Role of basolateral carbonic anhydrase in proximal tubular fluid and bicarbonate absorption

SHUICHI TSURUOKA,1 ERIK R. SWENSON,2 SNEZANA PETROVIC,3 AKIO FUJIMURA,1 AND GEORGE J. SCHWARTZ3

1Department of Clinical Pharmacology, Jichi Medical School, Kawachi, Tochigi 329-0498, Japan; 2Medical Service, Veterans Affairs Puget Sound Health Care System, University of Washington, Seattle, Washington 98108; and 3Department of Pediatrics, University of Rochester School of Medicine, Rochester, New York 14642

Received 2 May 2000; accepted in final form 19 September 2000

Tsuruoka, Shuichi, Erik R. Swenson, Snezana Petrovic, Akio Fujimura, and George J. Schwartz. Role of basolateral carbonic anhydrase in proximal tubular fluid and bicarbonate absorption. Am J Physiol Renal Physiol 280: F146–F154, 2001.—Membrane-bound carbonic anhydrase (CA) is critical to renal acidification. The role of CA activity on the basolateral membrane of the proximal tubule has not been defined clearly. To investigate this issue in microperfused rabbit proximal straight tubules in vitro, we measured fluid and HCO3− absorption and cell pH before and after the extracellular CA inhibitor p-fluorobenzyl-aminobenzolamide was applied in the bath to inhibit only basolateral CA. This inhibitor was 1% as permeant as acetazolamide. Neutral dextran (2 g/dl, molecular mass 70,000) was used as a colloid to support fluid absorption because albumin could affect CO2 diffusion and rheogenic HCO3− efflux. Indeed, dextran in the bath stimulated fluid absorption by 55% over albumin. Basolateral CA inhibition reduced fluid absorption (~30%) and markedly decreased HCO3− absorption (~60%), both reversible when CA was added to the bathing solution. In the presence of luminal CA inhibition, which reduced fluid (~16%) and HCO3− (~66%) absorption, inhibition of basolateral CA further decreased the absorption of fluid (to 74% of baseline) and HCO3− (to 22% of baseline). CA inhibition also alkalinized cell pH by ~0.2 units, suggesting the presence of an alkaline disequilibrium pH in the interspace, which would secondarily block HCO3− exit from the cell and thereby decrease luminal proton secretion (HCO3− absorption). These data clearly indicate that basolateral CA has an important role in mediating fluid and especially HCO3− absorption in the proximal straight tubule.

in vitro microperfusion; acidification; cell pH; inulin; dextran; para-fluorobenzyl-aminobenzolamide; hydratase assay

CARBONIC ANHYDRASE (CA) is a renal enzyme that is critical to acid-base homeostasis. Up to 5% of renal CA activity is membrane bound, much of which corresponds to CA IV, whereas more than 95% is primarily cytosolic CA II (4, 16, 43, 44). However, in CA II-deficient patients and mice, inhibition of CA activity (presumably membrane-bound CA) diminishes renal acid excretion, indicating a major role in urinary acidification (3, 34). Functional studies in isolated nephron segments have clearly shown the presence of membrane-bound CA activity along the apical membranes of proximal tubules (14) and collecting ducts from the inner stripe of the outer medulla (18, 37) and inner medulla (42).

With respect to the proximal tubule, CA activity has been identified in brush-border and basolateral membranes (3, 16, 24, 43), and these findings have been confirmed by histochemistry in CA II-deficient mice (21). Using a variety of antibodies to membrane-bound CA IV, we (30, 31) and others (5) have detected CA IV on both apical and basolateral membranes of proximal tubules; labeling was heavier in straight (S2) than in convoluted (S1) segments.

The role of basolateral membrane CA in transepithelial fluid and HCO3− transport by the proximal tubule has not been functionally characterized. It was the purpose of this study to examine whether inhibition of basolateral membrane-bound CA affected proximal tubular handling of H+/HCO3−. We hypothesized that the following three properties would be sensitive to inhibition of basolateral CA in isolated perfused proximal straight tubules: 1) fluid absorption, 2) H+ secretion (HCO3− absorption), and 3) cell pH. By increasing pH in the vicinity of the basolateral membrane and intercellular space, HCO3− exit via the Na+-HCO3− cotransporter would be inhibited and HCO3− would accumulate in proximal tubule cells. The results indicate that inhibition of basolateral CA results in an increase in cell pH and a decrease in fluid and HCO3− absorption; the decrease in HCO3− absorption far exceeded the decrease in fluid absorption. These effects were completely reversed by adding CA (with the inhibitor) to the basolateral medium.

