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Research Article

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Role of the Na^+/H^+ Antiporter in Rat Proximal Tubule Bicarbonate Absorption

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Abstract

Amiloride and the more potent amiloride analog, 5-(*N*-*t*-butyl) amiloride (*t*-butylamiloride), were used to examine the role of the Na^+/H^+ antiporter in bicarbonate absorption in the in vivo microperfused rat proximal convoluted tubule. Bicarbonate absorption was inhibited 29, 46, and 47% by 0.9 mM or 4.3 mM amiloride, or 1 mM *t*-butylamiloride, respectively. Sensitivity of the Na^+/H^+ antiporter to these compounds in vivo was examined using fluorescent measurements of intracellular pH with (2',7')-bis(carboxyethyl)-(5,6)-carboxyfluorescein (BCECF). Amiloride and *t*-butylamiloride were shown to be as potent against the antiporter in vivo as in brush border membrane vesicles. A model of proximal tubule bicarbonate absorption was used to correct for changes in the luminal profiles for pH and inhibitor concentration, and for changes in luminal flow rate in the various series. We conclude that the majority of apical membrane proton secretion involved in transepithelial bicarbonate absorption is mediated by the Na^+-dependent, amiloride-sensitive Na^+/H^+ antiporter. However, a second mechanism of proton secretion contributes significantly to bicarbonate absorption. This mechanism is Na^+-independent and amiloride-insensitive.

Introduction

Amiloride-sensitive Na^+/H^+ antiporter activity has been demonstrated in brush border membrane vesicles from rabbit and rat renal cortex (1–10). In addition, an H^+-ATPase has been demonstrated in endosomes derived from renal cortex and in brush border membranes (11–17). The contribution of Na^+/H^+ exchange to apical membrane proton secretion in the intact proximal tubule has been investigated in several microperfusion studies by examining the effect of removing luminal and capillary Na^+ or inhibiting the Na^+/K^+ ATPase on the rate of bicarbonate absorption. Most of these studies found that 80–100% of bicarbonate absorption is dependent on the presence of Na^+ and Na^+ absorption (18–23).

The interpretation of these studies is complicated by the fact that the major basolateral membrane H^+/HCO_3^- transport mechanism in the proximal tubule is Na^+-coupled and rheogenic (carrying negative charge) (24–30). Removal of Na^+ from the luminal and capillary fluids inhibits basolateral membrane bicarbonate exit, which alkalinizes the cell (25, 26, 31), and can secondarily inhibit any apical membrane proton secretory mechanism. Inhibition of Na^+ transport by inhibition of the Na^+/K^+ ATPase will depolarize the cell, which has been shown (25) to decrease the rate of the basolateral exit step, and lead to a cell alkalinization. Indeed, Wang et al. (32) have confirmed that inhibition of the Na^+/K^+ ATPase with ouabain alkalinized the cell.

To more directly examine the role of the Na^+/H^+ antiporter in bicarbonate absorption, both Chan and Giebisch (21) and Howlin et al. (33) used luminal amiloride, and found less inhibition of bicarbonate absorption than would be predicted if Na^+/H^+ antiport were the sole apical membrane mechanism of proton secretion. Determination of the contribution of Na^+/H^+ exchange to bicarbonate absorption was complicated in these studies by a number of problems and concerns: (*a*) the possibility that amiloride was a less potent inhibitor of Na^+/H^+ exchange in the in vivo tubule than in vesicles; (*b*) the question of amiloride absorption along the length of the tubule; (*c*) the possibility that inhibition of Na^+/H^+ exchange acidified the cell and secondarily stimulated apical membrane proton secretion; (*d*) the effect of a change in the axial luminal bicarbonate concentration profile when bicarbonate absorption is inhibited; and (*e*) an inability to get higher, more potent concentrations of amiloride into physiological solutions, so that a larger percent of bicarbonate absorption could be manipulated. However, these studies raised the possibility that a sodium-independent mechanism of proton secretion existed in this epithelium.

In the present studies, we examined the effect of a more potent amiloride analog, 5-(*N*-*t*-butyl) amiloride (*t*-butylamiloride),[1] and a higher concentration of amiloride on volume, glucose, and bicarbonate absorption in the in vivo microperfused rat proximal convoluted tubule. Perfused and collected concentrations of amiloride and *t*-butylamiloride were measured. In addition, the effects of these agents on cell pH, and their inhibitory potency on apical membrane Na^+/H^+ exchange activity in vivo were determined using the fluorescent measurement of cell pH. The results demonstrate the most, but not all, of apical membrane proton secretion is mediated by Na^+/H^+ exchange, whereas the remaining fraction is mediated by a Na^+-independent, amiloride-insensitive process. Based on the findings by others of H^+-ATPase activity in brush border membrane vesicles and endosomes derived from the renal cortex (11–17), we suggest that this second mechanism is a H^+-ATPase.

Methods

General methods for microperfusion studies. Male Wistar rats (Charles River Breeding Laboratories, Wilmington, MA) weighing 200–300 g

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1. *Abbreviations used in this paper:* BCECF, (2',7')-bis(carboxyethyl)-(5,6)-carboxyfluorescein; DCCD, dicyclohexylcarbodiimide; SITS, 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonate; *t*-butylamiloride, 5-(*N*-*t*-butyl)amiloride.

were prepared for in vivo microperfusion as previously described (34). Briefly, the rats were anesthetized with an intraperitoneal injection of Inactin (100 mg/kg body wt with additional dosage if needed) and placed on a heated table that maintained body temperature at 36.5–37°C. The right femoral artery was catheterized for monitoring blood pressure (transducer, Gould Inc., Oxnard, CA, and monitor, Stentor Co., Kansas City, MO) and obtaining blood samples. The left jugular vein was catheterized for continuous infusion of Ringers' bicarbonate (in mM: NaCl, 105; $NaHCO_3$, 25; Na_2HPO_4, 4; KCl, 5; $MgSO_4$, 1; $CaCl_2$ 1.8) at 1.5 cc/h. The left kidney was exposed by a flank incision and immobilized in a Lucite cup. The ureter was cannulated (PE-50) to ensure the free drainage of urine. Proximal tubule transit time was measured from an intravenous injection of 0.02 ml of 10% lissamine green dye. Only those kidneys with transit times of < 12 s were accepted for study.

