This might occur if one or two of the bicarbonate-binding sites had a high affinity for bicarbonate, probably in the micromolar range. However, not all of the bicarbonate-binding sites can have such a high affinity, because the transporter would become saturated at low concentrations and its rate would be insensitive to changes in cell and bath bicarbonate concentrations from 25 to 5 mM.

In conclusion, our studies demonstrate the presence of a stilbene-sensitive Na/base cotransporter on the basolateral membrane of the rabbit PCT. This cotransporter has an absolute requirement for CO$_2$/HCO$_3^-$, which suggests that HCO$_3^-$ is at least one of the transported base species.

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