METHODS

Isolation of proximal straight tubules. Kidneys were obtained from female New Zealand White rabbits weighing
1.5–2.5 kg and maintained on standard laboratory chow plus free access to tap water. Death was accomplished using intracardiac injection of 130 mg pentobarbital sodium after premedication with ketamine (44 mg/kg) and xylazine (5 mg/kg).

Coronal slices (1–2 mm) of the kidneys were transferred to chilled dissection medium containing (in mM) 145 NaCl, 2.5 K2HPO4, 2 CaCl2, 1.2 MgSO4, 5.5 d-glucose, 1 trisodium citrate, 4 sodium lactate, and 6 l-alanine, pH 7.4, 290 ± 2 mosmol/kgH2O (40). From the medullary rays in the mid-cortex, proximal straight tubule segments were isolated. Length was restricted to <1.5 mm to minimize axial differences along the proximal tubule.

In vitro microperfusion. In vitro microperfusion was performed according to the method of Burg and Green (7), with modifications (27, 29, 40). An isolated proximal straight tubule segment was rapidly transferred to a 1.2-ml temperature- and environmentally controlled specimen chamber mounted on an inverted microscope and perfused with Burg’s solution containing (in mM) 120 NaCl, 25 NaHCO3, 2.5 K2HPO4, 2 CaCl2, 1.2 MgSO4, 5.5 d-glucose, 1 trisodium citrate, 4 sodium lactate, and 6 l-alanine, 290 ± 2 mosmol/kgH2O, and gassed with 94% O2-6% CO2 to maintain the pH at 7.4 at 37°C. The bathing solution was usually comprised of Burg’s solution plus neutral dextran (molecular mass 70,000); initial control studies also used defatted bovine serum albumin at 6 g/dl (7). The specimen chamber was continuously suffused with 94% O2-6% CO2 to maintain the bath pH at 7.4. Bathing solution was continuously exchanged by a peristaltic pump at a rate of 14 ml/h to maintain constant solute concentration.

Transmural voltage (Vw) was measured using the perfusion pipette as an electrode. The voltage difference between calomel cells connected via 3 M KCl agar bridges to perfusing and bathing solutions was measured using a high-impedance electrometer (Duol 773, WPI).

Fluid absorption. The collecting end was sealed into a holding pipette using Sylgard 184 (Dow Corning, Midland, MI). The length of each segment was measured using an eyepiece micrometer. Tubules were equilibrated for 20 min. [14C]inulin was added to the perfusate at 10 µCi/ml, yielding ~30 counts·min⁻¹·nl⁻¹ and equilibrated another 20 min. Samples (47 nl) were collected under water-saturated mineral oil by timed filling of a calibrated volumetric pipette. Collections were obtained in triplicate, placed in 1 ml of water plus 6 ml of scintillation solution containing 4 mg Omnifluor (Packard Bioscience, Groningen, The Netherlands) per milliliter in toluene-Triton X-100 (2:1 vol/vol), and the beta emission of 14C was counted (Beckman LSC-3500; Aloka, Tokyo, Japan) (39). Samples of perfusate were handled similarly with the same pipette. Fluid absorption rate (J) was calculated as

\[ J = \frac{V_c (C_o - C_i) - 1}{L} \]

where \( V_c \) is collection rate (in nl/min), \( L \) is tubular length (in mm), and \( C_o \) and \( C_i \) are concentrations of [methoxy-14C]inulin in the collected fluid and perfusate, respectively. Perfusion rates were usually 6–8 nl/min.

This protocol was designed to compare the effect of dextran and BSA on fluid absorption by proximal straight tubules. In the first period, baseline readings were obtained using BSA (6 g/dl) in the bath. Dextran (2 g/dl, 70,000 molecular mass, Sigma) was substituted for albumin in the second period. This concentration was chosen as being low enough to support fluid absorption without being harmful to the perfused segment. In the third period, we tested the effect of inhibiting basolateral CA by adding the impermeant inhibitor p-fluorobenzyl-aminobenzolamide (38), 1 µM to the bathing solution in the presence of dextran. This inhibitor has been shown to be sevenfold less permeant in human red blood cells than benzolamide, the classical impermeant CA inhibitor (38) and would therefore be expected to inhibit only CA that is resident in the basolateral membrane.

