Electroneutral K^+/HCO_3^- Cotransport in Cells of Medullary Thick Ascending Limb of Rat Kidney

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Abstract

The renal medullary thick ascending limb (MTAL) of the rat absorbs bicarbonate through luminal H^+ secretion and basolateral HCO_3^- transport into the peritubular space. To characterize HCO_3^- transport, intracellular pH (pHi) was monitored by use of the pH-sensitive fluorescent probe (2',7')-bis-(carboxyethyl)-(5,6)-carboxyfluorescein in fresh suspensions of rat MTAL tubules. When cells were preincubated in HCO_3^-/CO_2 -containing solutions and then abruptly diluted into HCO_3^-/CO_2 -free media, the pHi response was an initial alkalization due to CO_2 efflux, followed by an acidification (pHi recovery). The pHi recovery required intracellular HCO_3^- , was inhibited by 10^{-4} M diisothiocyanostilbene-2-2'-disulphonic acid (DIDS), and was not dependent on Cl^- or Na^+ . As assessed by use of the cell membrane potential-sensitive fluorescent probe 3,3'-dipropylthiadicarbocyanine, cell depolarization by abrupt Cl^- removal from or addition of 2 mM barium into the external medium did not affect HCO_3^- -dependent pHi recovery, and the latter was not associated per se with any change in potential difference, which indicated that HCO_3^- transport was electroneutral. The HCO_3^- -dependent pHi recovery was inhibited by raising extracellular potassium concentration and by intracellular potassium depletion. Finally, as measured by use of a K^+ -selective extracellular electrode, a component of K^+ efflux out of the cells was HCO_3^- dependent and DIDS sensitive. The results provide evidence for an electroneutral K^+/HCO_3^- cotransport in rat MTAL cells. (*J. Clin. Invest.* 1992; 90:869–878.) Key words: barium • diisothiocyanostilbene-2-2'-disulphonic acid (DIDS) • intracellular pH • membrane potential • ouabain

Introduction

Bicarbonate absorption by Henle's loop of the kidney, which represents as much as ~ 15% of the bicarbonate filtered load in

euvoletic rats (1, 2) and is thus of importance for bicarbonate excretion in final urine, probably results in large part from the activity of the thick ascending limb (TAL).¹ In fact, both the medullary (MTAL) and cortical (CTAL) segments of the rat TAL absorb bicarbonate at relatively high rates (3). This TAL ability requires carbonic anhydrase activity (4) and, indeed, rat TAL cells (5), like human TAL cells (6), are rich in carbonic anhydrase. In addition, the TAL is a site of regulation of the renal acid-base handling in that chronic metabolic acidosis and increased sodium intake are associated with adaptive increases in the ability of the rat MTAL to absorb bicarbonate (7). The knowledge of the membrane H^+/HCO_3^- transport mechanisms is a prerequisite for understanding the processes involved in these adaptations of TAL cells.

Bicarbonate absorption by any nephron segment is secondary to proton secretion within the tubular lumen and bicarbonate transport across the basolateral membrane from cell to peritubular space. Evidence has been recently provided in our laboratory that the rat MTAL may secrete protons by both Na^+/H^+ antiporter and proton-adenosine triphosphatase (H^+ -ATPase) activities (8). The membrane mechanism of basolateral bicarbonate exit in the rat MTAL is unknown at present. Yet basolateral bicarbonate transport may be of crucial importance in both transepithelial bicarbonate absorption and intracellular pH (pHi) regulation, as has been shown in the proximal tubule (9). Arguments, derived from pHi measurements, have been put forward to suggest the presence of a $Na^+/(HCO_3^-)_n$ cotransporter in the rat CTAL (10) and in the mouse MTAL (11).

Thus the aim of the present work was to determine the membrane transport mechanism of bicarbonate exit from rat MTAL cells, the direction of transport that normally occurs. To this purpose, we have monitored pHi in suspensions of fresh rat MTAL tubules by use of the pH-sensitive fluorescent probe (2',7')-bis-(carboxyethyl)-(5,6)-carboxyfluorescein (BCECF). Also, variations in cell membrane potential difference (PD) were assessed by use of the PD-sensitive fluorescent probe 3,3'-dipropylthiadicarbocyanine [DiS-C3-(5)], and potassium transport out of the cells was estimated by use of a highly selective extracellular K^+ electrode. The results provide evidence that bicarbonate transport takes place in rat MTAL cells by a mechanism that was not previously described in another cell type, to our knowledge, i.e., by electroneutral K^+/HCO_3^- cotransport.

1. Abbreviations used in this paper: BCECF, (2',7')-bis-(carboxyethyl)-(5,6)-carboxyfluorescein; CTAL, cortical thick ascending limb; DIDS, 4,4'-diisothiocyanostilbene-2-2'-disulfonic acid; DiS-C3-(5), 3,3'-dipropylthiadicarbocyanine; MTAL, medullary thick ascending limb; NMG, N-methyl-D-glucamine; PD, potential difference; pHi, intracellular pH; TAL, thick ascending limb; TMA, tetramethylammonium.

