Regulation of Intracellular pH in the Rabbit Cortical Collecting Tubule

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Abstract

The cortical collecting tubule (CCT) is an important nephron segment for Na^+, K^+, water and acid-base transport. Differential loading characteristics of the pH sensitive dye 2',7'-bis-(2-carboxyethyl)-5(and-6)carboxyfluorescein (BCECF) and basolateral Cl^- removal were used to identify and study intracellular pH (pH_i) regulation in each of three cell types involved in this transport. Both principal cells and β-intercalated cells were found to have a basolateral Na^+/H^+ exchanger based on the Na^+ and amiloride sensitivity of pH_i recovery from acid loads.

Intercalated cells demonstrated abrupt pH_i changes with basolateral Cl^- removal. α-intercalated cells alkalinized; β-intercalated cells acidified. In the β-intercalated cells, luminal Cl^- removal blocked changes in pH_i in response to changes in luminal HCO_3^- or peritubular Cl^-, providing direct evidence for a luminal Cl^-/HCO_3^- exchanger. In principal cells, brief removal of either peritubular or luminal Cl^- resulted in no change in pH_i; however, return of peritubular Cl^- after prolonged removal resulted in a rapid fall in pH_i consistent with a basolateral Cl^-/HCO_3^- exchanger, which may be relatively inactive under baseline conditions. Therefore, Cl^-/HCO_3^- exchange is present in all three cell types but varies in location and activity. (*J. Clin. Invest.* 1990. 85:274–281.) 2',7'-bis-(2-carboxyethyl)-5(and-6)carboxyfluorescein (BCECF) • Cl^-/HCO_3^- exchange • intercalated cell • Na^+/H^+ exchange • principal cell

Introduction

The collecting tubule of the mammalian nephron is the segment of final modulation of urinary excretion of Na^+, K^+, water, and acid-base equivalents. In the cortical collecting tubule (CCT)[1] regulation of the excretion or reabsorption of each of these occurs. Corresponding with the diversity of functions, the CCT is a heterogenous tissue composed of multiple cell types: principal cells and at least two types of intercalated cells (1, 2).

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Received for publication 9 June 1989 and in revised form 12 September 1989.

1. *Abbreviations used in this paper:* ADH, antidiuretic hormone; BCECF, 2',7'-bis-(2-carboxyethyl)-5(and-6)carboxyfluorescein); BCECF-AM, acetoxymethyl ester of BCECF; CCT, cortical collecting tubule; pH_i, intracellular pH.

J. Clin. Invest.

0021-9738/90/01/0274/08 $2.00
Volume 85, January 1990, 274–281

Principal cells are the most numerous cell type and appear to function primarily to reabsorb Na^+, secrete K^+, and modulate arginine-vasopressin induced water reabsorption (3, 4). Principal cell pH_i regulation may be important in the regulation of salt and water transport by this cell. Intracellular acidosis appears to decrease Na^+ transport in the CCT and analogous epithelia (5–9). In addition, pH_i may alter the hydroosmotic response to antidiuretic hormone (ADH) and cAMP (10–12). Conversely, ADH appears to activate numerous acid-base transporters in both renal (13, 14) and nonrenal tissues (15, 16). ADH may also have effects on urinary acidification (17).

Intercalated cells are interspersed between principal cells, make up ~ 35–40% of the total cells of the CCT (1, 2), and both secrete (18) and reabsorb HCO_3^- (19). At least two subtypes of intercalated cells are present. The α-intercalated cell secretes H^+ and reabsorbs luminal HCO_3^-. An electrogenic, H^+ translocating ATPase is present at the luminal membrane (20). HCO_3^- exits the cell via a basolateral Cl^-/HCO_3^- exchanger. The Cl^-/HCO_3^- exchanger is sensitive to the disulfonic stilbenes (21) and is immunologically similar to the Cl^-/HCO_3^- exchanger of the mammalian red blood cell, band 3 protein (22, 23).

The second type of intercalated cell is the β-intercalated cell. The β-intercalated cell is postulated to be responsible for HCO_3^- secretion by the CCT. This transport is modeled to occur via a luminal Cl^-/HCO_3^- exchanger and a basolateral H^+-ATPase. This cellular model has been derived in large part by indirect inferences from transepithelial flux measurements. The basolateral location of H^+-ATPase has been confirmed by immunocytochemical studies (20). However, direct confirmation of the luminal Cl^-/HCO_3^- exchanger has been difficult. Antibodies to band 3 protein do not stain the apical membrane (22, 23) and luminal disulfonic stilbenes do not inhibit bicarbonate secretion (21, 24). As a result, direct confirmation of the luminal Cl^-/HCO_3^- exchanger in the β-intercalated cell has been lacking.