HCO3⁻ and fluid absorption. The concentrations of inulin and total CO2 (assumed to be equal to that of HCO3⁻) in perfusate and collected fluid were measured in a continuous flow microfluorimeter (Nanoflo; WPI, Sarasota, FL) (45). Three 47-nl collections were made per period and stored under water-saturated mineral oil. Aliquots (15 nl) of each collection were analyzed for total CO2 on the day of the experiment and for inulin on the following day, using procedures specified by the manufacturer. Samples of perfusate were processed similarly. Perfusion rate was generally 5–8 nl/min. HCO3⁻ transport rate (JHCO3) was calculated as

\[ J_{HCO3} = \frac{V_c [HCO3_i] - V_v [HCO3_o]}{L} \]

where \( V_c \) and \( V_v \) are perfusion rate and collection rate, respectively, [HCO3_i] and [HCO3_o] are HCO3⁻ concentrations in the perfusate and collected fluid, respectively, and \( L \) is tubular length in millimeters. Positive values of \( J_{HCO3} \) indicate net HCO3⁻ absorption.

This experiment was designed to test the effect of inhibiting basolateral CA on both fluid and HCO3⁻ absorption. After equilibration, baseline readings were obtained. Then 10 µM p-fluorobenzyl-aminobenzolamide (38) was added to the bathing solution, and collections were begun 10 min later for the second period. This higher concentration of p-fluorobenzyl-aminobenzolamide was used to inhibit most of the basolateral enzyme [I50 at 25°C for CA IV is 2 µM (E. R. Swenson, unpublished observations)]. In the third period, 1 mg/ml CA (Sigma) was added with the p-fluorobenzyl-aminobenzolamide to the bathing solution. This concentration (33 µM) was chosen to exceed by threefold the concentration of p-fluorobenzyl-aminobenzolamide and thereby ensure complete binding of the drug and its removal from the bathing solution. Reversal of the inhibition of the second period would prove that it is specific to blocking extracellular CA activity. It would also confirm that there is no residual cytosolic CA inhibition, which could not be reversed by adding CA to the bathing solution.

An additional experiment was performed to show that p-fluorobenzyl-aminobenzolamide did not permeate the proximal tubule cells and thereby did not inhibit cytosolic CA. The purpose of this experiment was to determine whether

---

1 In one preliminary experiment in a medullary collecting duct from the inner stripe, we titrated the effect of p-fluorobenzyl-aminobenzolamide on net HCO3⁻ absorption. Previous studies (40) have shown that benzolamide at 1 µM inhibited 96% of HCO3⁻ absorption, and this was reversed to 88% of control levels by adding CA to the perfusate. The baseline HCO3⁻ absorptive rate in our medullary collecting duct was 12.6 pmol·min⁻¹·mm tubular length⁻¹, and this was reduced to 5.6 pmol (45% of baseline) with 1 µM p-fluorobenzyl-aminobenzolamide and to 0.7 pmol (5% of baseline) with 10 µM p-fluorobenzyl-aminobenzolamide. Simultaneous perfusion of 10 µM p-fluorobenzyl-aminobenzolamide and 1 mg/ml CA restored the flux to 11.2 pmol·min⁻¹·mm tubular length⁻¹ (89% of baseline). Voltage studies confirmed the decrease in electrogenic H⁻ secretion by inhibiting CA: baseline, 3.5 mV; 1 µM inhibitor, 2.7 mV; 10 µM inhibitor, 1.7 mV. The addition of CA (1 mg/ml) to the 10 µM inhibitor restored the voltage to 2.8 mV. These data allowed us to choose a concentration of 10 µM p-fluorobenzyl-aminobenzolamide to inhibit basolateral CA and determine its role in mediating HCO3⁻ absorption by proximal straight tubules.
addition of the CA inhibitor to the bath decreases fluid and HCO$_3^-$ absorption in the presence of luminal p-fluorobenzyl-aminobenzolamide. After equilibration, baseline measurements of $J_e$ and $J_{HCO_3}$ were made. Then, 10 $\mu$M p-fluorobenzyl-aminobenzolamide were added to the luminal fluid. In the third period, 10 $\mu$M p-fluorobenzyl-aminobenzolamide were added to both the luminal and bathing fluids. Finally, in the last period, in the presence of 10 $\mu$M luminal and basolateral p-fluorobenzyl-aminobenzolamide, 5 mg/ml CA were added to the bath. This concentration (165 $\mu$M) should have completely bound the drug and removed it from the bathing solution, thereby reversing the inhibition of basolateral p-fluorobenzyl-aminobenzolamide.

**Cell pH.** After equilibration, a proximal straight tubule was perfused at 5 cm water pressure for 10–15 min with 5–10 $\mu$M 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-acetoxyethyl ester or 2',7'-bis-(3-carboxypropyl)-5- (and-6)-carboxyfluorescein-acetoxyethyl ester (Molecular Probes, Eugene, OR). Diffusion of the ester into the cytosol followed by de-esterification results in intracellular fluorescence. The distal end of the tubule was allowed to stick to the coverslip, which had been coated with poly-1-lysine (Sigma, St. Louis, MO); this maneuver reduced tubular movement and optimized fluorescent imaging. In general we focused on cells that were 100–200 $\mu$m from the tip of the perfusion pipette.