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Methods

Isolation of rat MTAL tubules. The method used was derived from that previously described by Trinh-Trang-Tan et al. (12, 13). Male Sprague-Dawley rats (200–300 g in body weight) were starved overnight but allowed free access to tap water until anesthetization with pentobarbital. For each experiment, six kidneys of three anesthetized rats were rapidly removed without any previous manipulation and immediately immersed into ice-cold dissecting solution, which took only a few seconds, to avoid anoxic damage to medullary tissues and improve cell viability; the kidneys were then cut into thin slices along the corticopapillary axis into ice-cold Hanks' solution containing (in mM): 137 NaCl, 5.4 KCl, 4.2 NaHCO₃, 0.3 Na₂HPO₄, 0.4 KH₂PO₄, 0.4 MgSO₄, 0.5 MgCl₂, 1.2 CaCl₂, 5 glucose, previously bubbled with 95% O₂-5% CO₂. Under a dissecting microscope, the inner stripe of outer medulla of each slice was excised and cut into uniform small pieces by gently forcing it through a piece of stretched medical gauze. The small tissue pieces were then serially subjected to collagenase digestion (0.40 g/liter) in Hanks' solution bubbled with 95% O₂-5% CO₂ at 37°C during six to eight 10–15-min periods. After each period, the cells- and tubule fragments-containing supernatants were collected after 1-min gravity sedimentation and stored into ice-cold Hanks' solution. After the last digestion, these supernatants were sieved through a 75-μm opening nylon mesh that retained the largest fragments of MTAL. These harvested fragments were then collected in Hanks' solution containing 10 g/liter bovine serum albumin (BSA), centrifuged 1 min at low speed (~80 g), and resuspended in an appropriate volume of the desired medium. As observed by light microscopy, the final suspension contained almost exclusively MTAL tubules (> 95%), occasional thin descending limb fragments, and no isolated cells or medullary collecting tubule segments, as was also observed by others (12). The lengths of the MTAL fragments ranged in general from 75 to 200 μm.

Measurements of pHi. To load MTAL cells with the pH-sensitive probe BCECF, tubule suspensions were incubated for 30–45 min at 37°C in a bicarbonate-containing medium bubbled with 95% O₂-5% CO₂ (solution A in Table I) containing 10 μM BCECF-acetoxymethyl ester (BCECF-AM, dissolved in dimethylsulfoxide (DMSO) and stored at -20°C). The BCECF-loaded tubules were then washed five times by gentle centrifugation to remove the extracellular dye and resus-

pended in the appropriate medium. Aliquots of the MTAL tubule suspension were diluted into glass cuvettes containing 3 ml of the experimental medium to reach a final cytocrit of ~1 vol%. BCECF fluorescence was monitored by use of a Shimadzu model RF-5000 spectrofluorometer (Shimadzu Corporation, Kyoto, Japan) equipped with a water-jacketed, temperature-controlled cuvette holder and magnetic stirrer. All experiments in the present work were performed at 25°C to avoid any contribution to the observed pHi changes of the plasma membrane H⁺-ATPase of MTAL cells (8). Fluorescence intensity was recorded at one emission wavelength, 530 nm, whereas the excitation wavelength alternated automatically at 2-s intervals between two wavelengths, 500 and 450 nm. Just before each run, the MTAL tubule suspension aliquot to be added into the spectrofluorometer cuvette was washed and centrifuged in the appropriate medium to further remove any residual extracellular dye. The rate of increase of extracellular fluorescence caused by leakage of BCECF out of the cells was low at 1.55±0.13%·min⁻¹ (pH 7.4, n = 3) and was neglected because experiments were performed for short time periods (< 2 min). The values of the fluorescence ratio F₅₀₀/F₄₅₀ were converted into pHi values with use of calibration curves. Calibration curves were established daily by one of two methods. First, the relationship between intracellular BCECF fluorescence and pHi was determined on a sample of the MTAL tubule suspension under conditions during which pHi and extracellular pH may be assumed to be equal, i.e., by placing the cells in a medium containing 115 mM potassium and 3.3 μM of the K⁺/H⁺ ionophore, nigericin; in this case, for the calibration and for each experimental run, the fluorescence of the extracellular medium at both excitation wavelengths was subtracted from the total fluorescence observed after addition of BCECF-loaded cells to determine the signal generated by the intracellular dye. Second, after each experimental run, the cells were permeabilized with Triton X-100 (0.25 g/liter) and the relationship between extracellular BCECF fluorescence and medium pH was established; this method was used particularly when some materials (e.g., high doses of amiloride) suspected to interfere with the fluorescence were present in the cuvette during experimental runs and when experiments were performed with potassium-depleted cells. We have observed that external calibration was not different from that with nigericin when both were performed on the same sample of cells.

Estimation of variations in membrane potential. Variations in MTAL cell PD were estimated by use of the PD-sensitive fluorescent

Table I. Composition of Experimental Solutions

	A	B	C	D	E	F	G	H	I
	mM								
Na ⁺	138	138	138	138	138	—	—	138	138
K ⁺	5	5	5	5	5	5	120	—	—
Mg ⁺⁺	1	1	1	1	1	1	1	1	1
Ca ⁺⁺	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
TMA ⁺	—	—	—	—	—	138	23	—	—
Barium ⁺⁺	—	—	—	—	2	—	—	—	—
Cl ⁻	115	144	144	—	150	144	144	77	137
HCO ₃ ⁻	25	—	—	—	—	—	—	60	—
PO ₄ ⁻⁻⁻	3	1	1	1	1	1	1	3	3
SO ₄ ⁻	1	1	1	1	—	1	1	1	1
Gluconate ⁻	—	—	—	144	—	—	—	—	—
Hepes	—	10	10	10	10	10	10	3	10
Glucose	5	5	5	5	5	5	5	5	5
L-Leucine	5	5	5	5	5	5	5	5	5
pH	7.4	7.4	8.1	7.4	7.4	7.4	7.4	7.8	7.4

Bicarbonate-containing solutions were bubbled with 95% O₂/5% CO₂; bicarbonate-free solutions were bubbled with 100% O₂ and adjusted to the indicated pH with Tris.

probe DiS-C3-(5), as described by Waggoner (14) and used by Zeidel et al. (15) in renal cells. The fluorescence of $0.25 \mu\text{M}$ DiS-C3-(5) in 2 ml of the appropriate solution in glass cuvettes equilibrated with the dye was monitored with a Shimadzu model RF 5000 spectrofluorometer before and after addition of a constant amount of cells within a given experiment, and the resulting quench in fluorescence was determined. Excitation wavelength was 643 nm and emission wavelength 666 nm (slits 10 nm). The validity of the method for rat MTAL cells was checked at 37°C by adding KCl-loaded cells (previously incubated in a solution containing 140 mM KCl, 10 mM Hepes, 1 mM MgSO_4 , 1 mM CaCl_2 , 5 mM glucose, 5 mM L-leucine, adjusted to pH 7.4 with Tris) into a K^+ -free solution containing the dye (KCl replaced in the above solution with nonpermeant *N*-methyl-D-glucamine [NMG]-Cl), which was followed by addition of $3 \mu\text{M}$ valinomycin and of small volumes of a 1 M KCl solution to sequentially increase the extracellular potassium concentration. A representative tracing is shown in Fig. 1;