In vivo microperfusion technique for flux studies. Proximal convoluted tubules were perfused with a thermally insulated microperfusion pump (Wolfgang Hampel, Berlin, FRG) as previously described (34).The perfusion pipette was placed in a proximal loop. An oil block was placed proximal to the perfusion pipette, and a hole was left proximal to the block for glomerular ultrafiltrate to escape. A collection pipette was placed in a late proximal loop, an oil block inserted distally, and a timed collection made. Collected samples were stored under Hepes-equilibrated paraffin oil (35). After the collection the perfused segment was filled with Microfil (Canton Bio-Medical Products, Boulder, CO). On a subsequent day the kidney was incubated in 6 N hydrochloric acid at 37°C for 70 min, allowing dissection of the Microfil casts and measurement of the length of the perfused segment.

In vivo microperfusion technique for fluorescence measurements. Pipettes were placed using a dissecting microscope (E. Leitz, Inc., Rockleigh, NJ). Peritubular capillaries were perfused as previously described (25) with a 12–14 μm tip pipette designed to allow rapid changes between two perfusion fluids.

The lumen of a proximal convoluted tubule was then perfused at 40 nl/min for 5–7 min using a thermally insulated microperfusion pump with a solution containing the acetoxymethyl derivative of (2',7')-bis(carboxyethyl)-(5,6)-carboxyfluorescein (BCECF), as previously described (36). After 5–7 min, this pipette was removed and a second luminal pipette was placed in a more distal loop of the same nephron. This second pipette, which was similar to that used in the peritubular capillary except that it had a smaller tip (7–9 μm), perfused the proximal tubular lumen in a retrograde fashion as previously described (36, 37). This method of lumen and capillary perfusion has been demonstrated to give complete control of luminal and capillary fluids (25, 36).

After placement of the pipettes, the dissecting microscope was moved out of position, and an epifluorescence microscope (MPV Compact system, E. Leitz, Inc.) was moved into position. Fluorescence intensity was measured as previously described (25) and used to calculate cell pH.

General methods for vesicle studies. The activity of five amiloride analogs against the antiporter was measured in rabbit renal brush border vesicles using the acridine orange method (6). The brush border vesicles were prepared by Mg^{2+} aggregation as previously described (8). Vesicles (400 μg of protein) in pH 6.0 buffer (50 mM Mes/Tris, 250 mM sucrose, 150 mM K gluconate) were added to 2 ml of pH 7.5 assay solution (50 mM Mes/Tris, 250 mM sucrose, 150 mM K glconate, 6 μM acridine orange, 20 μg valinomycin) and assayed for Na^+/H^+ exchange in an SLM 8225 spectrofluorometer as previously described (8).

Amiloride hyrochloride dihydrate and its analogs 5-(*N*-*t*-butyl)-amiloride, 5-(*N*,*N*-dimethyl)amiloride, 5-(*N*-ethyl-*N*-propyl)amiloride and 5-(*N*-amino-*N*-methyl)amiloride were prepared for this study by the procedures described previously (38). Using similar methods, 5-(*N*-methyl-*N*-methallyl)amiloride was synthesized. This compound has a melting point of 206–207°C.

Perfusion solutions

In vivo microperfusion flux studies: rates of volume, bicarbonate, and glucose absorption. In these studies tubules were perfused with a plasma ultrafiltrate–like solution (Table I, luminal perfusion solution No. 1), except when 4.3 mM amiloride was added to the perfusate. Then it was necessary to remove sulfate and phosphate from the luminal perfusate to increase the solubility of amiloride (Table I, luminal perfusion solution No. 2). This substitution did not affect bicarbonate absorption when compared with tubules perfused without the substi-

Table I. Perfusion Solutions

	Luminal solutions						Capillary solutions		
	1	2	3	4	5	6	1	2	3
	mM	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*
NaCl	120	122	120	122	127	—	120	122	142
$NaHCO_3$	25	25	25	25	25	—	25	25	5
KCl	5	5	5	5	5	5	5	5	5
$MgSO_4$	1	—	1	—	—	—	1	—	0
$CaCl_2$	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8
Na_2HPO_4	1	—	1	—	—	—	1	—	0
Glucose	5	5	5	5	—	—	5	5	5
Alanine	5	5	5	5	—	—	5	5	5
Urea	5	5	5	5	5	5	5	5	5
$MgCl_2$	—	1	—	1	1	1	—	1	1
Choline Cl	—	—	—	—	—	127	—	—	—
Choline HCO_3	—	—	—	—	—	25	—	—	—
SITS	—	—	—	—	—		—	—	1
Amiloride	±0.9	±4.3	±0.9	±3.6	±3.6	±3.6	—	—	—
t-Butylamiloride	±1	—	—	±0.35	±0.35	±0.35	—	—	—

All solutions bubbled with 7% CO_2 and 93% O_2.

tutions (125±5 [n = 8] vs. 124±13 [n = 4] pmol/mm · min [$P > 0.5$]). In the t-butylamiloride studies, it was necessary to dissolve this inhibitor in dimethylsulfoxide (DMSO) before adding it to the perfusion solution. The final concentration of DMSO in the perfusate was 0.1%, which did not affect the rate of bicarbonate absorption when compared to that measured in the absence of DMSO (125±5 [n = 8] vs. 127±8 [n = 3] pmol/mm · min [$P > 0.5$]).

To determine the rate of glucose absorption, we added tracer amounts (0.0041 mmol) of [^{14}C]glucose (New England Nuclear, Boston, MA) to the above perfusates. In addition, the perfusates contained 0.05% FD and C green dye No. 3 (Warner Jenkinson, St. Louis, MO), to identify the perfused segment, and dialyzed [methoxy-^{3}H]inulin (New England Nuclear) as a volume marker. All solutions were bubbled with 7% CO_2 and 93% O_2.

