Bicarbonate Absorption Stimulates Active Calcium Absorption in the Rat Proximal Tubule

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Abstract

To evaluate the effect of luminal bicarbonate on calcium reabsorption, rat proximal tubules were perfused in vivo. Perfusion solution contained mannitol to reduce water flux to zero. Total Ca concentration was measured by atomic absorption spectrometry, Ca ion concentration in the tubule lumen (Ca_L^{2+}) and the peritubular capillary (Ca_P^{2+}), and luminal pH (pH_L) with ion-selective microelectrodes and transepithelial voltage (V_{TE}) with conventional microelectrodes.

When tubules were perfused with buffer-free Cl-containing solution, net Ca absorption (J_{Ca}) averaged 3.33 pmol/min. Even though V_{TE} was 1.64 mV lumen-positive, Ca_L^{2+}, 1.05 mM, did not fall below the concentration in the capillary blood, 1.07 mM. When 27 mM of Cl was replaced with HCO_3, there was luminal fluid acidification. Despite a decrease in V_{TE} and Ca_L^{2+}, J_{Ca} increased to 7.13 pmol/min, indicating that the enhanced J_{Ca} could not be accounted for by the reduced electrochemical gradient, $\Delta\tilde{C}_{Ca}$. When acetazolamide or an analogue of amiloride was added to the HCO_3 solution, J_{Ca} was not different from the buffer-free solution, suggesting that HCO_3-stimulated J_{Ca} may be linked to acidification. To further test this hypothesis, we used 27 mM Hepes as the luminal buffer. With Hepes there was luminal fluid acidification and J_{Ca} was not different from the buffer-free solution but $\Delta\tilde{C}_{Ca}$ was significantly reduced, indicating enhanced active calcium transport.

We conclude from the results of the present study that HCO_3 stimulates active Ca absorption, a process that may be linked to acidification-mediated HCO_3 absorption.

Introduction

Bicarbonate administration is known to decrease urinary calcium excretion in acidotic (1–3) and nonacidotic states (4). Sutton et al. (2) showed that infusion of HCO_3 to dogs did not enhance proximal Ca reabsorption and concluded that the decreased urinary Ca excretion was the result of a direct HCO_3 effect to increase Ca absorption beyond the late proximal tubule. In that study HCO_3 administration was associated with decreased plasma ultrafilterable Ca and decreased proximal fluid reabsorption. Both of these effects would alter proximal Ca transport and could mask any direct effects of luminal HCO_3.

In the rat proximal tubule, Ullrich et al. (5) also did not find a direct effect of HCO_3 on active Ca transport. However, in that study direct measurements of transepithelial voltage (V_{TE}) and calcium ion concentration in the lumen and capillary blood were not made, rendering the interpretation of the data difficult. With the recent introduction of Ca^{2+} ion selective microelectrodes direct in situ measurements are now possible (6).

The present study was designed to reexamine the effects of luminal HCO_3 on proximal Ca transport, while keeping transepithelial water flow near zero and accounting for changes in the transepithelial electrochemical driving forces by in situ measurements of transepithelial voltage and calcium ion concentration. The results show that, in the absence of net transepithelial water flow, luminal HCO_3 stimulates calcium absorption by an active transport process that appears to be linked to tubule fluid acidification.

Methods

Male Sprague-Dawley rats (Bantin & Kingman, Freemont, CA) weighing 250–300 g were anesthetized by intraperitoneal injection of 5-sec-butyl-2-ethyl thiobarbituric acid (Inactin, Byk-Gulden, Konstanz, Federal Republic of Germany) (100 mg/kg body weight) and were prepared for in vivo micropuncture and microperfusion as previously described (6, 7). A continuous intravenous infusion of solution containing 140 mM NaCl and 4.0 mM KCl at a rate of 0.1 ml/min per kg body weight was administered and arterial blood pressure was monitored throughout the experiment. The surface of the kidney was bathed with solution that contained (in mM) Na 140, Cl 117, HCO_3 30, K 4.0, and Ca 1.5 and was warmed to 37°C.

Calcium transport. Composition of perfusion fluids is given in Table I. To identify proximal tubules having four or more surface segments, a solution containing 0.5 g/liter Brilliant Blue FCF dye (Pfaltz & Bauer, Inc., Stamford, CT) was injected into a tubule segment by means of a micropipette (3–5 μm o.d.) and was observed as it flowed downstream. A microperfusion pipette (5–7 μm) was attached to a pump (K. Effenberger, Munich, FRG) and was inserted into the second most proximal tubule identified. The perfusion rate was set at 18 nl/min. The most proximal surface segment was filled with bone wax injected from a micropipette (10–12 μm o.d.) attached to a hydraulic microdrive (model 3-0590, Trent Wells, South Gate, CA). Enough pressure was applied to inject a wax column up to the perfusion site but not beyond it. The wax pipette was then withdrawn, permitting the glomerular filtrate to escape through the hole in the tubule wall proximal to the wax block. Contamination of perfusion fluid by glomerular filtrate was thus avoided. After 1–2 min a micropipette (8–10 μ o.d.) containing colored (Sudan black) mineral oil was inserted into one of the visible segments (three to four segments distal to the perfusion site). Oil was injected at this site and then held in this place distal to the puncture by collecting all luminal fluid for 5–6 min. At the end of the collection period the collection pipette was quickly removed from the tubule, and a small amount of oil was drawn into the pipette to prevent evaporation of the collected fluid. The volume of each collected fluid sample was measured using a precalibrated constant-bore glass tubing (0.1 mm i.d.). A measured volume of the sample was then transferred for [^{14}C]inulin radioactivity measurements. After the collection was completed, the perfusion pipette was removed and a latex compound (General Biological, Chicago, IL) was injected