Figure 1. Effect of extracellular potassium concentration on DiS-C3-(5) fluorescence. *Top:* Cells, preincubated in 140 mM KCl solution, were added into NMG-Cl solution (final K^+ concentration 3 mM) containing $0.25 \mu\text{M}$ DiS-C3-(5), the fluorescence of which was quenched; then, after addition of the potassium ionophore valinomycin (Val.), small amounts of 1 M KCl solution were added to reach 8, 12, and 25 mM external potassium concentration. *Bottom:* Relative fluorescence (value after valinomycin expressed in percentage of that before valinomycin) is plotted against the potassium equilibrium potential ($E_K = -61.5 \times \log([K]_i/[K]_o)$, in which $[K]_i = 140 \text{ mM}$); each point is mean $\pm$ SE of 8–10 determinations in three separate MTAL suspensions.

addition of KCl loaded-cells into the K^+ -free solution caused a large decrease or quench in DiS-C3-(5) fluorescence owing to accumulation of the dye within the cells; subsequent addition of valinomycin further decreased the fluorescence due to cell membrane hyperpolarization caused by the K^+ ionophore; then, sequentially increasing the extracellular potassium concentration progressively increased the fluorescence because of progressive cell membrane depolarization and attendant exit of the dye from the cells. As shown on the bottom panel of Fig. 1, the relative fluorescence (difference between values after and before valinomycin expressed in percentage) was linearly related ($P < 0.0001$) to the potassium equilibrium potential (i.e., the PD in the presence of valinomycin) over a large range of values. Intracellular potassium concentration of KCl-loaded cells was assumed to equal 140 mM because valinomycin had no effect on the DiS-C3-(5) fluorescence when KCl-loaded cells were added into the same KCl-rich medium containing the dye. From Fig. 1, it can be seen that KCl-loaded cells had a PD of $\sim -80 \text{ mV}$ when diluted into the K^+ -free solution. In the experiments to be described hereafter, no attempt was made to determine the absolute values of MTAL cell PD under the various experimental conditions; rather, variations in the quench in DiS-C3-(5) fluorescence were determined within 10 s after addition of a constant amount of cells to semiquantitatively estimate changes in the cell PD between paired experiments.

Measurement of K^+ efflux. Net K^+ efflux out of rat MTAL cells were estimated under defined experimental conditions with use of a solid-state extracellular K^+ electrode (POT-1, World Precision Instruments, Inc., New Haven, CT) and a reference calomel electrode (type K701, Radiometer, Copenhagen, Denmark) containing a secondary salt-bridge filled with 1 M NMG-chloride. The electrodes were fixed into a mechanically stirred chamber and were connected to a high-impedance electrometer (model PHM 84, Radiometer) and to a chart recorder (model 500, Linear Instruments Corporation, Reno, NV). For each experiment, the K^+ electrode was calibrated before and at the end of the runs, which revealed no difference between the two calibration lines; the slope of the electrode was $52 \pm 1 \text{ mV/decade } \text{K}^+$ concentration and was "linear" between 10^{-4} and $10^{-2} \text{ M } \text{K}^+$. The selectivity of K^+/Na^+ was $10^4:1$. Rat MTAL tubules were isolated as described above and abruptly diluted into $500 \mu\text{l}$ of various experimental media at a final density of 1.0–2.4 mg of cell protein/ml in the electrodes-containing chamber; the experimental media contained agents (ouabain, barium, furosemide) that inhibit known potassium transport mechanisms, which is derived from protocols previously described by others (16, 17); at the end of each run, the exact cell protein amount was measured by the method of Lowry and net K^+ efflux was expressed in nanomoles per milligram of cell protein.

Materials. Collagenase CH grade II was obtained from Boehringer Mannheim France SA, Meylan, France; Hanks' solution from Gibco, Paisley, Scotland; BCECF-AM from HSC Research Development Corporation, Toronto, Canada; DiS-C3-(5) from Molecular Probes, Inc., Eugene, OR.; tetramethylammonium (TMA)-chloride from Aldrich-Chimie S.A.R.L. Strasbourg, France; amiloride, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), acetazolamide, DMSO, L-leucine, nigericin, valinomycin, monensin, ouabain, and all other chemicals were obtained from Sigma-Chimie S.A.R.L., La Verpillière, France. *t*-butyl acetazolamide was a gift from Lederle Laboratories, Pearl River, NY.

Statistics. Results are expressed as means $\pm$ SE. Statistical significance between experimental groups was assessed by one-way analysis of variance, completed by Dunnett's *t* test. Comparison of two regression lines was made by analysis of covariance.