The purpose of the present studies was to characterize the distribution of the major mechanisms of pH_i regulation in two of the cell types (principal cell and β-intercalated cell) present in the rabbit CCT. Evaluation of the mechanisms of pH_i regulation in the CCT has been difficult due to the cellular heterogeneity of this segment of the nephron. We have recently described a technique whereby principal cell and intercalated cell pH_i can be separately measured in the in vitro, microperfused rabbit CCT using the fluorescent, pH sensitive dye 2',7'-bis-(2-carboxyethyl)-5(and-6)carboxyfluorescein (BCECF) (25). Therefore, the specific purpose of these studies was to characterize the distribution of Na^+/H^+ and Cl^-/HCO_3^- exchangers in the rabbit CCT.

Methods

Microperfusion. In vitro microperfusion of cortical collecting tubules was performed using standard techniques (26). Dissection of individ-

*Table I. Solutions**

	1	2	3	4	5	6	7	8
NaCl	119.2	–	–	119.2	–	144.2	119.2	–
Choline chloride	–	119.2	–	–	119.2	–	–	–
Sodium gluconate	–	–	119.2	–	–	–	20.0	139.2
$NaHCO_3$	25	–	25	–	–	–	5	5
Choline bicarbonate	–	25	–	–	–	–	–	–
Hepes	–	–	–	25	25	–	–	–
KCl	3	2	–	3	2	3	3	–
Potassium gluconate	–	–	3	–	–	–	–	3
Sodium acetate	1	–	1	1	–	1	1	1
Potassium acetate	–	1	–	–	1	–	–	–
$CaCl_2$	1.2	1.2	–	1.2	1.2	1.2	1.2	–
Calcium gluconate	–	–	3.4	–	–	–	0.5	5.2
KH_2PO_4	2	2	2	2	2	2	2	2
$MgSO_4$	1	1	1	1	1	1	1	1
Alanine	5	5	5	5	5	5	5	5
Glucose	8.3	8.3	8.3	8.3	8.3	8.3	8.3	8.3

* All concentrations expressed in millimoles per liter. Osmolality adjusted to 285–295 mosmol/kg by addition of the major salt. Solutions 4 and 5 bubbled with 100% O_2, all others with 95% O_2/5% CO_2.

ual CCT was performed in cold solution 1 (for studies with CO_2 containing solutions) or in solution 4 (for studies with CO_2 free solutions) (see Table I) containing 5% fetal calf serum. Tubules were studied in a 1-ml chamber thermostatically controlled to 37°C; the peritubular bathing solution was continuously exchanged at a rate of ~ 3 ml/min. CO_2-impermeable Saran tubing (Clarkson Equipment & Controls, Detroit, MI) was used to deliver the bathing solution. Bath pH was continuously monitored with a flexible pH electrode (model MI-508; Microelectrodes, Inc., Londonderry, NH).

Solutions. The components of the various solutions used in the study are shown in Table I. Solutions 1–3, 6–8 were used for experiments performed in the presence of CO_2; solutions 4 and 5 were used for experiments performed in the nominal absence of CO_2. CO_2 containing solutions were bubbled with 95% O_2/5% CO_2. CO_2-free solutions were bubbled with 100% O_2. CO_2-free solutions were adjusted to pH 7.40 with tetramethylammonium hydroxide instead of sodium hydroxide in order to minimize the Na^+ concentration of Na^+ free solutions. Total Ca^{2+} was increased in solutions 3, 7, and 8 to compensate for complexing of Ca^{2+} by gluconate. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

Fluorescent dyes. The acetoxymethyl ester of BCECF (BCECF-AM), was obtained from Molecular Probes, Inc. (Eugene, OR) and maintained at −20°C as a 30-mM stock solution in DMSO. On the day of an experiment 15 μM (for luminal loading, see below) or 5 μM (for basolateral loading, see below) solutions were made by diluting with either solution 1 (for CO_2/HCO_3^- containing experiments) or solution 4 (for CO_2/HCO_3^- free experiments).