In vivo microperfusion fluorescence studies: effect of inhibitor on cell pH. In these studies both the lumen and capillaries were perfused with plasma ultrafiltrate–like solutions (Table I, capillary perfusate No. 1 with luminal perfusate No. 3, or capillary perfusate No. 2 with luminal perfusate No. 4). The substitutions made in the second set of solutions were necessary to keep the higher concentrations of amiloride and t-butylamiloride in solution. All solutions were bubbled with 7% CO_2 and 93% O_2.

In vivo microperfusion fluorescence studies: in vivo rate of Na^+/H^+ exchange and amiloride and t-butylamiloride inhibition. Capillaries were perfused with the same plasma ultrafiltrate–like solution described above, except that 20 mM bicarbonate was replaced with 20 mM chloride, and 1 mM SITS was added (Table I, capillary perfusate No. 3). These substitutions/additions were made to allow changes in the luminal Na^+/H^+ antiporter rate to have a maximum effect on cell pH (36). The luminal perfusate was either No. 5 or No. 6. Again, all solutions were bubbled with 7% CO_2 and 93% O_2.

Analysis

In vivo microperfusion flux studies: volume measurements. The total collected volume was measured using calibrated constant-bore pipettes. From this volume an aliquot was removed for measurement of total CO_2 and inhibitor concentration. The remaining volume was placed in a vial containing a 1:4 mixture of acetic acid:Aquasol (New England Nuclear) for liquid scintillation counting (Tri-Carb 460 C, Packard Instrument Co., Inc., Downers Grove, IL). Because the solutions contained both [^{3}H]inulin and [^{14}C]glucose, appropriate corrections were made for cross-counting between the isotope windows.

Total CO_2 concentration. The total CO_2 concentration in both the perfusate and collected fluid was measured using microcalorimetry (39).

Inhibitor concentration in perfusate and collected fluid. The concentration of amiloride or t-butylamiloride in nanoliter samples of perfusate and collected fluid was measured by fluorescence intensity in an 8225 spectrofluorometer (SLM Instruments Co., Urbana, IL) using single photon counting. To increase the signal-to-noise ratio, all measurements were made in ethanol where amiloride and t-butylamiloride exhibit a 10-fold increase in fluorescence intensity. Measurements were made at λ_{ex} = 360, λ_{em} = 420 to avoid the Raman peaks of water (408 nm) and ethanol (400 nm) at λ_{ex} = 360. 12.5-nl samples of perfusate or collected fluid were added to 500 μl ethanol in 2-ml plastic tubes to facilitate mixing. The solution was then transferred to 500-μl quartz cuvettes (Uvonic Instruments, Plainview, NY). Excitation slits were 16 nm, emission 8 nm. On each day of measurements, standards were performed using 12.5-nl samples of reference solutions ranging in concentration from 0.05 to 1 mM t-butylamiloride (1 mM t-butylamiloride studies), 0.04 to 0.9 mM amiloride (0.9 mM amiloride studies), or 0.9 to 4.3 mM amiloride (4.3 mM amiloride studies). Over these ranges, fluorescence was a linear function of concentration for both compounds (r value ranged from 0.960 to 0.999 for all studies). The limit of detection under these conditions is $\sim 10^{-9}$ M, or in the undiluted tubular fluid samples, 50×10^{-6} M.

In vivo microperfusion fluorescence measurements. As described previously (25), fluorescence was measured alternately at 500 and 450 nm excitation (emission 530 nm) using an epifluorescence microscope with interference filters (Corion Corp., Holliston, MA). All results were corrected by subtracting background. The fluorescence excitation ratio (F_{500}/F_{450}) was calculated as the mean of the two 500-nm excitation measurements divided by the 450-nm excitation measuremnt. Use of the fluorescence excitation ratio provides a measurement unaffected by changes in dye concentration.[2] To convert fluorescent excitation ratios to an apparent cell pH value, we used results of our previously reported intracellular calibration (25).

Calculations

Volume measurements. The perfusion rate, V_p nl/min, was calculated as $V_p = (I_c/I_p)(V_c)$, where I_c and I_p are the collected fluid and perfusate inulin concentrations, respectively, and V_c is the collection rate.

Volume flux per tubular length, J_v nl/mm · min, was calculated as $J_v = (V_p - V_c)/L$, where L is the perfused length.

Total CO_2 flux measurements. The rate of bicarbonate absorption, J_{tCO_2} pmol/mm · min, was calculated as $J_{tCO_2} = [(C_pV_p) - (C_cV_c)]/L$, where C_p and C_c are the total CO_2 concentrations of the perfusate and collected fluid, respectively, as determined by microcalorimetry.

Glucose absorption. The rate of glucose absorption, J_{glu} pmol/mm · min, was calculated as $J_{glu} = 5\text{ mM}\ \{V_p - [V_c \times (G_c/G_p)]\}/L$, where G_c/G_p is the ratio of counts per minute of [^{14}C] in the collected fluid and perfusate, respectively, and 5 mM is the concentration of cold glucose in the perfusate.

Log mean inhibitor concentration. Log mean concentration of either t-butylamiloride or amiloride was calculated as: log mean concentration = $(A_p - A_c)/\ln(A_p/A_c)$, where A_p and A_c are the concentration of inhibitor in the perfusate and the mean concentration of inhibitor in the collected fluid, respectively.

Initial rate of cell pH change. In some fluorescence studies, the initial rate of cell pH change was calculated, as described previously (25). During a fluid change, fluorescence was followed with 500 nm excitation on a chart recorder. The slope of a line drawn tangent to the initial deflection [$d(F_{500})/dt$] defined the initial rate of change of 500 nm fluorescence. We have previously demonstrated that fluorescence with 450 nm excitation is not measurably affected by cell pH, and thus can be considered constant (25). The rate of change in the fluorescence excitation ratio [$(F_{500}/F_{450})/dt$] can then be calculated using the formula: $d(F_{500}/F_{450})/dt = [d(F_{500})/dt]/F_{450}$, where F_{450} represents the calculated 450 nm excitation fluorescence corrected for background at the time of the fluid change (interpolated from the measurements before and after the fluid change).