Results

When MTAL cells were incubated into a medium containing 25 mM bicarbonate (solution A in Table I), pHi was 7.24 ± 0.04 ($n = 6$) and intracellular bicarbonate concentration was $17.4 \pm 2.0 \text{ mM}$. To characterize the membrane mechanism

Figure 2. Effect of HCO_3^- efflux from rat MTAL cells on pH_i . Cells, preincubated in 25 mM HCO_3^- solution (●) or alkalized in HCO_3^- free solution at pH 8.1 (○), were diluted into $\text{HCO}_3^-/\text{CO}_2$ free solution, pH 7.4; HCO_3^- loaded cells initially alkalized due to rapid CO_2 exit. Initial rate of acidification was lower without (0.075 ± 0.25 pH unit/min) than with (2.03 ± 0.016 , $P < 0.001$) bicarbonate within the cells.

of HCO_3^- efflux, the general approach was to study the evolution of pH_i after transferring these HCO_3^- loaded cells into a nominally $\text{HCO}_3^-/\text{CO}_2$ free medium (solution B in Table I), which was derived from the method first described by Zeidel et al. (18). After abruptly diluting 200 μl of the bicarbonate solution containing HCO_3^- loaded cells into 3 ml of $\text{HCO}_3^-/\text{CO}_2$ free solution in the spectrofluorometer cuvette, extracellular PCO_2 and bicarbonate concentration fell from 40 to ~ 2.5 mmHg and from 25 to ~ 1.6 mM, respectively. Thus sudden outward-directed gradients for CO_2 and HCO_3^- were generated. During the following 60 s, the pH_i time course could be divided into two phases (Fig. 2). An almost instantaneous cellular alkalization first occurred, due to very rapid CO_2 exit from the cells and equilibration to the final value of ~ 2.5 mmHg. The peak value could not be recorded and the first cell pH value measured at the 6th s was 7.78 ± 0.03 ($n = 47$ from 16 different suspensions). This was followed by a secondary cell acidification phase (hereafter referred to as pH_i recovery), and the pH_i value was 7.45 ± 0.02 after 60 s; this relatively rapid pH_i recovery was mainly due to bicarbonate efflux from the cells for the following reasons. First, when cells were alkalized to a similar

extent by preincubation in an alkaline $\text{HCO}_3^-/\text{CO}_2$ free medium, pH 8.1 (solution C in Table I), and then abruptly diluted into a $\text{HCO}_3^-/\text{CO}_2$ free medium buffered at pH 7.4 (solution B in Table I), cells did not acidify as rapidly as they did when they contained bicarbonate (Fig. 2). Second, when the absolute values of the initial rates of pH_i recovery ($d\text{pH}_i/dt$)² were plotted against the corresponding intracellular bicarbonate concentrations (calculated from the final PCO_2 and the pH_i value measured at the 6th s), a positive relationship was obtained which tended to plateau at high intracellular concentrations of bicarbonate (Fig. 3), which indicated that the HCO_3^- transport mechanism was a saturable process. To obtain high intracellular bicarbonate concentration values, some cells were preincubated in a solution with bicarbonate concentrations and pH values up to 55 mM and 7.70, respectively. Lineweaver-Burk plot of these data led to an apparent intracellular $K_m(\text{HCO}_3^-)$ of ~ 16 mM (Fig. 3). Third, in the presence of 10^{-4} M DIDS in the spectrofluorometer cuvette, the pH_i recovery was inhibited; as shown in Fig. 4, the $d\text{pH}_i/dt$ values were lower by 40–50% in the presence than in the absence of DIDS for any intracellular bicarbonate concentration ($P < 0.05$). Also, we observed that, after 10 min of preincubation, 10^{-4} M DIDS increased the resting pH_i of MTAL cells incubated in the bicarbonate solution by 0.15 ± 0.04 pH unit ($n = 4$, $P < 0.01$).

Taken together, these findings suggest that bicarbonate exit from rat MTAL cells occurs by a carrier-mediated DIDS-sensitive transport mechanism. Similar results were obtained by Kikeri et al. (11) in suspensions of mouse MTAL tubules. Finally, initial rates of pH_i recovery were not significantly af-

2. The time course of pH_i recovery from alkalization was curvilinear but could be well fitted from the 6th to the 20th s to the following linear equation form: $\text{pH}_i = C - k \times \ln(t)$ (Eq. 1), in which C is pH_i at time 1 and the rate of change in pH_i at any time t_i is $d\text{pH}_i/dt = -k/t_i$ (Eq. 2); r values from these linear fits were ≥ 0.90 . Initial rate of pH_i recovery at the 6th s, i.e. at the time of the first measured pH_i value, was thus defined from Eq. 2 as $d\text{pH}_i/dt = -k/6$ in pH unit/s and expressed in Results in pH unit/min. Fitting the first 20 s of the pH_i time course to a linear function relating pH_i to $\ln(t)$ requires no assumption regarding the mechanisms of the pH_i response, but merely provides a straightforward means of quantitatively comparing experimental groups.

Figure 3. (A) Initial rate of pH_i recovery from alkalization (HCO_3^- loaded cells diluted in $\text{HCO}_3^-/\text{CO}_2$ free solution) in function of corresponding intracellular HCO_3^- concentration. (B) Lineweaver-Burk plot of the data gave a straight line ($y = 2.28x + 0.14$, $P < 0.01$) from which was derived the value of ~ 16 mM for intracellular $\text{HCO}_3^- K_m$.

Figure 4. Effect of DIDS on HCO_3^- efflux. Left panel: HCO_3^- -loaded cells were diluted in HCO_3^-/CO_2 -free medium in presence (●) or absence (○) of 10^{-4} M DIDS. Right panel: Initial rate of pHi recovery was lowered by DIDS at any intracellular bicarbonate concentration ($P < 0.05$, as assessed by analysis of covariance after linearization by Lineweaver-Burk analysis).

ected by 10^{-4} M acetazolamide (1.80 ± 0.48 pH unit/min, $n = 5$) or 10^{-4} M *t*-butyl-acetazolamide (compound devoid of anti-carbonic anhydrase activity; 2.54 ± 0.34 , $n = 5$) as compared with own controls (2.45 ± 0.26 , $n = 4$); these results indicate that carbonic anhydrase-dependent processes do not contribute importantly to pHi recovery, and that acetazolamide does not inhibit directly HCO_3^- transport in rat MTAL cells.