Cell pH was determined by excitation ratiofluorometry (490 nm/445 nm excitation; 520 nm emission), using an intracellular calibration with 10 $\mu$M nigericin-high potassium phosphate buffer (9, 27, 32) at the end of the experiment. Fluorescence was detected using a DAGE model 68 SIT camera, a Deltascan dual-monochrometer, and a quartz bifurcated fiberoptic illuminating system, using software provided by the manufacturer (Photon Technology, S. Brunswick, NJ). The system allowed us to examine multiple cells in duplicate 1-s readings by applying “regions of interest” to the captured images and to subtract background fluorescence from these images. By examining cells in focus close to the perfusion line we were able to avoid movement and contaminating fluorescent signals were minimized.

Twenty to thirty minutes after washout of the dye, baseline readings were obtained. These were followed by readings 10–15 min after the addition of 10 $\mu$M p-fluorobenzyl-aminobenzolamide to the bathing solution (second period). In the third period the inhibitor was removed and baseline readings were obtained again to prove reversibility. In one experiment, CA (1 mg/ml) was added with the inhibitor to show reversibility.

Viability. Evidence for viability was derived from the stability of $V_{te}$, a lack of inulin leak, and the absence of damaged cells assessed from the inclusion of 0.15 mg/ml Fast green dye. The experiment was discarded if there was any loss of voltage exceeding 1 mV, if leak of inulin exceeded 2%, or if there were green-staining cells in the wall of the tubule.

CA inhibitor assay. To determine whether basolaterally applied p-fluorobenzyl-aminobenzolamide enters the cell, we performed a CA inhibitor assay. Proximal straight tubules (1.5–2.8 mm) were microdissected in PBS containing calcium and transferred into a bath of PBS containing 100 $\mu$M of either the putatively impermeant inhibitor p-fluorobenzyl-aminobenzolamide or the very permeant inhibitor acetazolamide. The incubation was performed at room temperature to assure that there was no metabolic uptake of the drugs. After incubation for 15 min in the inhibitor, each tubule was briefly rinsed twice in PBS and transferred to a microcentrifuge tube. The tubules were freeze dried and then resuspended in a 1–2 $\mu$l of distilled water. The suspension was heated to 100°C to denature tubular CA activity and further lyse the cells.

The concentration of CA inhibitor was measured using a modification of the micro-method of Maren (15). In brief, one enzyme unit of purified bovine red cell CA was added to 60 $\mu$l of distilled water containing 25 mg/l bromthymol blue indicator at -10°C in a reaction vessel that was continuously gassed with 100% CO$_2$. The low temperature was used to assure stronger binding of the inhibitor to the cytosolic CA II in the reaction vessel to increase the assay’s detection of small amounts of inhibitor. The catalyzed CO$_2$ hydration reaction was initiated by the addition of 40 $\mu$l of 50 mM barbitral buffer at pH 7.9, and the time was recorded to obtain a color change of the indicator, which occurs at $pH$ 6. Known amounts of the two inhibitors or the unknowns were preincubated with CA in the reaction vessel for 2 min with corresponding reductions in the amount of distilled water to maintain a final volume of 100 $\mu$l after the addition of 40 $\mu$l barbital buffer. The reaction times in the presence of known concentrations of CA inhibitor were used to construct a standard curve from which the unknown concentrations in the tubular lysate were measured.

**Statistics.** Data are presented as means ± SE. Paired comparisons for each tubule were analyzed by paired $t$-test using statistical software (Microsoft Excel). Significance was asserted when $P < 0.05$.

**RESULTS**

**Fluid absorption.** Nine proximal straight tubules averaging 1.0 ± 0.1 mm in length were examined for the effect of dextran and basolateral CA inhibition on fluid absorption (Fig. 1). Fluid absorption in the presence of 6 g/dl bovine serum albumin in the bath averaged 0.42 ± 0.02 nl·min$^{-1}$·mm tubule length$^{-1}$. The baseline voltage varied in a range of 6–8 mV. When albumin was replaced by 2 g/dl dextran, fluid absorption was increased 55% to 0.66 ± 0.02 nl·min$^{-1}$·mm tubule length$^{-1}$ ($P < 0.010$). There was no significant change in $V_{te}$ (−3.8 ± 0.1 mV). When 1 $\mu$M p-fluorobenzyl-aminobenzolamide was added to the dextran bath, fluid absorption decreased 23% to 0.50 ± 0.02 nl·min$^{-1}$·mm$^{-1}$ ($P < 0.01$) with no change in $V_{te}$.