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Table I. Composition of Perfusion Solutions

Solute	Solution I	II	III	IV	V
			mM		
NaCl	142	115	115	115	115
KCl	4.0	4.0	4.0	4.0	4.0
$CaCl_2$	1.5	1.5	1.5	1.5	1.5
$MgCl_2$	1.0	1.0	1.0	1.0	1.0
Na_2HPO_4	1.0	1.0	1.0	1.0	1.0
$NaHCO_3$		27	27	27	
Na Hepes					27
ATZ			1.0		
MIA				0.01	
Mannitol	30	26	32	26	28

All solutions contained 0.5 g/liter brilliant blue FCF dye and 10 μCi/ml [^{14}C]inulin.

into the tubule from the collection site. When all collections were completed the kidney was removed and digested in 6 M HCl at 25°C for 24 h. The latex casts of the tubules were teased free from the unfilled tubules and the lengths of the perfused segments were measured.

The in vivo perfusion rate averaged 18.0±0.3 nl/min (n = 150) and was determined in each tubule from the relation $V_o = V_L[In]_L/[In]_o$, where V_L is the collection rate of tubule fluid at the end the perfused segment, and $[In]$ is the [^{14}C]inulin radioactivity in the collected (L) and the initial (0) perfusion fluid. The rate of water transport (J_v) was calculated as the difference between V_o and V_L. Sufficient concentration of mannitol was added to each solution to reduce J_v near zero (6–8). Net transport of calcium (J_{Ca}) or sodium (J_{Na}) was calculated as $J_i = V_o[i]_o - V_L[i]_L$, where $[i]_o$ and $[i]_L$ are the total calcium or sodium concentration in the perfusion and collection fluids. Total calcium and sodium concentration in perfusion and collection fluids were measured by flameless atomic absorption spectrometry (model 655/951, Instrumentation Laboratory, Inc., Lexington, MA) (6, 7). Perfusion and collection fluids were diluted 1:2,000 with deionized water in 1.5-ml Eppendorf tubes, and 10 μl of the diluted samples (Eppendorf ultramicropipettor) was used to measure total calcium and 1 μl to measure total sodium concentration.

Voltage, calcium activity, and pH measurements. Measurements of V_{TE}, aCa_L, and pH_L were made in a different group of animals from the group used for ion transport studies. A modification of the perfusion technique used for ion transport experiments was used in which two perfusion pipettes were positioned in the same tubule segment. One pipette had a solution containing 140 mM NaCl and 4.0 mM KCl and contained no dye. The second pipette contained one of the perfusion solutions in Table I. When fluid was being pumped from the second pipette, the perfusion segments appeared green; when fluid was being pumped from the first pipette, the green fluid was cleared from the tubule. The transepithelial voltage (V_{TE}), luminal calcium activity (aCa_L), and pH (pH_L) were measured by means of microelectrodes placed in the last visible segment of the perfused tubule (three to four surface segments distal to the pump). The intraluminal position of the microelectrodes was assured by switching from one solution to the other and observing the colored fluid appear in or wash out of the tubule at the same time that a change was observed in the microelectrode voltage reading. In most of the experiments, measurements of V_{TE} and aCa_L were taken simultaneously in the same tubule segment. pH measurements were made in a different group of animals without measuring V_{TE}.

The microelectrodes used for transepithelial voltage (V_{TE}) measurements had beveled tips (3–5 μm o.d.) and were filled with 3 M KCl in 2% agar. These electrodes have relatively low resistances (< 1 MΩ) and, with the perfusates used, have negligible tip potentials (8). The electrode was connected to one of the two channels of a high-impedance electrometer (model FD-223, W-P Instruments, New Haven, CT) via a Ag-AgCl wire. The reference side of the circuit was connected through a polyethylene tube filled with 3 M KCl in 2% agar and placed in calibration or perfusion solution. For in vivo measurements, the polyethylene 3 M KCl in 2% agar bridge was placed in the same Ringer's solution as the cut tail of the rat.

Calcium activity in the perfusion fluids, tubule lumen, and peritubular capillaries was measured with calcium-sensitive microelectrodes (6, 7). Micropipettes were pulled from 2-mm o.d. borosilicate glass tubing. The pipettes were beveled (tip 3–4 μm o.d.) and were siliconized with tri-*N*-butylchlorosilane (Pfaltz & Bauer, Inc.) vapor at 200°C. The pipettes were backfilled with 100 mM $CaCl_2$ solution and a neutral carrier liquid ion exchanger (IE 200, W-P Instruments) was aspirated through the tip to form a column 300–600 μm in length. Ca^{2+} microelectrodes were connected via Ag-AgCl wire to the second channel of the high-impedance electrometer.