Chloride and sodium independence, and electroneutrality of HCO_3^- transport. When HCO_3^- -loaded cells were abruptly diluted into a HCO_3^-/CO_2 -free solution that was nominally chloride-free (solution D in Table I, chloride isoosmotically replaced with gluconate), the pHi recovery kinetics were not different from those observed under control conditions (Fig. 5). In the latter experiments, however, the final extracellular chloride concentration was ~ 7.5 mM and thus might have been sufficient to drive a possible Cl^-/HCO_3^- antiport. Therefore, we have performed other experiments in which the extracellular chloride concentration was reduced to zero by preincubating and washing the cells in a chloride-free bicarbonate solution; in the latter case also, the pHi recovery kinetics during HCO_3^- efflux were not different from controls ($n = 5$). These results clearly exclude Cl^-/HCO_3^- antiport as the mechanism of bicarbonate transport in rat MTAL cells. It must be emphasized that acutely removing chloride from the extracellular medium is known to largely depolarize TAL cells because of the chloride conductance present in the basolateral membrane of these cells (see reference 19 for review). Also, 2 mM barium, which inhibits potassium conductances and depolarizes TAL cells (19), did not affect the HCO_3^- efflux kinetics, as shown in

Fig. 5. Thus these results suggest that the bicarbonate transport mechanism in rat MTAL cells is electroneutral because unaltered by maneuvers known to cause cell membrane depolarization.

To confirm this point, acute variations in the cell membrane PD were directly estimated by use of the PD-sensitive fluorescent probe DiS-C3-(5), as described in Methods. The effects on the quench in DiS-C3-(5) fluorescence of removing chloride from and addition of 2 mM barium into the extracellular medium in the absence of bicarbonate are shown in B of Fig. 6. These maneuvers reduced the quench in DiS-C3-(5) fluorescence by $56 \pm 3\%$ ($P < 0.001$) and $12 \pm 4\%$ ($P < 0.05$), respectively. This corresponded to cell membrane depolarization that was particularly important with acute reduction of extracellular chloride. Thus these results demonstrate that concomitant substantial cell depolarization does not affect bicarbonate efflux in MTAL cells (Fig. 5). Also, we observed that bicarbonate efflux per se was not associated with any change in PD (A of Fig. 6). Furthermore, when HCO_3^- -loaded cells were diluted into a HCO_3^-/CO_2 -free and Na^+ -free solution (solution F in Table I), a moderate $14 \pm 5\%$ increase in the quench in DiS-C3-(5) fluorescence was observed (A of Fig. 6), which corresponded to hyperpolarization instead of an expected depolarization if $Na^+/(HCO_3^-)_n$ cotransport were to support bicarbonate efflux. The origin of the slight hyperpolarization caused by abrupt sodium removal (conductive sodium pathway) was not investigated in the present study aimed to determine the bicarbonate transport mechanism.

To further rule out the presence of $Na^+/(HCO_3^-)_n$ cotrans-

Figure 5. Lack of effect of chloride removal and barium on HCO_3^- efflux. Left panel: HCO_3^- -loaded cells were diluted in HCO_3^-/CO_2 -free medium containing 144 mM chloride (●), or that was chloride-free (chloride replaced with gluconate, □), or that contained 2 mM barium (○). Right panel: Initial rate of pHi recovery was not different in control, chloride-free, and barium situations at any intracellular HCO_3^- concentration.

Figure 6. Variations in MTAL cell PD evaluated from changes of quench in DiS-C₃-(5) fluorescence after addition of a constant amount of cells. *Left panel:* HCO₃⁻-loaded cells were diluted into 25 mM HCO₃⁻ (HCO₃⁻), or HCO₃⁻-CO₂-free (HCO₃⁻-free), or HCO₃⁻-CO₂- and sodium-free (HCO₃⁻ & Na free) solutions; slight hyperpolarization ($P < 0.05$) occurred in HCO₃⁻ and Na-free solution. *Right panel:* Cells were preincubated and diluted in HCO₃⁻/CO₂-free solution containing 144 mM chloride (C), or that was chloride-free (Cl Free), or that contained 2 mM barium (Ba); depolarization occurred in Cl-free ($P < 0.001$) and Ba ($P < 0.05$) conditions.

port, we examined whether an imposed outward-directed sodium gradient stimulates bicarbonate exit from rat MTAL cells. To this purpose, HCO₃⁻-loaded cells preincubated in a 138 mM sodium solution were diluted into a HCO₃⁻/CO₂- and sodium-free solution (solution F in Table I). However, in the latter case, it is necessary to avoid any contribution of variations in sodium-coupled H⁺ movements to the observed changes in pHi by use of amiloride at doses sufficient to inhibit both the Na⁺/H⁺ antiporter and a possible sodium channel. This necessity is demonstrated in Fig. 7 that summarizes the effects of amiloride on the pHi recovery of alkalinized cells containing or not bicarbonate; 10⁻³ M amiloride significantly enhanced the initial rate of pHi recovery both in the absence (0.75±0.25 vs. 0.075±0.023 pH unit/min, $P < 0.01$) and in the presence (3.27±0.50 vs. 2.03±0.16 pH unit/min, $P < 0.001$) of bicarbonate inside the cell. Note that the amiloride-induced increases in pHi recovery rates after intracellular alkalinization were of similar degrees in both situations. These

results clearly show that some proton efflux that is inhibited by amiloride, and thus also by abrupt removal of external sodium, slows down the rate at which MTAL cells recover from intracellular alkalinization. Thus in the presence of amiloride in both control and experimental runs, as shown in Fig. 8, an abrupt decrease in extracellular sodium concentration from 138 to 9 mM had no effect on pHi recovery caused by bicarbonate efflux. Note that the kinetics of pHi recovery in the sodium-free medium were the same, as expected, whether amiloride was present or not (not shown) because Na⁺/H⁺ exchange was inhibited in both cases.

Thus these results exclude Cl⁻/HCO₃⁻ antiport and Na⁺/(HCO₃⁻)_n cotransport as mediating bicarbonate efflux and demonstrate that the main bicarbonate transport mechanism is electroneutral (no major HCO₃⁻ conductance) in rat MTAL cells.