Statistics

The results are expressed as means±SE. In the flux studies statistical significance was assessed by the Student's t test using an analysis of covariance. In the in vivo fluorescence studies examining the effect of the inhibitors on cell pH, statistical significance was assessed by the Student's t test for paired data. In the fluorescence studies determining the potency of amiloride and t-butylamiloride in vivo, statistical significance was assessed by the Student's t test for unpaired data.

2. In these studies BCECF was excited alternately at 500 and 450 nm and emission was measured at 530 nm. These excitation wavelengths are a fair distance from the excitation wavelength of amiloride (360 nm), and thus it is unlikely that there is significant amiloride fluorescence. In addition two observations were made in the in vivo studies. One is that the lumen of the tubules do not visibly fluoresce when amiloride is added to the luminal perfusate, suggesting that little or no fluorescent light is being emitted from the amiloride. The second is that in the studies done to examine the effect of amiloride on cell pH, the addition of amiloride or t-butylamiloride to the perfusate did not change the fluorescent ratio or the intensity of 500 or 450 nm fluorescence, again suggesting that the inhibitors are not emitting any substantial amount of fluorescence.

Table II. Microperfusion Flux Data

	Controls (n = 15)	0.9 mM Amiloride (n = 7)	4.3 mM Amiloride (n = 7)	1 mM t-Butylamiloride (n = 10)
J_v (*nl/mm · min*)	2.13±0.2	2.02±0.4	1.16±0.2*	0.97±0.2§
J_{tCO_2} (*pmol/mm · min*)	125±4	90±14‡	68±8§	67±10§
J_{Glu} (*pmol/mm · min*)	30.7±1.3	29.0±2.5	26.5±2.1	20.9±1.8§
Perfusion rate (*nl/min*)	16.7±0.2	16.6±0.5	16.8±0.2	16.0±0.4
Length (*mm*)	1.87±0.1	2.32±0.2	1.94±0.2	1.99±0.3
Collected tCO_2 (*mM*)	12.5±0.8	15.6±1.6	17.1±1.4*	18.0±1.1§

* $P < 0.01$ control vs. inhibitor. ‡ $P < 0.005$ control vs. inhibitor. § $P < 0.001$ control vs. inhibitor.

Results

In vivo microperfusion flux studies. In the initial set of studies tubules were perfused in vivo with a plasma ultrafiltrate–like solution containing 25 mM bicarbonate (Table I, luminal perfusate No. 1). Under these conditions J_v was 2.13±0.2 nl/mm · min, J_{tCO_2} was 125±4 pmol/mm · min, and J_{glu} was 30.7±1.3 pmol/mm · min (Table II). When 0.9 mM amiloride was added to the perfusate J_v and J_{tCO_2} were inhibited by 5.2% ($P > 0.1$) and 28.7% ($P < 0.005$), respectively, and J_{glu} was unaffected (30.7±1.3 vs. 29.0±2.5 pmol/mm · min, $P > 0.5$). This degree of inhibition is similar to that observed by Howlin et al. (33). Because of the small degree of inhibition of bicarbonate absorption we next examined the effect of higher (4.3 mM) amiloride concentrations (Table I, luminal perfusate No. 2). When compared with control tubules, J_v was inhibited 45.5% ($P < 0.01$) and J_{tCO_2} was inhibited 45.9% ($P < 0.001$) (Table II). Again, J_{glu} was not affected ($P > 0.1$). This degree of inhibition of bicarbonate absorption was less than that predicted by kinetics from vesicle studies assuming that all of apical membrane proton secretion is effected by Na^+/H^+ antiport.

Brush border membrane vesicle studies: amiloride analogs. To find a more potent inhibitor of Na^+/H^+ exchange, we screened a group of analogues of amiloride in which the protons of the 5-amino group were replaced by one or two alkyl groups. To facilitate the screening process, we measured activity against Na^+/H^+ exchange in isolated rabbit renal brush border membrane vesicles. Inhibitory potency of amiloride and its 5-(*N,N*-dimethyl)- (1), 5-(*N*-ethyl-*N*-propyl)- (2), 5-(*N*-methyl-*N*-methallyl)- (3), 5-(*N*-amino-*N*-methyl)- (4), and 5-(*N*-*t*-butyl)- (5) derivatives were examined. Compounds 2, 3, and 5 all had affinity constant for inhibitor (K_i) = 2–5 μM against the renal Na^+/H^+ antiporter, whereas compounds 1 and 4 were only about twice as potent as amiloride (K_i = 25 μM [8]). Unfortunately, compounds 2 and 3 were only slightly soluble in aqueous solutions and could not be used for in vivo experiments at high concentrations. Thus, among the compounds examined, only t-butylamiloride exhibited the combination of high aqueous solubility and potency against the Na^+/H^+ antiporter.

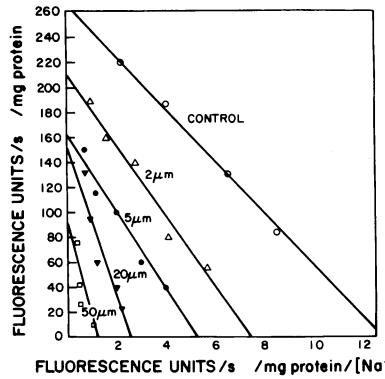

Figure 1. Inhibition of Na^+/H^+ exchange by t-butylamiloride. Concentration-response curve for Na^+ (10–200 mM) was obtained at each of the indicated concentrations of t-butylamiloride. Data plotted in Eadie-Hofstee form as mean±SE for n = 3.

Before perfusing tubules with t-butylamiloride, we examined its potency against the Na^+/H^+ antiporter in more detail (Fig. 1). These experiments showed that t-butylamiloride is a mixed inhibitor of Na^+/H^+ exchange as described for amiloride (8) with a K_i = 2.5 μM. To show that t-butylamiloride could inhibit Na^+/H^+ exchange completely under physiological conditions, we measured Na^+/H^+ exchange with 200 mM Na^+ and 200 μM t-butylamiloride. Na^+/H^+ exchange was inhibited at 96±3% (n = 4) under these conditions.