We elected to use dextran in the bath for subsequent experiments, realizing that albumin may facilitate CO$_2$ diffusion in solutions by providing a mobile buffer (12). This facilitated flux of CO$_2$ might partially offset the effect of CA inhibition and minimize a real change in transport or cell pH. In addition, the negative charge of albumin could, in principle, inhibit rheogenic HCO$_3^-$ absorption across the basolateral membrane and thereby reduce overall transepithelial fluid and HCO$_3^-$ absorption.

**Fluid and HCO$_3^-$ absorption.** We examined nine proximal straight tubules averaging 1.2 ± 0.1 mm in length for the effect of basolateral CA inhibition on absorption of fluid (Fig. 2A) and HCO$_3^-$ (Fig. 2B). The baseline data using a 2 g/dl dextran bath revealed a $J_V$ of 0.65 ± 0.04 nl·min$^{-1}$·mm$^{-1}$ and a HCO$_3^-$ absorption rate ($J_{HCO_3}$) of 75 ± 1 pmol·min$^{-1}$·mm$^{-1}$. In the presence of 10 $\mu$M p-fluorobenzyl-aminobenzolamide in the bath, there was a 31% decrease in fluid absorption (to 0.45 ± 0.04 nl·min$^{-1}$·mm$^{-1}$, $P < 0.01$).
and a 62% decrease in HCO$_3^-$ absorption (to 29 ± 3 pmol·min$^{-1}$·mm$^{-1}$, $P < 0.01$). In addition, $V_{te}$ became slightly more negative with CA inhibition (from $-3.5 \pm 0.1$ to $-3.8 \pm 0.1$ mV, $P < 0.01$). When CA (1 mg/ml) was added to the bath with the inhibitor, transport was restored to baseline ($J_v$, 0.75 ± 0.06 nl·min$^{-1}$·mm$^{-1}$, and $J_{HCO_3^-}$, 76 ± 2 pmol·min$^{-1}$·mm$^{-1}$), as was $V_{te}$ ($-3.5 \pm 0.1$).

**Luminal and basolateral CA inhibition.** We determined in four tubules averaging 1.1 ± 0.1 mm in length the additive effects of 10 μM p-fluorobenzyl-aminobenzolamide added to the lumen and then to the bath on the absorption of fluid (Fig. 3A) and HCO$_3^-$ (Fig. 3B). The baseline data using a 2-g/dl dextran bath revealed a $J_v$ of 0.61 ± 0.01 nl·min$^{-1}$·mm$^{-1}$ tubule length and a $J_{HCO_3^-}$ of 72 ± 2 pmol·min$^{-1}$·mm$^{-1}$. In the presence of luminal p-fluorobenzyl-aminobenzolamide, there was a 16% decrease in fluid absorption (to 0.51 ± 0.01 nl·min$^{-1}$·mm$^{-1}$, $P < 0.01$) and a 66% decrease in HCO$_3^-$ absorption (to 25 ± 3 pmol·min$^{-1}$·mm$^{-1}$, $P < 0.01$) compared with baseline period. *$P < 0.01$ compared with albumin period. #$P < 0.01$ compared with dextran period.

Fig. 1. Effect of dextran and basolateral carbonic anhydrase (CA) inhibition on fluid absorption rate ($J_v$ in nl·min$^{-1}$·mm tubule length$^{-1}$) by proximal straight tubules. In 9 experiments 2 g/dl neutral dextran (molecular mass 70,000) in the bath caused a 55% increase in fluid absorption compared with a standard bath of 6 g/dl bovine serum albumin. The addition of 1 μM p-fluorobenzyl-aminobenzolamide to the dextran-containing bath reduced fluid absorption by 23%, nearly back to baseline. *$P < 0.01$ compared with albumin period. #$P < 0.01$ compared with dextran period.