Potassium dependence of HCO₃⁻ transport. To determine whether the electroneutral HCO₃⁻ transport mechanism in rat MTAL cells is dependent on potassium, we carried out two series of experiments. First, possible effects on bicarbonate efflux of acutely raising the extracellular potassium concentration were evaluated by diluting HCO₃⁻-loaded cells into a HCO₃⁻/CO₂-free solution containing 5 (solution F in Table I) or 120 mM (solution G in Table I) potassium. In both cases, the experimental solution was nominally sodium-free (NaCl replaced with KCl and/or TMA-Cl) to avoid any difference in the transmembrane sodium gradient. As shown in Fig. 9, the initial rate of pHi recovery caused by bicarbonate efflux was significantly inhibited by a high extracellular potassium concentration (1.78±0.35 vs. 2.77±0.33 pH unit/min, $P < 0.05$). Since bicarbonate transport is electroneutral, this result suggests the presence in these cells of a K⁺/HCO₃⁻ cotransport mechanism. Second, we tested for the dependence of bicarbonate transport on the intracellular potassium concentration. MTAL cells were potassium-depleted by several washings in a potassium-free solution containing 60 mM bicarbonate (solution H in Table I) during 30 min of preincubation. These potassium-depleted and sodium-enriched cells (Na⁺-K⁺-ATPase inhibition) were further HCO₃⁻-loaded by adding to the medium 10 μM monensin (a Na⁺/H⁺ ionophore) to set up the pHi at ~ 7.42 with a mean intracellular bicarbonate concentration of ~ 26 mM which is a value slightly higher than that of control cells. Then, after monensin was removed by BSA, bicarbonate efflux was imposed by diluting these cells into a potassium- and

Figure 7. Effect of amiloride on pHi recovery from alkalinization. (A) Cells alkalinized by preincubation at pH 8.1 in HCO₃⁻-free solution were diluted into same medium but at pH 7.4 in absence (■, dpHi/dt = 0.075±0.023 pH unit/min) or presence of 10⁻³ M amiloride (□, dpHi/dt = 0.75±0.25 pH unit/min, $P < 0.01$). (B) HCO₃⁻-loaded cells were diluted into HCO₃⁻/CO₂-free medium in absence (●, dpHi/dt = 2.03±0.16 pH unit/min) or presence of 10⁻³ M amiloride (○, dpHi/dt = 3.27±0.50 pH unit/min, $P < 0.001$).

Figure 8. Lack of effect of external sodium removal on HCO_3^- efflux. HCO_3^- -loaded cells were diluted into $\text{HCO}_3^-/\text{CO}_2$ -free solution containing 138 mM sodium ($\circ$) or that was sodium-free (Na replaced with TMA, $\square$). Amiloride (2×10^{-3} M) was present in the two conditions.

$\text{HCO}_3^-/\text{CO}_2$ -free solution (solution I in Table I). As shown in Fig. 10, pH_i recovery caused by bicarbonate efflux was markedly inhibited in potassium-depleted cells as compared with that in control cells, with 2×10^{-3} M amiloride present in both situations; intracellular potassium depletion lowered the dpH_i/dt for any initial intracellular bicarbonate concentration (right panel of Fig. 10, $P < 0.0001$). A quite similar result was obtained when MTAL cells were potassium-depleted by preincubation during 30 min in a 60 mM bicarbonate-solution (solution A in Table I, but containing 60 mM HCO_3^- , pH 7.8) containing 10^{-3} M ouabain (Fig. 10); indeed, ouabain has been shown to cause potassium depletion in TAL cells (20).

Thus it is clear that electroneutral bicarbonate efflux from rat MTAL is inhibited by lowering the intracellular and raising the extracellular potassium concentration, which is consistent with the presence in these cells of a $\text{K}^+/\text{HCO}_3^-$ cotransporter.

Bicarbonate dependence of K^+ transport. To directly demonstrate the presence in rat MTAL cells of a HCO_3^- -dependent K^+ transport, we measured net K^+ efflux out of the cell by use of an extracellular K^+ electrode as described in Methods. To impose an outward-directed K^+ gradient and improve the quantification of K^+ addition to the extracellular medium, cells were preincubated in a HCO_3^- -medium containing 5 mM K^+ and abruptly diluted into low- K^+ media (0.36 ± 0.01 mM final K^+ concentration); the latter media contained 10^{-3} M ouabain, 10^{-4} M furosemide, and 10 mM barium chloride to inhibit Na^+/K^+ -ATPase, $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter, and

K^+ channels, respectively; note that a possible K^+/Cl^- cotransporter should also have been inhibited since this transporter is sensitive to both barium and furosemide (21, 22). The results are summarized in Fig. 11. When cells were preincubated and diluted into 25 mM HCO_3^- -containing media, the net K^+ efflux rate (dK_e^+/dt) was relatively low at 23.1 ± 0.6 nmol/mg cell protein per min ($n = 16$); when an outward-directed HCO_3^- gradient was imposed by diluting HCO_3^- -loaded cells into a low- HCO_3^- medium (~ 0.65 mM final HCO_3^- concentration), dK_e^+/dt was augmented to 49.0 ± 0.9 nmol/mg cell protein per min ($n = 13$, $P < 0.001$ as compared with the value in the absence of outward-directed HCO_3^- gradient) (Fig. 11 A). As shown in Fig. 11 B, this HCO_3^- -stimulated K^+ efflux was lowered by 10^{-4} M DIDS to 38.1 ± 1.2 nmol/mg cell protein per min ($n = 9$, $P < 0.001$). At the end of each run, digitonin was added to permeabilize the cell plasma membrane and completely release the cytosolic potassium into the medium; by subtracting the basal medium potassium content from the post-digitonin value, estimates of cytosolic K^+ contents were obtained that were not different between the various experimental conditions; the mean cell K^+ content was 340 ± 11 nmol per mg cell protein ($n = 38$). Thus it is clear that a HCO_3^- -dependent DIDS-sensitive K^+ transport takes place in rat MTAL cells.