In vivo microperfusion flux studies: 1 mM t-butylamiloride. Next we examined the effect of t-butylamiloride on bicarbonate absorption. When 1 mM of inhibitor was added to the perfusate (Table I, luminal perfusate No. 1), J_v was inhibited 54.5% ($P < 0.001$) and J_{tCO_2} was inhibited 46.9% ($P < 0.001$) (Table II). In these studies, however, J_{glu} was inhibited significantly when compared with control values,[3] (30.7±1.3 vs. 20.9±1.8 pmol/mm · min, $P < 0.001$). Once again, J_{tCO_2} was inhibited less than would be expected if the Na^+/H^+ antiporter mediated all of apical membrane proton secretion. Possible causes for this discrepancy in all series include loss of inhibitor from the luminal fluid, resistance of the antiporter to the inhibitors in vivo, and secondary effects of the inhibitors on luminal and cell pH.

Presence of inhibitor along the perfused segment. To ensure that the inhibitor was present along the entire perfused segment, the concentrations of amiloride and t-butylamiloride in the perfusate and collected fluid were measured by fluorescence intensity (see Methods). As can be seen in Table III, both

3. An effect on glucose transport was only observed in the t-butylamiloride studies. We feel it likely that this is a direct effect on the Na/glucose transporter, as amiloride analogues have been shown to have a higher affinity for the transporter than does amiloride (40). However, if t-butylamiloride inhibited the Na^+/K^+-ATPase, this would further inhibit bicarbonate absorption (18–23) and overestimate the role of Na^+/H^+ antiporter.

Table III. Concentration of Inhibitor in Perfusate and Collected Fluid

	Measured concentration			Log mean concentration*	Inhibitor permeability
	Perfusate	Collected fluid	Mean collected		
	mM	*mM*	*mM*	*mM*	*cm/s*
0.9 mM amiloride	0.8±0.0	0.4–0.8	0.5±0.1	0.7	11.0
4.3 mM amiloride	4.0±0.1	2.9–3.6	3.2±0.1	3.6	6.1
1 mM *t*-butylamiloride	0.99±0.0	0.02–0.18	0.08±0.04	0.35	43.5

* Calculated from the equation: log mean concentration = $(A_p - A_c)/\ln (A_p/A_c)$.

amiloride and *t*-butylamiloride were absorbed from the luminal fluid, *t*-butylamiloride to a much greater extent, consistent with its more hydrophobic nature. Loss of inhibitor could possibly explain some of the difference between the observed inhibition of J_{fCO_2} and that predicted from vesicle studies if the Na^+/H^+ antiporter mediated all of apical membrane proton secretion. We corrected for the loss of inhibitor by using a model of proximal bicarbonate absorption.

In vivo rate of Na^+/H^+ exchange activity and inhibition by amiloride and t-butylamiloride. Previous studies had left open the possibilities that in vivo the rat epithelium was less sensitive to amiloride than in the vesicle studies or that amiloride was not gaining access to the antiporter, and thereafter that use of these compounds underestimates the role of Na^+/H^+ exchange in bicarbonate absorption (33). To rule out these possibilities, we examined the rate of Na^+/H^+ exchange activity in the presence and absence of the inhibitors in vivo. The protocol for these studies was similar to that previously described by us (36). The peritubular capillaries were perfused with a solution containing 5 mM bicarbonate and 1 mM SITS (Table I, capillary perfusate No. 3). We have previously shown that with this capillary perfusate the basolateral $Na^+/(HCO_3^-)_{n>1}$ transporter is inhibited, but that cell pH is not alkalinized enough to suppress the luminal membrane Na^+/H^+ antiporter (36). This maneuver, then, allows changes in the rate of the luminal membrane Na^+/H^+ antiporter to have the greatest effect on cell pH (36).

The luminal perfusate was changed between one containing 152 mM Na^+ and one with zero Na^+ (Table I, luminal perfusates No. 5 and No. 6). Mean luminal inhibitor concentrations from the flux studies were used. In the control condition when the luminal Na^+ concentration was decreased from 152 to 0 mM, cell pH decreased at a rate of 2.51±0.35 pH units/min (Table IV). When luminal Na^+ was returned to 152 mM, cell pH increased at a rate of 1.78±0.34 pH units/min. When 3.6 mM amiloride was added to the luminal perfusate, the rate of change in cell pH, upon luminal Na^+ removal and replacement, was reduced 87 and 91%, respectively (Table IV).

In similar studies with *t*-butylamiloride (luminal perfusates No. 5 and No. 6, and capillary perfusate No. 3) (Table I), 0.35 mM inhibitor reduced the rate of cell pH change by 86 and 89%, respectively (Table IV). A typical tracing is shown in Fig. 2. In general, these studies demonstrate that the Na^+/H^+ antiporter is very sensitive to amiloride and its analogue in vivo.

Effect of amiloride and t-butylamiloride on intracellular pH. An effect of the inhibitors on cell pH would affect the interpretation of the observed inhibition of bicarbonate absorption. To rule out this possibility, the effect of amiloride and *t*-butylamiloride on cell pH was determined in vivo. Peritubular capillaries were perfused with a plasma ultrafiltrate–like solution (Table I, capillary perfusate No. 1 or No. 2). The tubular lumens were perfused with a plasma ultrafiltrate–like solution with sulfate and phosphate removed to increase inhibitor solubility (Table I, luminal perfusates No. 3 or No. 4). The inhibitor was added to the luminal fluid only, at amiloride concentrations of 0.9 or 3.6 mM, or a *t*-butylamiloride concentration of 0.35 mM. As can be seen in Table V, cell pH was not affected significantly in any of the studies, suggesting that

Table IV. Potency of Inhibitors In Vivo

Luminal [Na] changes	Rate of change of cell pH	Percent inhibition*
	pH units/min	
Control (n = 8)		
Na^+ 152 to 0	2.51±0.35	
Na^+ 0 to 152	1.78±0.34	
3.6 mM amiloride (n = 7)		
Na^+ 152 to 0	0.33±0.06	87
Na^+ 0 to 152	0.16±0.07	91
0.35 mM *t*-butylamiloride (n = 6)		
Na^+ 152 to 0	0.36±0.05	86
Na^+ 0 to 152	0.19±0.03	89

* Calculated as inhibited/control rate of change in cell pH.