Voltage measured with the calcium-sensitive microelectrode in luminal fluid (E_L) and calibration solutions (E_C) are given by Nernst equation (9):

$$E_L = S \log_{10} [(aCa_L)/(aCa)] + E'_o; \quad (1)$$

$$E_c = S \log_{10} [(aCa_c)/(aCa)] + E''_o, \quad (2)$$

where S is the slope of the Ca^{2+} voltage response, aCa_L and aCa_C are calcium activities in luminal fluid and in one of the calibration solutions, aCa is the activity in the solution filling the Ca^{2+}-sensitive microelectrode; E'_o and E''_o is the sum of the junction potentials and is equal to the voltage measured with a conventional voltage microelectrode. The junction potential in the calibration solutions can be offset to zero by adjusting the DC position of the electrometer, $E''_o = 0$. This way, the voltage reading with a conventional voltage microelectrode, $E'_o = V$, is equal to the change in junction potential, in going from the calibration solutions to the surface of the kidney plus the transepithelial voltage. The difference in junction potential between the calibration solutions and the surface of the kidney is, in part, generated in the circuit going from the reference 3 M KCl in 2% agar bridge through the tail and the body of the rat to the surface of the kidney. The difference in junction potentials was in the range 2–10 mV and stayed the same during the course of an experiment. By subtracting Eq. 2 from Eq. 1 and then rearranging, we obtain an equation which was used to calculate aCa in the tubule lumen:

$$aCa_L = aCa_c \exp_{10}[(E_L - E_c - V)/S]. \quad (3)$$

Slope of the Ca^{2+} microelectrode voltage response was calculated from

$$S = (E'_c - E''_c)/\log_{10} (aCa'/aCa''), \quad (4)$$

where aCa' and aCa'' are calcium activities in two calibration solutions calculated as the product of calcium activity coefficient (γ_{Ca}) and total calcium concentration [Ca], $aCa = \gamma_{Ca}[Ca]$. Calcium activity coefficients were calculated using the Debye-Hückle (10) equation which for Ca has the following form:

$$-\log_{10} \gamma_{ca} = 2.05\sqrt{I}/(1 + 1.98\sqrt{I}), \quad (5)$$

where I is ionic strength (in molar). The Ca^{2+} microelectrode response slope was linear and averaged 29.0±0.2 mV/$\log_{10}$ aCa (n = 28 electrodes), ranging from 27.0 to 31.1 mV/$\log_{10}$ aCa at 37°C. Three calibration solutions were used containing 0.1, 1.0, and 10 mM $CaCl_2$ plus 150 mM NaCl, pH 6.02. Calibration of the microelectrodes were done after one to three measurements using two of the calibration solutions. Measurement of calcium activity in perfusion fluids (aCa_o) was determined using the same equations (3 and 4), except that $V = 0$ because there is no difference in junction potentials in going from the calibration to the perfusion solutions. Free Ca ion concentration (Ca^{2+}) in the perfusion solutions, tubule lumen, and peritubular capillaries was cal-

culated from $Ca^{2+} = aCa/\gamma_{Ca}$. The same $\gamma_{Ca} = 0.36$, determined from Debye-Hückel equation, was used for all solutions.

To determine if pH has an effect on calcium activity and/or Ca^{2+} microelectrode reading, we varied pH in solutions containing 1.5 mM $CaCl_2$. As shown in Table II, there was a trend for the voltage reading to be slightly higher at lower pH but in the range of pH values used in the present study the effect would be < 5%.

pH in the perfusion fluids, tubule, and capillary lumen was measured using liquid membrane pH microelectrodes (11). The electrodes were constructed in the same way as described above for Ca^{2+} microelectrodes except they were filled with 100 mM Na citrate-100 mM NaCl, pH 6.00, and contained H^+-selective liquid in the tip (010 W-P Instruments). pH in the tubule lumen was determined from the following equation:

$$pH_L = (*E_c - *E_L + V)/*S + pH_c, \quad (6)$$

where pH_C is the known pH of one of the pH calibration solutions, $*E_C$ and $*E_L$ are pH microelectrode voltage readings in one of the calibration solutions and in the luminal fluid, V is the difference in junction potential between the calibration solution and the surface of the kidney measured before and after each experiment with a conventional microelectrode, $*S$ is the slope of the pH microelectrode calculated from

$$*S = (*E' - *E'')/(pH'' - pH'), \quad (7)$$

where pH'' and pH' is the known pH of the calibration solutions and $*E''$ and $*E'$ are the pH voltage readings in two calibration solutions. The pH voltage response was linear in the range pH 5–10 and the slope averaged 63.7±1.2 mV/pH units (n = 8 electrodes), ranging from 58.1 to 68.0 mV/pH units at 37°C. Calibration solutions were prepared by blending Trizma-HCl with Trizma-base. pH of the perfusion fluids was determined using Eqs. 6 and 7, except there was no difference in junction potentials so that $V = 0$. For aCa and pH measurements in perfusion fluids, solutions were extensively bubbled with 97%/7% O_2/CO_2 gas mixture to achieve P_{CO_2} level present in renal cortex, ~ 60 mmHg (11).

Steady-state voltage, calcium activity, and pH measurements. The steady-state measurements were carried out using the split oil-droplet method (5). Briefly, a long column of mineral oil was injected into a surface segment of proximal tubule. The mineral oil pipette was withdrawn and the hole created in the tubule wall was large enough to permit the glomerular filtrate to escape; thus the movement of the oil column was prevented. A pipette (3–4-μm tip) containing one of the perfusion fluids was inserted in the middle of the oil column and enough solution was injected to split the oil droplet one surface segment apart. The perfusion pipette was then replaced by an ion-selective or voltage microelectrode. Calcium activity and voltage measurements were made in different tubules of the same kidney. pH measurements were taken in a different group of animals. Readings were taken after ~ 5 min to allow enough time to reach a steady state. For each tubule, to correct the Ca microelectrode voltage reading for the transepithelial voltage the average V_{TE} was used in the calculation of stop-flow aCa_L (Eq. 3). For stop-flow pH measurements, no correction was made because of the small contribution of V_{TE} to the pH microelectrode voltage reading. As in the case of the flow experiments, in the stop-flow studies Ca and pH microelectrode voltage readings were corrected for the difference in junction potential between the calibration solution and the surface of the kidney.