Discussion

The key observations of this study of suspensions of rat MTAL tubules were the following: (a) bicarbonate exit from the cells occurred via a saturable process that was inhibited by DIDS; (b) bicarbonate transport was not dependent on chloride or sodium; (c) cell membrane depolarization by abrupt removal of chloride from or the presence of barium into the extracellular medium did not alter bicarbonate transport, and bicarbonate efflux per se did not change the cell membrane PD; (d) bicarbonate transport out of the cell was inhibited by a rise in the extracellular potassium concentration and by intracellular potassium depletion; and (e) a component of potassium transport out of the cell was bicarbonate dependent and DIDS sensitive. Taken together, these findings provide evidence for the presence in rat MTAL cells of a chloride- and sodium-independent, stilbene-sensitive, electroneutral $\text{K}^+/\text{HCO}_3^-$ cotransporter, which is described for the first time in the present work

Figure 9. Effect of extracellular potassium concentration on HCO_3^- efflux. HCO_3^- -loaded cells were diluted into $\text{HCO}_3^-/\text{CO}_2$ -free and Na-free (NaCl replaced with KCl and/or TMA-Cl) solution containing 5 mM ($\circ$; $\text{dpH}_i/\text{dt} = 2.77 \pm 0.33$ pH unit/min) or 120 mM potassium ($\blacktriangle$; $\text{dpH}_i/\text{dt} = 1.78 \pm 0.35$, $P < 0.005$).

Figure 10. Effect of intracellular potassium depletion on HCO_3^- efflux. Cells were K^+ -depleted by preincubation and several washings in K^+ -free solution (Δ) or by preincubation with 10^{-3} M ouabain (∇) during 30 min; these K^+ -depleted cells were HCO_3^- -loaded in 60 mM HCO_3^- solution and then transferred into $\text{HCO}_3^-/\text{CO}_2$ -free solution. *Left panel:* Evolution of pH_i is shown and compared with that in control cells ($\circ$), with 2×10^{-3} M amiloride present in the three conditions. *Right panel:* Initial rate of pH_i recovery was lower in K^+ -depleted vs. control cells for any corresponding intracellular HCO_3^- concentration ($P < 0.0001$, as assessed by analysis of covariance after linearization by Lineweaver-Burk analysis).

to our knowledge. The luminal or basolateral localization of this new transporter cannot be assessed in our tubule suspension; however, because $\text{K}^+/\text{HCO}_3^-$ cotransport is the only mechanism of bicarbonate efflux that was detected in the present work, and because transepithelial bicarbonate absorption takes place in the rat MTAL (3), it seems reasonable to assume that the $\text{K}^+/\text{HCO}_3^-$ cotransporter is located in the basolateral membrane while both Na^+/H^+ antiport and vacuolar-type H^+ -ATPase may mediate proton secretion through the luminal membrane of the cells of this segment (8).

The method used in the present study to analyze the bicarbonate transport mechanism of the rat MTAL, i.e., dilution of $\text{HCO}_3^-/\text{CO}_2$ -loaded cells into a low- $\text{HCO}_3^-/\text{CO}_2$ medium, has been previously documented (18). Because outward-directed gradients were established simultaneously for HCO_3^- and CO_2 , dissipation of the HCO_3^- gradient could occur by direct efflux of HCO_3^- or by conversion of HCO_3^- to H_2CO_3 and subsequent

diffusion of CO_2 out of the cell. Clearly, the latter mechanism that consumes protons was responsible for the initial intracellular alkalization, which ended when the PCO_2 inside and outside the cell was equilibrated at ~ 2.5 mmHg. Then the secondary cell acidification phase was due specifically to HCO_3^- efflux as discussed in Results. Indeed, the acidification response was not reproduced with intracellular alkalinity alone (in the absence of bicarbonate, Fig. 2), exhibited saturability with respect to intracellular bicarbonate concentration (Fig. 3), and was inhibited by DIDS (Fig. 4); DIDS raised by ~ 0.15 pH unit the resting pH_i of MTAL cells incubated in a bicarbonate-medium.

The electroneutrality of the HCO_3^- transport mechanism of rat MTAL cells is firmly established in the present work. The use of DiS-C3-(5) fluorescence for estimating variations in the cell membrane PD, which was previously applied with success in renal cells (15), provided a very sensitive method in this study also (Fig. 1). Experimental maneuvers that depolarized the cell membrane, i.e., addition of barium and particularly abrupt removal of external chloride, did not modify the bicarbonate efflux kinetics, and acute bicarbonate exit out of the cell per se did not alter the cell PD. Note, for comparison, that in studies dealing with an electrogenic $\text{Na}^+/(\text{HCO}_3^-)_n$ cotransporter in the proximal tubule, depolarizing the cell membrane inhibited bicarbonate efflux and imposing acute bicarbonate exit produced major cell depolarization (reviewed in reference 9). In addition in the present work, abrupt sodium removal from the extracellular medium did not stimulate bicarbonate transport out of the cell in the presence of amiloride (Fig. 8). These results clearly rule out an electrogenic HCO_3^- pathway such as a HCO_3^- conductance and particularly a $\text{Na}^+/\text{HCO}_3^-$ cotransport in rat MTAL cells.