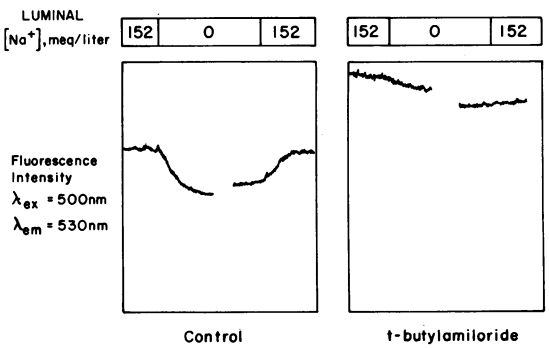

Figure 2. Inhibition of Na^+/H^+ exchange by *t*-butylamiloride in vivo: typical tracing. Fluorescence intensity with 500 nm excitation (pH-sensitive wavelength) as a function of time during luminal Na^+ removal and readdition.

Table V. Effect of Inhibitor on Cell pH

	Cell pH		
	−inhibitor*	+inhibitor	*P* value
0.9 mM amiloride (n = 7)	7.28±0.02	7.28±0.02	>0.5
3.6 mM amiloride (n = 7)	7.28±0.06	7.28±0.06	>0.2
0.35 mM t-butylamiloride (n = 7)	7.23±0.04	7.21±0.04	>0.2

* These values are the means of the pre- and post-control values.

the inhibition of bicarbonate absorption that was observed was not secondarily altered by changes in cell pH. (In these studies there was a progressive cell alkalinization that has been observed by us previously (36). To determine the effect of the inhibitors on cell pH, we meaned the pre- and post-control values for comparison with the experimental period during which the inhibitor was present.) This finding is somewhat expected as we have previously shown that the basolateral membrane $Na^+/(HCO_3^-)_{>1}$ transporter in the major determinant of cell pH under physiological conditions (36).

Role of Na^+/H^+ exchange in bicarbonate absorption. In the microperfusion flux studies, 4.3 mM amiloride and 1 mM *t*-butylamiloride only inhibited J_{tCO_2} 46 and 47%, respectively. However, inhibition of the Na^+/H^+ antiporter did increase the pH and bicarbonate concentration of the collected fluid (Table II). This increase in the luminal bicarbonate axial concentration profile would be expected to stimulate bicarbonate absorption (35). To correct the rates of Na^+/H^+ exchange activity for the varying bicarbonate concentration profiles, we used a modification of our previously published model of bicarbonate absorption in the proximal tubule (41). The details of the model calculations are presented in the Appendix. In addition to correcting for changes in the bicarbonate concentration profile, the model also adjusted the magnitude of bicarbonate inhibiton for the varying inhibitor concentration profile. The results of the model are presented in Table VI. The observed inhibition in column one is the data from reference 33 and Table II. The predicted inhibition of bicarbonate absorption (second column) is calculated from the model determined inhibition of J_{tCO_2} assuming that all of J_{tCO_2} is mediated by the antiporter. This rate of J_{tCO_2} has been adjusted for both changes in the axial bicarbonate concentration profile and for the loss of inhibitor along the perfused segment. The contribution of Na^+/H^+ exchange to J_{tCO_2} can be determined from the ratio of these two values (third column). As shown, 64.1–86.4% of bicarbonate absorption was mediated by the antiporter. The 65% Na^+/H^+ antiporter contribution calculated with 4.3 mM amiloride and 1 mM *t*-butylamiloride are the more accurate estimates.

Table VI. Contribution of Na^+/H^+ Exchange to J_{tCO_2}

	Observed inhibition of J_{tCO_2}	Predicted inhibition of Na^+/H^+*	Conribution of Na^+/H^+ exchange to J_{tCO_2}‡
	(%)	(%)	(%)
0.9 mM amiloride	21.4§–28.7‖	31.3–33.2	68.4–86.4
4.3 mM amiloride	45.9	68.1	67.4
1.0 mM *t*-butylamiloride	46.9	73.2	64.1

* Calculated from model presented in Appendix. Corrects for difference in axial luminal bicarbonate concentration profile between the studies, and for the varying inhibitor concentration along the perfused segment.

‡ Calculated as the observed inhibition/predicted inhibition. See text for further description.

§ Data are from reference 33.

‖ Data are from Table II.

Discussion

To examine the role of the apical membrane Na^+/H^+ antiporter in bicarbonate absorption, we perfused proximal convoluted tubules in vivo with 4.3 mM amiloride and a more potent amiloride analogue, *t*-butylamiloride. In the search for an amiloride analogue that could be used to achieve greater inhibition of Na^+/H^+ exchange in vivo, we had two major problems. First, because the more potent amiloride analogues have hydrophobic groups on the 5-amino group, they have greater lipid/water partition coefficients and decreased aqueous solubility when compared with amiloride. Second, although *t*-butylamiloride was more soluble than some analogues, it exhibited significant uptake into tissues and left the luminal compartment. However, even at the measured mean luminal concentration, Na^+/H^+ exchange activity was inhibited as predicted. While it is conceivable that bulky hydrophilic groups on the 5-amino nitrogen atom could overcome these difficulties, the compound of this class which we have examined (5-[*N*-amino-*N*-methyl]amiloride) is not much more potent than amiloride against the Na^+/H^+ exchanger.