Fig. 2. Effect of basolateral CA inhibition on fluid (A) and HCO$_3^-$ absorption rate ($J_{HCO_3^-}$ in pmol·min$^{-1}$·mm tubule length$^{-1}$, B) by proximal straight tubules. In 9 experiments the addition of 10 μM p-fluorobenzyl-aminobenzolamide to the bath caused a 31% decrease in fluid absorption and a 62% decrease in HCO$_3^-$ absorption. The addition of 1 mg/ml CA to the p-fluorobenzyl-aminobenzolamide to the bath completely reversed the inhibition of fluid and HCO$_3^-$ absorption. *$P < 0.01$ compared with baseline period. #$P < 0.01$ compared with inhibitor period.
In addition, $V_{te}$ became less negative with luminal CA inhibition (from $-3.6 \pm 0.1$ to $-3.4 \pm 0.1$ mV, $P < 0.01$). Then 10 $\mu$M $p$-fluorobenzyl-aminobenzolamide were added to the bathing solution so that both luminal and basolateral CA were inhibited. There was a further decrease in fluid absorption (to $0.45 \pm 0.01$ nl/min/mm, $P < 0.05$), $HCO_3^-$ absorption (to $16 \pm 2$ pmol/min/mm, $P < 0.01$), and $V_{te}$ (to $-3.2 \pm 0.1$ mV, $P < 0.01$). With inhibition of both luminal and basolateral CA, the residual rates of transport were 74% for fluid absorption and 22% for $HCO_3^-$ absorption.

Cell $pH$. Twenty-five cells in five tubules showed a baseline $pH$ of 7.15 $\pm$ 0.03 units (Fig. 4A). The addition of 1 $mg/ml$ CA to the bath in the presence of $p$-fluorobenzyl-aminobenzolamide almost completely restored cell $pH$ back to the baseline (baseline, 7.15 $\pm$ 0.02; CA inhibitor, 7.38 $\pm$ 0.02; inhibitor + CA, 7.21 $\pm$ 0.02, each change was significant).

Comparison of $p$-fluorobenzyl-aminobenzolamide and acetazolamide permeabilities. Proximal straight tubules were exposed to 100 $\mu$M $p$-fluorobenzyl-aminobenzolamide or acetazolamide for 15 min and assayed for total cellular CA inhibitor (Table 1). Six tubules averaging 1.8 mm and exposed to acetazolamide had a mean concentration of 96 $\pm$ 4 $\mu$M, similar to the bathing concentration and suggesting complete equilibration of the tubular cytoplasm. Seven tubules averaging 1.8 mm and exposed to $p$-fluorobenzyl-aminobenzolamide had a mean concentration of 1.4 $\pm$ 0.2 $\mu$M. Two of these tubules had concentrations below 0.8 $\mu$M, the limit of the assay, but in the calculations were considered to equal 0.8 $\mu$M, the limit of the assay, but in the calculations were considered to equal 0.8 $\mu$M, the mean concentration of $p$-fluorobenzyl-aminobenzolamide was at most 1.5% of that achieved with acetazolamide, indicating that the permeability of $p$-fluorobenzyl-aminobenzolamide was $\approx 1/100$ of that of acetazolamide.
Previous experiments in convoluted and straight proximal tubules (7, 17) using the permeant CA inhibitor acetazolamide showed nearly comparable results: only 30–40% inhibition of $J_v$ and nearly complete inhibition of $J_{HCO_3^-}$. The nearly complete inhibition of $J_{HCO_3^-}$ (compared with 78% inhibition after both membrane CAs were inhibited) is probably explained by the concomitant inhibition of cytosolic CA, as well as membrane CAs. Presumably, these findings relate to the dependence of $HCO_3^-$ absorption on proton secretion via the $Na^+ / H^+$ exchanger, which requires cytosolic CA to generate protons and $HCO_3^-$ within the cell, according to

$$H_2O + CO_2 \rightleftharpoons H_2CO_3 \rightleftharpoons H^- + HCO_3^-$$

Cytosolic CA catalyzes the hydration of $CO_2$, and the carbonic acid generated rapidly dissociates into protons and $HCO_3^-$. Inhibition of the cytosolic enzyme would impair the generation of protons for secretion into the lumen. In contrast, fluid absorption is mediated by a variety of sodium-coupled ion transporters, only one of which is the above-noted $Na^+ / H^+$ exchanger.

The reversal of CA inhibition, by adding CA to the inhibitor in the bathing solution, indicates that only basolateral and not cytosolic CA was inhibited. What would be the consequence of inhibiting basolateral CA? To answer this question requires one to examine the $HCO_3^-$ exit step across the basolateral membrane. This exit is primarily via the $Na^+ - HCO_3^-$ cotransporter (22, 28, 36). It is likely that this cotransporter involves the cotransport of $Na^+, CO_3^2 -, and HCO_3^-$ on distinct sites (36).