To avoid the problem of KCl leakage from 3 M KCl/2% agar microelectrodes into the small volume of the tubule stationary fluid column, the voltage microelectrodes were filled with the same solution that was injected into the lumen. Using the electrometric titration method (8), we determined that the 3 M KCl/2% agar microelectrodes leak 2×10^{-11} mol of chloride per minute. In a droplet of the size of the fluid column used to split the oil droplet (0.2 nl) the leak from the electrode would increase chloride and potassium concentration by 100 mM. In contrast, when tubules are perfused at 20 nl/min, inserting the 3 M KCl/2% agar electrode would increase the chloride and potassium concentration by only 1.0 mM and thus have no significant effect on V_{TE}.

Calculation of electrochemical driving force. The transepithelial electrochemical gradient ($\Delta\tilde{C}_{Ca}$) was calculated as the driving-force term in the Goldman-Hodgkin-Katz equation (9):

$$\Delta\tilde{C}_{Ca} = zFV_{TE}/RT[\overline{Ca_L}^{2+} - Ca_P^{2+}\exp(-zFV_{TE}/RT)]/$$
$$[1 - \exp(-zFV_{TE}/RT)], \quad (8)$$

where zF/RT is 0.0748 mV^{-1}, Ca_P^{2+} is the group average calcium ion concentration in peritubular capillaries, and $\overline{Ca_L}^{2+}$ is the logarithmic mean of the calcium ion concentration along the perfused segment calculated by the following (12):

$$\overline{Ca_L}^{2+} = (Ca_o^{2+} - Ca_L^{2+})/\ln(Ca_o^{2+}/Ca_L^{2+}), \quad (9)$$

where Ca_o^{2+} and Ca_L^{2+} is the calcium ion concentration in the perfusion fluid and the group mean concentration at the end of the perfused segment. To calculate the voltage contribution to $\Delta\tilde{C}_{Ca}$, we used the group mean V_{TE} at the end of the perfused segment assuming, as was the case with the HCO_3 solution (8), that the voltage remained approximately constant along most of the perfused segment.

The results are expressed as means±SE; significance of difference between values was assessed by means of a modified t statistics (analysis of variance). Values for the differences were calculated using the Bonferroni method (13). $P < 0.05$ determined by the two-tailed test was considered significant.

Table II. Ca^{2+} Microelectrode Measurements in Solutions of Different pH

pH	4.81	6.73	7.15	7.57	8.07
$E_{Ca^{2+}}$, mV	8.8	8.5	8.3	8.1	8.2
aCa, mM	0.544	0.520	0.522	0.515	0.518

Each solution contained 1.5 mM $CaCl_2$ plus 150 mM NaCl. pH was adjusted by blending Trizma-HCl with Trizma-Base buffer (Sigma Chemical Co.). Each value represents a single measurement.

Results

Calcium activity averaged 0.384±0.011 mM (calcium ion concentration 1.07±0.03 mM) in 46 peritubular capillaries of nine rats.

The measured values for net fluid transport, total calcium concentration in perfusion and collection fluids, and net calcium fluxes are given in Table III. Results of voltage, calcium activity, and ion concentrations are given in Table IV, and pH in Table V. Because the perfusion solutions contained 26–32 mM mannitol, net fluid absorption was reduced to near zero with all perfusates (7).

When tubules were perfused with the control Cl-containing solution, solution I, Ca absorption averaged 3.33±0.65 pmol/min (n = 45) and as result total Ca concentration along the perfused segment decreased. Although V_{TE} at the end of the perfused averaged 1.64±0.19 mV (n = 32) (lumen-positive), Ca_L^{2+} was 1.05±0.03 mM (n = 44), and thus did not fall below the value in the capillaries. The calculated electrochemical gradient with this solution was 0.28 mM.

When a portion of the Cl (27 mM) was replaced with HCO_3, solution II, net Ca absorption more than doubled and averaged 7.13±0.56 pmol/min (n = 64). Compared to the buffer-free Cl solution, luminal HCO_3 had no measurable effect on net Na transport; total Na concentration in the col-

Table III. Results of Ion Transport Experiments

Solution		J_v	$[Ca]_o$	$[Ca]_L$	J_{Ca}
	n	*nl/min*	*mM*	*mM*	*pmol/min*
I. Cl	45	0.12±0.25	1.47±0.04	1.30±0.03	3.33±0.65
II. HCO_3	64	0.17±0.23	1.50±0.12	1.11±0.02*	7.13±0.56*
III. HCO_3 + ATZ	18	−0.56±0.17	1.49±0.04	1.32±0.04	2.78±1.08
IV. HCO_3 + MIA	8	−0.24±0.43	1.44±0.03	1.25±0.04	3.17±1.36
V. Hepes	15	−0.18±0.31	1.44±0.02	1.24±0.03	3.48±0.57