The latter conclusion is at variance with that of Kikeri et al. (11) favoring the presence of an electrogenic $\text{Na}^+/(\text{HCO}_3^-)_n$ symporter in mouse MTAL cells studied with the same experimental protocol as that in the present study. The presence of a $\text{Na}^+/(\text{HCO}_3^-)_n$ symporter was assumed from the observation that an abrupt reduction of the extracellular sodium concentration stimulated the intracellular acidification phase following the transfer of HCO_3^- -loaded cells into a $\text{HCO}_3^-/\text{CO}_2$ -free medium, which was not inhibited by amiloride (11). In fact, it is shown in the present work that the acidification response of

Figure 11. Net K^+ efflux. Cells were preincubated in 5 mM- K^+ medium and transferred in low- K^+ media containing 10^{-3} M ouabain, 10^{-4} M furosemide, and 10 mM barium; after a 15-s mixing time, net addition of K^+ into the extracellular medium was expressed as ΔK_e . (A) Net K^+ efflux was higher when extracellular HCO_3^- was ~ 0.65 mM ($\bullet$) than 25 mM ($\circ$) ($P < 0.001$). (B) 10^{-4} M DIDS (Δ) significantly lowered net K^+ efflux in presence of ~ 0.65 mM extracellular HCO_3^- ($P < 0.001$).

alkalinized MTAL cells is on the contrary stimulated by amiloride whether the cells contain or not bicarbonate (Fig. 7), which may therefore be explained by inhibition by amiloride of proton efflux out of the cells through Na^+/H^+ antiport. Results in the present study (Fig. 8) show that abrupt sodium removal does not stimulate bicarbonate efflux from MTAL cells when the latter is studied in the presence of amiloride in both control and experimental conditions. Moreover, in direct contradiction, Kikeri et al. (11) also reported that abrupt removal of external chloride, which is shown in the present work to greatly depolarize MTAL cells in suspension, did not affect HCO_3^- efflux from mouse MTAL cells. Yet, the sensitivity of the $\text{Na}^+/(\text{HCO}_3^-)_n$ cotransporter to imposed modifications of the cell PD is considered as one of the major criteria for identifying this transporter (23). Thus these considerations strongly raise question about the presence of a $\text{Na}^+/(\text{HCO}_3^-)_n$ cotransporter and suggest that electroneutral $\text{K}^+/\text{HCO}_3^-$ cotransport also takes place in mouse MTAL cells.

That a $\text{Na}^+/(\text{HCO}_3^-)_n$ cotransporter is present in the basolateral membrane of rat CTAL cells seems to have been well documented by Krapf (10). However, it may be noted that, in the latter study (10), increasing the bath potassium concentration significantly alkalinized the cells in the presence of bicarbonate; this was interpreted to result from cell depolarization because the potassium-induced cell alkalinization was reduced by 2 mM barium. However, the potassium-induced cell alkalinization was not abolished by barium in the absence of chloride (25% of control alkalinization) and thus it appears that the presence of a $\text{K}^+/\text{HCO}_3^-$ cotransporter, which was not tested for specifically, is not ruled out in the cortical segment also of the rat TAL.

Because results in the present study had established that the HCO_3^- transport mechanism of rat MTAL cells was electroneutral and did not depend on sodium or chloride, we raised the hypothesis that it could depend on potassium, the major intracellular cation. We found that raising the extracellular potassium concentration (Fig. 9) or lowering intracellular potassium by two different maneuvers (Fig. 10) inhibited an imposed bicarbonate efflux from the cells. Moreover, potassium efflux, as measured with a K^+ -selective extracellular electrode, had a component that was bicarbonate-dependent and DIDS-sensitive (Fig. 11); it must be emphasized that hypothetical indirect effects of bicarbonate on potassium efflux cannot be invoked to explain the latter result since the other potassium transport mechanisms were inhibited by the appropriate agents. Thus, these results demonstrate the presence of an electroneutral $\text{K}^+/\text{HCO}_3^-$ cotransport in the rat MTAL. In this respect, no comparison can be made with other studies since a $\text{K}^+/\text{HCO}_3^-$ cotransporter has not been described previously in mammalian cells. In a recent study, however, published in abstract form, Boron and Hogan (24) have suggested that the pHi recovery from an alkali load may involve the cotransport of K^+ and HCO_3^- in the squid giant axon. Thus the $\text{K}^+/\text{HCO}_3^-$ cotransporter may take place in cell types other than the MTAL cell and may be important in pHi regulation as well as in transepithelial bicarbonate transport. Note that we found an apparent intracellular $K_{m(\text{HCO}_3^-)}$ of ~ 16 mM (Fig. 3) which is very close to the physiological intracellular bicarbonate concentration and may make the $\text{K}^+/\text{HCO}_3^-$ cotransporter very sensitive to small variations in the latter. Because this study was performed at 25°C , additional work is needed at 37°C to specify

the physiological characteristics of the $\text{K}^+/\text{HCO}_3^-$ cotransporter with respect to HCO_3^- and K^+ . It can be already accepted, however, that electroneutral $\text{K}^+/\text{HCO}_3^-$ cotransport realizes a very efficient secondary active HCO_3^- transport out of the cells since it is driven by the outward-directed K^+ concentration gradient normally created and maintained by $\text{Na}^+/\text{K}^+-\text{ATPase}$ activity.

In the present study, inhibition of the $\text{K}^+/\text{HCO}_3^-$ cotransporter by DIDS alkalinized MTAL cells incubated in a bicarbonate solution, which suggests a role of this transporter in pHi regulation. Indeed, $\text{K}^+/\text{HCO}_3^-$ cotransport was the main mechanism by which MTAL cells recovered from intracellular alkalinization under the conditions of this study. With respect to transepithelial bicarbonate transport, Good (25) has observed in the isolated rat MTAL perfused in vitro that increasing external potassium concentration from 4 to 24 mM had no effect on steady-state bicarbonate absorption; the latter result is, however, difficult to analyze with respect to the $\text{K}^+/\text{HCO}_3^-$ cotransporter described in the present study because potassium concentration was raised in both the perfusate and bath, which could have resulted in various direct, indirect, and possible opposite effects on basolateral bicarbonate transport and luminal proton secretion in the steady state. Selective large variations of the potassium concentration in the medullary interstitium, which may occur in vivo due to potassium recycling depending on the state of potassium balance (26–28), may modulate the $\text{K}^+/\text{HCO}_3^-$ cotransporter and thus bicarbonate absorption by the rat MTAL. The latter possibility could contribute to the acid-base disorders that are observed in a variety of pathologic conditions that include potassium imbalance (29).

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