Electrophysiological analyses (2, 6, 25) have indirectly shown that membrane-bound CA is involved in passive rheogenic $HCO_3^-$ transfer across the peritubular membrane. In addition, $HCO_3^-$ transport across basolateral membrane vesicles can be inhibited by acetazolamide (11, 20, 35). This finding suggests a role for membrane-bound CA in facilitating the rheogenic transfer of $HCO_3^-$ across the peritubular membrane (6, 35). Inhibition of CA causes alkalinization of proximal tubular cells (13), which would in turn inhibit luminal $H^+$ secretion. Indeed, we have observed decreased

![Graph](http://ajprenal.physiology.org/)

**DISCUSSION**

*Functional basolateral CA.* These studies have examined in four different ways the functional presence of basolateral CA in the proximal straight tubule. We have made use of a newly developed impermeant CA inhibitor, which has been recently synthesized (38). In the first series we have shown that basolateral $p$-fluorobenzyl-aminobenzolamide inhibited fluid absorption. In the second, $p$-fluorobenzyl-aminobenzolamide inhibited the absorption of fluid and $HCO_3^-$ to a greater extent. The decrement in $J_v$ was 31%, whereas the decrease in $J_{HCO_3^-}$ was 62%, twice that for $J_v$. In the third series we have shown an additive inhibition when $p$-fluorobenzyl-aminobenzolamide was first applied to the perfusate and then to the bathing fluid, indicating the functional presence of CA on each membrane. The overall decrement in $J_v$ was only 26%, whereas the decrease in $J_{HCO_3^-}$ was 78%, nearly three times that for $J_v$. The sequential decreases with luminal and basolateral $p$-fluorobenzyl-aminobenzolamide indicate separate roles for both luminal and basolateral CA in mediating fluid and $HCO_3^-$ transport. In addition, this latter study shows that the luminal and basolateral inhibitors are confined to the membrane of application and do not permeate the cells.

### Table 1. Estimated concentrations of CA inhibitor in proximal straight tubules

<table>
<thead>
<tr>
<th>Acetazolamide</th>
<th>$p$-Fluorobenzyl-Aminobenzolamide</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Length</strong></td>
<td><strong>[ACZ]</strong></td>
</tr>
<tr>
<td>2</td>
<td>85</td>
</tr>
<tr>
<td>2.8</td>
<td>97</td>
</tr>
<tr>
<td>1.4</td>
<td>89</td>
</tr>
<tr>
<td>1.6</td>
<td>103</td>
</tr>
<tr>
<td>1.6</td>
<td>110</td>
</tr>
<tr>
<td>1.5</td>
<td>93</td>
</tr>
</tbody>
</table>

Length of proximal straight tubule, mm; concentration of carbonic anhydrase (CA) inhibitor, $\mu$M. [ACZ], acetazolamide concentration; [pF-AB], $p$-fluorobenzyl-aminobenzolamide concentration.
HCO$_3^-$ absorption (H$^+$ secretion) in the presence of basolateral CA inhibition (see Fig. 2B). The mechanism for this decrease in proximal tubular HCO$_3^-$ absorption in the setting of basolateral CA inhibition has been reviewed by Seki et al. (33) and may result from development of a disequilibrium CO$_3^{2-}$ (alkaline pH disequilibrium) in the basal labyrinth due to the accumulation of large amounts of CO$_3^{2-}$ via the basolateral Na$^+$-HCO$_3^-$ cotransporter (20). Because the CO$_3^{2-}$ equilibrium concentration in the physiological Ringer solution is only 20–80 µM, most of the CO$_3^{2-}$ generated must be converted to HCO$_3^-$, according to

$$\text{CO}_3^{2-} + \text{H}^+ \rightleftharpoons \text{HCO}_3^-$$

However, the physiological H$^+$ concentration is ~0.04 µM, and therefore, in the absence of basolateral CA activity, the local pH will immediately rise (alkaline disequilibrium pH). Further consumption of CO$_3^{2-}$ activity, the local pH will immediately rise (alkaline pH disequilibrium) in the basal labyrinth due to the accumulation of large amounts of CO$_3^{2-}$ via the basolateral Na$^+$-HCO$_3^-$ cotransporter (20). The CO$_3^{2-}$ equilibrium concentration in the physiological Ringer solution is only 20–80 µM, most of the CO$_3^{2-}$ generated must be converted to HCO$_3^-$, according to

At present it is not possible to reconcile these discrepancies between apical and basolateral membranes, but one possibility is that the CA activity on the latter membrane is not immunologically CA IV. The recent finding of other membrane-bound CAs (CA XII (41) and CA XIV (19)) suggests the possibility that one of these could be resident on the basolateral membrane. They could be present in addition to CA IV or could be cross-reacting with antibodies generated against CA IV. Further studies will be necessary to determine the molecular identity of the basolateral CA activity.