Values are means±SE. *n* is number of tubules. J_v, net fluid transport (+, absorption; −, secretion); $[Ca]_o$ and $[Ca]_L$, total calcium concentration in perfused and collected fluids; J_{Ca}, net transepithelial calcium transport. Probability of no difference from result with solution I is indicated by * for $P < 0.001$ (analysis of variance).

lected fluid and J_{Na} averaged 142.0±2.0 mM and 201±40 pmol/min (n = 19) for HCO_3, and 142.9±1.8 mM and 294±31 pmol/min (n = 19) for the Cl solution. Therefore, the effect of HCO_3 on Ca transport appears to be dissociated from the net transepithelial Na flux. With HCO_3, pH of the perfusion solution was 7.25 and as a result of acidification it fell along the perfused segment to 7.00±0.03 (n = 36). Compared to HCO_3, with Cl solution, perfusion fluid, 5.34, and luminal pH at the end of the perfused segment, 6.61±0.06 (n = 6), were significantly lower (Table V). Even though V_{TE} was less lumen-positive, 0.78±0.16 mV (n = 17), calcium ion concentration along the perfused segment fell significantly below the value in the capillary and averaged 0.88±0.03 mM (n = 32) ($P < 0.001$ compared with capillary). Because with HCO_3 solution $\Delta\tilde{C}_{Ca}$ was only 0.03 mM, the enhanced calcium absorption could not be accounted for by the electrochemical gradient.

To determine whether the enhanced Ca absorption with the HCO_3 containing solution was the result of HCO_3 absorption or the presence of luminal HCO_3 per se, we next perfused tubules with HCO_3 solution which contained 1.0 mM carbonic anhydrase inhibitor acetazolamide (ATZ),[1] solution III. ATZ has a high cell permeability and inhibits both luminal and cellular carbonic anhydrase (14). With ATZ, luminal pH averaged 6.87±0.06 (n = 16), a value that is not statistically different from the group mean of all tubules perfused with HCO_3 perfusate ($P < 0.1$, analysis of variance). However, when we compare pH_L with ATZ, 6.87±0.06, to the values obtained in the same kidney with the HCO_3 solution, 6.94±0.05, the results are statistically different ($P < 0.001$, n = 16). This is consistent with our previous study in which we also showed that ATZ lowers luminal pH (8). Results in the present study are similar to the pH values of 6.95 for HCO_3 and 6.84 for HCO_3 + ATZ solution obtained by Lucci et al. (14) who measured four surface segments from the perfusion site. These results were not statistically different but with 10^{-4} benzolamide (carbonic anhydrase inhibitor with a low cell permeability) luminal pH was significantly lower (14). In their study both drugs inhibited HCO_3 absorption equally, > 90%. We have previously found that ATZ decreased HCO_3 absorption from 224 to 67 pmol/min and increased luminal HCO_3 concentration from 16.4 to 25.0 mM (8). Therefore, in a flow situation, ATZ does not prevent the establishment of a transepithelial H^+ gradient, but by inhibiting luminal carbonic anhydrase it prevents the catalized buffering of secreted protons by the HCO_3/CO_2 system. As a result, HCO_3 absorption and the total amount of acid secreted are inhibited.

As shown in Table III, with ATZ, calcium absorption averaged 2.78±1.08 pmol/min (n = 18), not different from the control. Compared to control, solution I, Ca_L^{2+} was not different, averaging 1.11±0.05 mM (n = 17), but because V_{TE} was lower, 0.31±0.19 mV (n = 17), the electrochemical driving force was likewise lower, 0.14 mM. The ATZ results suggest that the stimulation of Ca absorption with HCO_3 was likely the result of acidification-mediated HCO_3 absorption and not due to the presence of luminal HCO_3 per se.

To further test if the stimulation of Ca absorption is linked to acidification, we perfused tubules with HCO_3 solution containing 10^{-5} M of an amiloride analogue 5-(*N*-methyl-*N*-isobutyl)-amiloride (MIA), an inhibitor of Na/H exchange (15). With MIA there was no acidification of tubule fluid and luminal pH at the end of the perfused segment was significantly higher compared with HCO_3 solution, averaging 7.24±0.04 (n = 11) (Table V). These results are consistent with the study by Howlin et al. (16) who showed that amiloride inhibits HCO_3 absorption. With MIA net Ca absorption averaged 3.17±1.36 pmol/min (n = 8), a rate that was not different from the control buffer-free Cl containing solution. With this perfusate Ca_L^{2+} averaged 0.99±0.05 mM (n = 7), V_{TE} 0.70±0.20 mV (n = 5), and $\Delta\tilde{C}_{Ca}$ was 0.08 mM. Because amiloride inhibited proximal acidification this result would further support the notion that acidification of tubule fluid with the HCO_3 perfusate stimulated Ca absorption.

If the enhanced Ca absorption was the result of proton secretion by the proximal tubule, then other buffers with pKa similar to that for HCO_3/CO_2 should show the same effect. To test this hypothesis, tubules were perfused with solution containing 27 mM Hepes (pKa = 7.5 solution V). As in the case with HCO_3, with Hepes solution there was acidification of perfusate along the tubule but compared with HCO_3 the perfusion fluid, 7.09, and luminal pH at the end of perfused segment, 6.81±0.06 (n = 11), were lower. Under the same conditions, we have previously found that with the same Hepes perfusate, there was significant accumulation of HCO_3 in the lumen, J_{HCO_3} = −317 pmol/min (17). With Hepes in the perfusate net Ca absorption averaged 3.48±0.57 pmol/min (n = 15) which was not different from the control buffer-free solution, but the transepithelial electrochemical driving force was close to zero due to the fact that both Ca_L^{2+} and V_{TE} were significantly lower, 0.88±0.03 mM (n = 18) and 0.61±0.40 mV (n = 13), respectively.