As shown in Table II, 0.9 mM amiloride, 4.3 mM amiloride, and 1 mM *t*-butylamiloride added to the luminal fluid inhibited J_{tCO_2} by 28.7, 45.9, and 46.9%, respectively. Before utilizing these data to determine the contribution of Na^+/H^+ exchange to bicarbonate absorption, we addressed four potential complications: (*a*) the possibility that the inhibitors leave the luminal compartment and therefore are not present along the entire length of the perfused segment, (*b*) the possibility that the inhibitors are not as potent against the antiporter in vivo as in vesicle preparations, (*c*) the possibility that the presence of the inhibitors changed cell pH, which secondarily affected bicarbonate absorption, and (*d*) the effect of changes in the axial luminal bicarbonate concentration profile when varying amounts of bicarbonate absorption have been inhibited.

Loss of inhibitor along the perfused segment. As shown in Table III, the proximal tubule is permeable to both amiloride and *t*-butylamiloride, more so to *t*-butylamiloride as is expected because of its more hydrophobic nature. To calculate a permeability for these inhibitors, a modification of our previously reported proximal tubule model was used (41) (see Appendix). Permeabilities were calculated iteratively and are shown in Table III. The apparent decrease in amiloride permeability at higher concentrations suggests the possibility that amiloride absorption is carrier-mediated and saturable. While an organic cation carrier system has been identified in renal cortical brush border membrane vesicles (42), further studies

are required to define the mechanisms of inhibitor efflux in vivo.

Potency of inhibitors in vivo. The potency of amiloride and *t*-butylamiloride was examined by comparing the ability of the inhibitors to reduce antiporter activity in vivo to that observed in vesicle studies. These studies examined the effect of luminal Na^+ removal on cell pH. As shown in Table IV, the rate of change in cell pH was reduced by 89% when 3.6 mM amiloride was added to the luminal fluid, and by 87.5% when 0.35 mM *t*-butylamiloride was added.

For amiloride, rat vesicle data have found a K_{Na} of 8.3 mM (43) and a K_i of 48 μm (44) for the Na^+/H^+ anitporter. Under the conditions of our amiloride studies, (Na^+ concentration of 152 mM and amiloride concentration of 3.6 mM), it can be calculated that 80% of antiporter activity should be inhibited. In our studies, the addition of 3.6 mM amiloride to the luminal perfusate reduced the effect of changing luminal Na^+ concentration by 89%, a value similar to 80%. For *t*-butylamiloride the K_{Na} in vesicles is 15 mM (rabbit brush border) and the K_i is 2.5 μM. Under the conditions of the in vivo studies (Na^+ concentration of 152 mM and an inhibitor concentration of 0.35 mM), it is predicted that 92.6% of antiporter activity would be inhibited. The same calculation using a K_{Na} of 8.3 mM (rat brush border vesicle studies [43]), would estimate that 87.9% of Na^+/H^+ activity would be inhibited. These numbers are similar to our results in vivo, in which 87.5% of antiporter activity was inhibited.

These studies suggest that the failure to obtain more complete inhibition of bicarbonate absorption in the flux studies cannot be attributed to decreased sensitivity of the epithelium to the inhibitors, or to an inability of the inhibitors to gain access to the antiporter. These studies also demonstrate that in this setting all of the effect of luminal Na^+ on cell pH is due to amiloride-sensitive Na^+/H^+ antiporter activity. This study rules out the presence of amiloride-insensitive Na^+/H^+ antiport in this preparation. If amiloride-insensitive Na^+/H^+ antiporter activity were present, then the effect of a luminal Na^+ concentration change on cell pH would not have been blocked as predicted in the presence of the inhibitors. Nakhoul and Boron (45) and Siebens and Boron (46) have recently reported an important role for amiloride-insensitive Na^+/acetate and Na^+/lactate cotransport in cell pH regulation in the proximal tubule. However, neither acetate nor lactate were present in our solutions.

Effect of inhibitors on cell and luminal pH. As shown in Table V, inhibition of the Na^+/H^+ antiporter by amiloride or *t*-butylamiloride did not affect cell pH in situations where the basolateral mechanism for bicarbonate exit is operational. As mentioned, this finding is somewhat expected as we have previously shown that the basolateral membrane $Na^+/(HCO_3^-)_{>1}$ transporter is the major determinant of cell pH (36).

As described in the Appendix, we accounted for changes in the axial luminal bicarbonate concentration profile by modifying a previous model. The results, as presented in Table VI, show that ~ 65% of bicarbonate absorption is mediated by the amiloride-sensitive Na^+/H^+ antiporter.

Comparison with previous studies. As mentioned above, previous in vivo and in vitro microperfusion studies have consistently found that 80–100% of bicarbonate absorption is inhibited by luminal and peritubular Na^+ removal (18–23). While these studies were initially interpreted to imply that 80–100% of apical membrane proton secretion is mediated by Na^+/H^+ exchange, this interpretation has recently been complicated by the demonstration of a Na^+-coupled basolateral membrane bicarbonate exit step (24–30). Based on the present results, the observed 80–100% inhibition of J_{tCO_2} in previous studies can be explained by a 65% inhibition of apical membrane proton secretion (Na^+/H^+ antiporter) and > 90% inhibition of basolateral membrane bicarbonate exit ($Na^+/(HCO_3^-)_{>1}$ symporter).

Microperfusion studies have also addressed the role of the apical membrane Na^+/H^+ antiporter by examining the effect of Na^+/K^+-ATPase inhibition on J_{tCO_2}. The observed inhibition, which has varied from 0 to 100% (18–23, 47), also cannot be specifically attributed to effects on an apical membrane Na^+/H^+ antiporter.

If a luminal membrane Na^+-independent, amiloride-insensitive mechanism of proton secretion exists, as suggested by the present studies, then one likely possibility is an H^+-ATPase. Evidence for such an ATPase has been found in brush border membrane vesicles and endosomes derived from the renal cortex (11–17). Also in support of an apical membrane H^+-ATPase in the rat is the finding by Fromter and Gessner of an acetazolamide-inhibitable lumen-positive potential difference (37). A lumen-positive potential difference, however, has not been observed in the rabbit proximal tubule (48–50).