Dextran and fluid absorption. Proximal tubular fluid absorption is known to be enhanced by adding albumin to the bathing solution (8, 10). However, our studies utilizing CA inhibition in the interspace were likely to lead to a buildup of carbonate and a possible disequilibrium alkaline pH. In view of the interrelationship between HCO$_3^-$ and CO$_3^{2-}$ and the rheogenic nature of HCO$_3^-$ exit, it seemed important to eliminate albumin from the bathing solution. Albumin is known to facilitate CO$_2$ diffusion by carrying protons in parallel with the diffusion of HCO$_3^-$ (12); this movement of albumin would substantially boost the diffusion constant of CO$_2$ diffusing across the interspace and might offset some of the inhibition of CA. In addition, the negative charge of albumin might have inhibitory effects on fluid and HCO$_3^-$ absorption due to the electrogenic nature of HCO$_3^-$ exit via the Na$^+$-HCO$_3^-$ cotransporter (36).

For these reasons we examined whether a comparatively sized dextran, a neutral molecule without substantial buffering capacity, would support fluid absorption by proximal straight tubules. To our knowledge, there are no previous studies of proximal tubular fluid transport comparing the effects of neutral dextran against albumin. Compared with 6 g/dl albumin, dextran at 2 g/dl concentration in the bath resulted in a 54% increase in the rate of fluid absorption. It is possible that the neutral dextran had no negative charges to oppose electrogenic HCO$_3^-$ absorption; further studies are needed to understand the mechanism for this increase in fluid transport.

In the presence of dextran, basolateral CA inhibition resulted in a 25–33% inhibition of fluid absorption, probably because of the buildup of carbonate in the interspace. The alkalinization of the interspace after CA inhibition could have increased tight junction permeability, resulting in a decrease in fluid absorption due to enhanced backflux. Evidence against this possibility is derived from the lack of change of the $V_{te}$. In the setting of increased leakiness of the tight junction one would have expected to observe a less negative $V_{te}$. However, we found that $V_{te}$ either became more negative or failed to change after basolateral CA inhibition.

Permeability of $p$-fluorobenzyl-aminobenzolamide. Our assay of CA-inhibitor permeability showed that $p$-fluorobenzyl-aminobenzolamide is 1% as permeable as is acetazolamide across the basolateral membrane of proximal straight tubules. It was assumed that all of the drug had diffused into cellular cytoplasm; however, there could be some binding to the basolateral CA as...
well. The contribution of membrane CA activity has been estimated to be at most 10% of total CA hydratase activity (4), so that the intracellular concentration is probably very close to the measured concentration. Because the transport and cell pH experiments were performed using 10 μM of $p$-fluorobenzyl-aminobenzolamide, the estimated maximal intracellular concentration would be $10^{-7}$ M, not high enough to substantially inhibit cytosolic CA activity. Thus these experiments are in agreement with other results obtained in this study, which showed that CA applied to the bath medium reversed the inhibitory effect of basolateral $p$-fluorobenzyl-aminobenzolamide. The reversal could not have occurred if cytosolic CA were also inhibited by the $p$-fluorobenzyl-aminobenzolamide. Thus $p$-fluorobenzyl-aminobenzolamide is an impermeant CA inhibitor.

In summary, we have presented four sets of experiments that show for the first time the functional presence of basolateral CA activity that mediates proximal tubular fluid and HCO$_3^-$ absorption and secondarily affects cell pH. These results are consistent with previous electrophysiological and membrane vesicle investigations, as well as with more recent histochemical and immunocytochemical studies. Alkalization of the interspace is likely to inhibit basolateral Na$^+$/HCO$_3^-$ cotransport, which would decrease active transport. Alkalization of the cell would decrease the concentration of protons available for luminal HCO$_3^-$ reabsorption via the apical Na$^+$/H$^+$ exchanger and decrease the cellular exit of chloride, the latter reducing electroneutral NaCl transport. These effects would lead to decreased fluid and HCO$_3^-$ reabsorption. The magnitude of the effect of inhibiting basolateral CA attests to the importance of membrane-bound CA in the region of the proximal tubule cell interspace. Further studies are needed to determine the molecular identity of the CA that is resident there.

We are grateful to A. Kittelberger for technical assistance, Dr. M. Imai for providing microperfusion equipment, and Dr. A. Weinstein for critically reviewing the manuscript.

This work was supported by the National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-50603 to G. J. Schwartz, National Heart, Lung, and Blood Institute Grant HL-45571 to E. R. Swenson, and a grant from the Ministry of Education, Science and Culture of Japan to S. Tsuruoka.

REFERENCES