1. *Abbreviations used in this paper:* ATZ, acetazolamide; MIA, 5-(*N*-methyl-*N*-isobutyl)-amiloride.

Table IV. Results of Voltage, Calcium Activity, and Calcium Ion Concentration

Solution	aCa_o	Ca_o^{2+}	aCa_L	Ca_L^{2+}	V_{TE}	$\Delta\tilde{C}_{Ca}$
	mM	*mM*	*mM*	*mM*	*mV*	*mM*
I. Cl	0.511	1.42	0.378±0.011 (44)	1.05±0.03 (44)	1.64±0.19 (32)	0.28
II. HCO_3	0.439	1.22	0.317±0.011* (32)	0.88±0.03* (32)	0.78±0.16‡ (17)	0.03
III. HCO_3 + ATZ	0.439	1.22	0.398±0.016 (17)	1.11±0.05 (17)	0.31±0.19* (17)	0.13
IV. HCO_3 + MIA	0.439	1.22	0.357±0.018 (7)	0.99±0.05 (7)	0.70±0.20 (5)	0.08
V. Hepes	0.454	1.26	0.317±0.012* (18)	0.88±0.03* (18)	0.61±0.40‡ (13)	0.04

Values are means±SE. Numbers in parentheses are numbers of tubules. aCa_o and aCa_L, calcium activity in perfused and luminal fluids measured with calcium sensitive microelectrodes. Ca_o^{2+} and Ca_L^{2+} are free ionic calcium concentrations calculated from calcium activity using actively coefficient 0.36 (Debye-Hückel equation). V_{TE}, transepithelial voltage (lumen relative to interstitium) $\Delta\tilde{C}_{Ca}$; electrochemical gradient calculated using free calcium ion concentration and V_{TE} (see Methods). For measurements of Ca^{2+} in perfusion fluids, solutions were extensively gassed with 93%/7% O_2/CO_2 gas mixture. Probability of no difference from results with solution I as indicated by * for $P < 0.005$ and ‡ for $P < 0.05$ (analysis of variance).

Because with solution II, the HCO_3-stimulated calcium absorption occurred despite a decrease in $\Delta\tilde{C}_{Ca}$, the results indicate that HCO_3 absorption mediates this effect via an active transport process. To confirm this, we carried out split-oil steady-state measurements of calcium ion concentration and voltage. When HCO_3 solution was injected, the steady-state Ca_L^{2+} averaged 0.86±0.03 mM (aCa_L = 0.310±0.012) (n = 8) and V_{TE} 0.50±0.20 mV (n = 4). In the absence of active transport process for the same transepithelial calcium ion concentration gradient to develop, we estimate, using the Nernst equation, $V_{TE} = RT/zF \ln (Ca_P^{2+}/Ca_L^{2+})$, that the equilibrium V_{TE} would have to be around 2.9 mV. Because the calculated V_{TE} is higher than the measured value the results suggest active transport component. With ATZ, solution III, the steady-state Ca_L^{2+} significantly increased and was not different from the value in the capillaries, 1.06±0.03 (n = 8) vs. 1.07 (aCa; 0.382±0.011 vs. 0.384 mM). This increase cannot be accounted for by V_{TE} which remained unchanged, 0.60±0.21 mV (n = 4). With HCO_3, the stop-flow luminal pH averaged 6.95±0.03 (n = 17), but in contrast to the flow studies, with HCO_3 + ATZ, luminal pH of the stationary fluid column was higher 7.14±0.04 (n = 17) ($P < 0.001$). The increase in stop-flow pH_L is likely the result of inhibition of cellular carbonic anhydrase involved in H^+ secretion (14). Because ATZ increased the stop-flow luminal calcium ion concentration, the results confirm presence of active calcium transport process, and because the increase in Ca^{2+} was associated with increased luminal pH, the results also suggest that active Ca absorption may be linked to acidification.

Table V. Results of pH Measurements

Solution	I Cl	II HCO_3	III HCO_3 + ATZ	IV HCO_3 + MIA	V Hepes
pH_o	5.34	7.25	7.25	7.25	7.09
pH_L	6.61* ±0.06 (6)	7.00 ±0.03 (36)	6.87 ±0.06 (16)	7.24* ±0.04 (11)	6.81‡ ±0.06 (11)

Values are means±SE. Numbers in parentheses are number of tubules. pH_o and pH_L were measured in perfusion solutions and tubule lumen using pH sensitive microelectrodes.
For pH measurements in perfusion fluids, solutions were extensively bubbled with 93%/7% O_2/CO_2 gas mixture (P_{CO_2} = 61.9 mmHg, blood gas analyzer, ABL-1, Radiometer, Copenhagen).
Probability of no difference from results with solution II as indicated for * for $P < 0.001$ and ‡ for $P < 0.02$ (analysis of variance).