Schwartz and Al-Awqati (51) have found further evidence for an apical membrane H^+-ATPase in the in vitro perfused proximal straight tubule. In their studies, CO_2 stimulated fusion of H^+-ATPase-containing intracellular vesicles with the luminal membrane, suggesting the possibility of a regulatory step for participation of these pumps in bicarbonate absorption in the proximal tubule. A role for these proton pumps in transepithelial transport is supported by the finding that dicyclohexylcarbodiimide (DCCD) inhibits bicarbonate absorption (52); however, the nonspecific effects of DCCD are of concern.

In summary, the addition of high concentrations of amiloride or more potent amiloride analogues to the luminal perfusate inhibited bicarbonate absorption by only 46% in the in vivo microperfused rat proximal convoluted tubule. After ruling out secondary effects on cell and luminal pH, the possibility that the epithelium is insensitive to the inhibitors in vivo, and the effects of varying inhibitor concentration profiles, we conclude that two mechanisms of proton secretion exist in the apical membrane and contribute to transepithelial bicarbonate absorption. The amiloride-sensitive Na^+/H^+ antiporter is probably the major mechanism. An additional mechanism is Na^+-independent and amiloride-insensitive. Based on other studies (11–17, 51, 52) the most likely mechanism for this proton secretion is a H^+-ATPase.

Appendix

The model used to calculate the rate of bicarbonate absorption per millimeter perfused tubule is a modification of our previously published model (41). Briefly, in the model the rates of bicarbonate transport were integrated along the length of the perfused segment using Euler's method. The proximal tubule was considered to be a cylinder of length equal to the mean length of perfused tubule (2 mm), and radius of 12 μm. The tubule was divided along its length into a number of small discs of 0.01 mm length. The rate of transcellular proton secretion within each integration interval (disc) was calculated from the effects of known determinants of proton secretion in this segment, e.g., luminal [HCO_3^-], peritubular [HCO_3^-], luminal flow rate, and rate

of volume absorption. The rate of transcellular proton secretion was assumed to be totally mediated by the apical membrane Na^+/H^+ antiporter. After calculating the antiporter rate (J_p), as a function of the above determinants, it was modified for the effect of the inhibitor using the following equation:

$$J_P^{INH} = \left[\frac{[Na^+]_x + K_{Na^+}}{[Na^+]_x + \{K_{Na^+} \cdot [1 + ([I]_x/K_i)]\}}\right] \cdot J_P \quad \text{(A1)}$$

where $[Na^+]_x$ is the luminal Na^+ concentration, $[I]_x$ is the inhibitor concentration entering the integration interval, K_{Na} is the affinity of the antiporter for Na (8.3 mM), and K_i is the affinity for the inhibitor (48 μM for amiloride and 2.5 μM for *t*-butylamiloride).[4] The use of this equation to determine the rate of the Na^+/H^+ anitporter assumes that there is only competitive inhibition of the antiporter by either inhibitor. If noncompetitive inhibition of the antiporter is also involved, then the model will underestimate the predicted inhibition of J_{fCO_2} and therefore overestimate the role of the Na^+/H^+ antiporter in mediating transepithelial bicarbonate absorption.

The initial boundary condition(s) for flow rate was the perfusion rate, and for luminal [HCO_3^-], [Na^+], and [inhibitor] were the perfusate concentrations of these substances. Peritubular HCO_3^- was the measured plasma bicarbonate concentration. After calculation of the rate of transcellular proton secretion, the rate of net bicarbonate absorption was calculated by correcting for paracellular HCO_3^- leak as previously described (41).

Because we have shown that both amiloride and *t*-butylamiloride are transported out of the luminal compartment, the rates of Na^+/H^+ exchange were corrected for the concentration profile of the inhibitor. To do this, we assumed that the inhibitors were absorbed from the tubule by a first-order process described by the equation:[5]

$$J_{INH} = P_{INH} \cdot [I]_x \quad \text{(A2)}$$

where J_{INH} is the rate of inhibitor loss from the luminal compartment and P_{INH} is the inhibitor permeability. P_{INH} was calculated by an iterative method using the measured perfused and collected fluid inhibitor concentrations. The calculated permeability was then used in the model along with Eq. A2 to determine the rate of inhibitor efflux. The concentration of inhibitor along the tubule was then determined using the mass balance equation:

$$[I]_{x+\Delta x} = \frac{V_x[I]_x - J_{INH}\Delta x}{V_{x+\Delta x}} \quad \text{(A3)}$$

where $[I]_{x+\Delta x}$ is the inhibitor concentration leaving the integration interval, $[I]_x$ is the inhibitor concentration entering the integration interval, Δx is the size of the integration interval, V_x is the luminal flow rate entering the integration interval, and $V_{x+\Delta x}$ is the luminal flow rate leaving the integration interval.

4. To justify the use of the kinetic constants defined from vesicle studies we tested the inhibitor potencies in vivo (see Table IV). While these in vivo studies were not sufficient to define a unique K_{Na} and K_i, they did confirm the abilities of the kinetic constants to predict the magnitude of inhibition at the concentration of Na and inhibitor used, which were similar to those used in the flux studies.

5. In these studies we assumed first-order kinetics for inhibitor efflux. The worst possible situation would be that the inhibitors left the luminal compartment at the beginning of the perfused segment and that the collected concentratioin was present along the entire perfused segment. Calculation of the predicted inhibition of the Na^+/H^+ antiporter under these conditions does not change the conclusion. In the 4.3 mM amiloride studies, the Na^+/H^+ antiporter would be inhibited 81.7% in the presence of 4.0 mM amiloride (measured perfusate concentration) and 78.1% in the presence of 3.2 mM amiloride (mean collected concentration). In the 1 mM *t*-butylamiloride studies, the Na^+/H^+ antiporter would be inhibited 95.5% in the presence of 0.99 mM *t*-butylamiloride (measured perfusate concentration) and 63.1% in the presence of 0.08 mM *t*-butylamiloride (mean collected concentration). Both 78.1 and 63% inhibition are still greater than the 46% inhibition of J_{fCO_2} that was observed in these two series.

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