Discussion

In the absence of net water movement, calcium transport by the proximal tubule is determined by the electrochemical driving forces and active transport (6). To evaluate the contribution of the transepithelial electrochemical gradient to the HCO_3 stimulated Ca absorption, we measured transepithelial voltage and calcium ion concentration in tubule fluid and in the peritubular capillary blood. Compared to the buffer-free control solution, with HCO_3 in the perfusate, V_{TE} and Ca_L^{2+} were significantly reduced and as a result $\Delta\tilde{C}_{Ca}$ was also reduced (Table IV). Because the enhanced Ca absorption occurred despite a decrease in the electrochemical driving force, the results indicate that HCO_3 stimulated an active Ca transport process.

In contrast to our observation, Sutton et al. (2) found that in dogs, relative to Na reabsorption, intravenous infusion of HCO_3 had no effect on proximal Ca reabsorption (2). The results showed that HCO_3 administration decreased urinary Ca excretion by a direct tubule effect beyond the late proximal tubule. In the study of Sutton et al. (2), the three groups of animals that received infusion of HCO_3 (metabolic acidosis, intact and thyroparathyroidectomized dogs) had a decrease in proximal fluid reabsorption and a decrease in ultrafiltrable Ca. It is possible, that because these changes would inhibit Ca reabsorption, the direct tubule effect of HCO_3 was not observed. In our study, we directly examined the effect of luminal HCO_3, while keeping net water movement near zero for all perfusates and taking into account the contribution of the electrochemical driving forces.

Ullrich et al. (5) using the stop-flow microperfusion technique with simultaneous capillary perfusion demonstrated ac-

tive calcium absorption, but in contrast to our study, it was not affected by HCO_3. There are at least two possible explanations for the discrepancy: (*a*) In the Ullrich study V_{TE} was not measured directly but was estimated from the Cl gradient across the tubule wall using the Nernst equation. With HCO_3 the luminal Ca concentration was 0.32 mM lower than in the capillary, a difference that diminished to 0.19 mM with buffer-free solution. Because the electrochemical gradient did not change, the authors concluded that this fall was the result of a less lumen positive V_{TE}. This conclusion may not be correct, because Cl transport by the proximal tubule is not all passive (8) and thus the calculated V_{TE} may not be an accurate estimate of the true V_{TE}. (*b*) In the Ullrich study the steady-state Ca ion concentration was not determined directly but was measured as ^{45}Ca counts. In HCO_3-containing solutions, HCO_3-Ca_{2+} soluble complexes (see Ca_o^{2+}, Table IV) (6) decrease calcium ion concentration; thus, using the ^{45}Ca radioactivity to calculate Ca^{2+} concentration gradient may not be appropriate.

To determine if, in our study, the HCO_3-stimulated calcium absorption was the result of luminal HCO_3 per se or the result of HCO_3 absorption, we perfused tubules with HCO_3-containing solution which also contained 10^{-3} M ATZ. With this perfusate, J_{Ca} was not different from the buffer-free control solution. Because ATZ inhibited HCO_3 absorption and increased luminal HCO_3 concentration (8), this result suggests that the enhanced active Ca absorption could not be accounted for by luminal HCO_3 per se but would be more likely the result of acidification-mediated HCO_3 absorption. Ullrich et al. (5) in the stop-flow microperfusion study also found that ATZ decreased Ca concentration gradient, a change which was attributed to V_{TE} rather than to active transport. Because V_{TE} determined from Cl concentration gradient may not be accurate, it is possible that the decreased Ca concentration gradient with ATZ in the study by Ullrich et al. (5) was, as in our case, the result of a decrease in active Ca transport. To elucidate the apparent discrepancy between the two studies on the effects of ATZ we performed similar stop-flow study and directly measured luminal Ca activity, pH and V_{TE}. We found that ATZ did not have a measurable effect on V_{TE} and yet decreased the transepithelial Ca^{2+} gradient from 0.29 to 0.01 mM. These data provide further evidence for ATZ-inhibitable active calcium absorption. Because in the stop-flow situation the increase in luminal Ca^{2+} concentration induced by ATZ was associated with an increase in luminal pH the data also suggest that active Ca^{2+} transport may be linked to acidification of proximal tubule fluid. Although a direct effect of luminal pH on aCa_L or Ca transport can not be ruled out, no differences in aCa_L or J_{Ca} were observed between control Cl, HCO_3 + ATZ, or HCO_3 + MIA solutions (Tables III and IV) even though the pH_L were different; 6.61, 6.87, and 7.24, respectively (Table V).

To further test the possibility that the enhanced Ca absorption is the result of acidification-mediated HCO_3 absorption, we perfused the tubules with HCO_3 solution that in addition contained Na/H exchange inhibitor, MIA. Because MIA inhibited luminal fluid acidification and Ca^{2+} absorption, the data support the notion that acidification of luminal fluid stimulates proximal active Ca^{2+} absorption.

When tubules were perfused with Hepes solution the rates of Ca transport were not different from the buffer-free control solution but V_{TE} was significantly less lumen-positive. We and others have shown that V_{TE} is an important driving force of Ca transport by the proximal tubule (6, 18, 19). Thus, based on the voltage change, we would predict that with Hepes, Ca absorption should have diminished when in fact there was no change. This result suggests to us, that as with HCO_3, acidification of Hepes containing tubule fluid resulted in stimulation of active Ca absorption. In Fig. 1 we plotted J_{Ca} against $\Delta\tilde{C}_{Ca}$ for each of the perfusates. The dashed line is based on our previous study that showed a nearly linear relationship between net calcium flux and the electrochemical driving forces (6) and was drawn here through the origin. In the absence of active transport one would predict that the points should fall on or near the line. It is apparent that both HCO_3 and Hepes points are well above the line indicating a component of active absorption. With both of the solutions the predicted J_{Ca} should be < 1 pmol/min or only 14% of the measured value for the HCO_3 perfusate and 30% of the measured value for Hepes. However, it is also apparent that the rate of Ca absorption with HCO_3 is twice as much as with Hepes, even though $\Delta\tilde{C}_{Ca}$ are close. The reason for this is unclear but at least two explanations seem possible. First, with HCO_3 and Hepes there was acidification of tubule fluid along the perfused segment but both the perfusion and luminal fluid pH was lower with Hepes (Table V). With a lower luminal fluid pH the rate of proton secretion may also be lower (20) and if Ca^{2+} transport is linked to acidification then the rates of Ca^{2+} absorption would also be lower. Secondly, with Hepes as a result of intraluminal generation and backflux, HCO_3 accumulates in the tubule lumen (16). HCO_3 backflux into the lumen may bring Ca^{2+} ions with it by virtue of soluble ion complex formation and thus decrease the net rate of Ca^{2+} absorption. Secretion of HCO_3-Ca^{2+} may also account for the fact that in Fig. 1 the point for buffer-free control solution appears to fall below the dashed line (i.e., more Ca^{2+} ions present in the lumen than can be accounted for by $\Delta\tilde{C}_{Ca}$).

In this study we present evidence for active Ca absorption that may be linked to acidification of luminal fluid by the

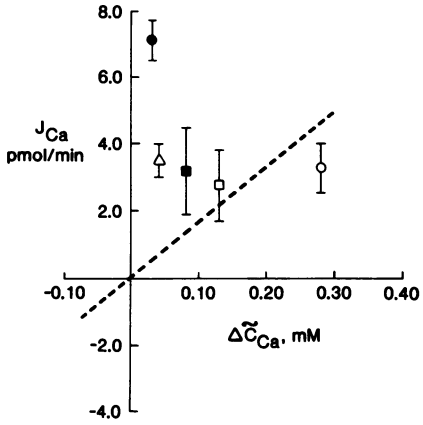

Figure 1. Net calcium fluxes (J_{Ca}) (Table III) plotted against transepithelial electrochemical concentration difference, ($\Delta\tilde{C}_{Ca}$) (Table IV). (○) Control, buffer-free solution, solution I; (●) HCO_3, solution II; (□) HCO_3 + ATZ, solution III; (■) HCO_3 + MIA, solution IV; (△) Hepes, solution V. The dashed line was drawn through the origin and represents the linear relationship that would exist between Ca flux and the electrochemical driving forces in the rat proximal tubule in absence of any active transport process (6). The slope of this line is 16.4 nl/min and was calculated as the product of calcium permeability of these tubules, 16.1×10^{-7} cm²/s (6), and the averaged perfused length in these experiments, 1.8±0.1 mm (n = 129).

proximal tubule cell. It is thought that because intracellular Ca ion concentration is 10^{-7} M and because cell membrane potential is negative, there exist a very high electrochemical gradient for passive Ca entry across the luminal membrane into the cell (21). Calcium is then actively transported across the basolateral membrane against similar but oppositely oriented electrochemical gradient. Studies with basolateral membrane vesicles from rat cortex showed that active calcium transport across this membrane can proceed via ATP-driven Ca pump or Na gradient driven Na/Ca exchange (22). The Na/Ca exchange process depends on basolateral Na/K ATPase which sets up the Na gradient. Therefore, it is possible that some of the effects we observed were the result of changes in intracellular Na concentration. In a system where Na/Ca exchange is the major process moving Ca^{2+} out of the cell, maneuvers that increase cell Na would lead to a decrease in Na^+-driven Ca^{2+} efflux. That could be the case with ATZ or MIA which may inhibit Na-K-ATPase resulting in increased cell Na^+ and thus lead to decreased Ca^{2+} absorption. Even though net transepithelial Na transport with HCO_3 compared to Cl solution was not different, we can not rule out differences in cell Na^+. With HCO_3, V_{TE} was less lumen-positive, suggesting that with this perfusate, compared to Cl solution, a larger fraction of net Na flux was active and transcellular. This would correspond to a situation of increased Na pumping across the basolateral membrane leading to decreased cell Na^+ concentration. A fall in intracellular Na^+ would enhance Na/Ca exchange-mediated Ca^{2+} pumping across the basolateral membrane, a situation that may be present with HCO_3 absorption.

Basolateral Ca^{2+}-ATPase is another process that potentially might mediate transcellular Ca transport, a process that may be affected by intracellular pH and possibly account for our findings. A recent study suggested that in reconstituted erythrocytes, electrogenic Ca^{2+}-translocating ATPase not only translocates Ca^{2+} but also transports H^+ in the opposite direction (23). If present in the basolateral membrane of the proximal tubule cell, such a process may explain our observations because transport of H^+ by the ATPase into the cell down the electrochemical gradient would add energy for the uphill Ca^{2+} pumping. There may be other possibilities for our observations but the true explanation may have to be derived from direct measurements of intracellular pH and Ca^{2+} in proximal tubule cells studied under the condition of the present study.

In summary, we found that luminal HCO_3 stimulates active calcium absorption by the rat proximal tubule, a process that may result from acidification-mediated HCO_3 absorption. The enhanced Ca absorption may account, at least in part, for the observation that HCO_3 administration lowers renal calcium excretion.

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