Loading with BCECF was performed as previously described (25). In brief, luminal BCECF-AM is selectively concentrated by intercalated cells, while peritubular BCECF-AM is homogeneously taken up by both principal and intercalated cells (25). As a result, intercalated cells were studied after loading with luminal BCECF-AM. Principal cells were studied by loading first with luminal BCECF-AM, identifying an area of the tubule without intercalated cells, and then loading with peritubular BCECF-AM. In all cases at least 5 min was allowed after loading BCECF-AM before measurement of pH_i.

Intracellular pH measurements. Fluorescence studies were performed on a Nikon Diaphot-TMD microscope modified for fluorescent use as previously described (25). pH_i measurements were made by exciting an area of ~ 5 μm diam. In general this field was positioned at the edge of the tubule to minimize fluorescence from cells above or below the plane of measurement. Use of a small excitation field centered at the edge of the tubule thereby allowed measurement of the pH_i of either a single intercalated cell or portions of approximately one to four principal cells. pH_i measurements were made by alternatively exciting at 500 and 450 nm. Fluorescence at 530 nm was measured by a Nikon P1 photometer. The analog output from the photometer was digitized (8232; Starbuck Data Co., Waltham, MA) and recorded on a personal computer for analysis at a later time. Background fluorescence was < 5% of dye fluorescence at both excitation wavelengths and was subtracted before calculation of fluorescence ratio. pH_i results were graphed using a moving 10–15-s time average technique. These tracings were then used for measurement of pH_i for analysis. Although amiloride is a fluorescent molecule, 1 mM peritubular amiloride had no effect on fluorescent ratio in cells which were clamped at a fixed pH_i using the high-K^+/Nigericin technique of Thomas et al. (27) (results not shown).

Changes in pH_i after acid loading are expressed (except where specifically noted) as the difference in pH_i between 1 and 5 min after the solution change; the initial 1-min time point was chosen to match the nadir of pH_i after acid loading and to ensure complete solution change.

A change in the luminal fluid required a period of ~ 1–3 min during which pH_i was not measured. After completion of the perfusate change, pH_i was measured for at least 5 min or until it had stabilized. The pH_i at the end of this period was used for analysis.

Calibration. Calibration of intracellular BCECF was performed using the method of Thomas et al. (27). The calibration solution contained (in mM) 120 KCl, 1.2 $CaCl_2$, 1.0 $MgCl_2$, 2.0 NaH_2PO_4, 25 Hepes, and 14 μm nigericin and was adjusted to pH 6.8, 7.0, 7.2, 7.4, 7.6, and 7.8 using NaOH and HCl. Least-squares, linear regression was performed to fit the calibration points to the equation: ratio (500/450nm) = $a + b \cdot pH_i$. The calibration curve was then used for conversion of calculated fluorescent ratio to pH_i. Separate calibration curves were performed for intercalated cells and principal cells. In most experiments a calibration was performed for the cell type studied in that experiment and used for conversion of calculated ratio to pH_i; in a few experiments mean calibration curves from prior experiments were used for conversion. Dye concentration during calibration, as measured by fluorescence at 450 nm excitation, was similar to that during experimental pH_i measurements.

Acid loading. Acid loading was achieved by changing the peritubular solution to one containing 10 mM NH_4Cl (for principal cells and some β-intercalated cells) or 20 mM NH_4Cl (for some β-intercalated

cells) for a period of 5 min; removal of NH_4Cl resulted in an abrupt acidification. Equimolar amounts of the principal salt in the NH_4Cl containing solutions were removed so that solution osmolality remained constant. Although 20 mM NH_4Cl resulted in slightly greater acidification, no qualitative differences in results were seen.

Statistics. Values are presented as mean±SEM. In general, statistical tests are performed using paired, two sided Student's *t* test. Unpaired, two-sided Student's *t* tests were used when appropriate and are noted in the text. Statistical significance is defined as at least $P < 0.05$. All pH_i results are reported as the mean of *n* tubules.

Results

Principal cell pH_i. Baseline pH_i for principal cells was obtained in tubules bathed and perfused with identical solutions for at least 30 min before any measurements. The baseline pH_i in CO_2 containing solutions (solution 1) was 7.36±0.05 ($n = 13$), while in the absence of CO_2 (solution 4) the baseline pH_i was significantly higher at 7.77±0.06 ($n = 13$) ($P < 0.001$ by unpaired *t* test).

Principal cell Na^+/H^+ exchange. The importance of Na^+/H^+ exchange on principal cell pH_i recovery after an acid load was investigated. All experiments evaluating principal cell Na^+/H^+ exchange were performed in CO_2/HCO_3^- free solutions in order to minimize the effect of HCO_3^- transporters on pH_i regulation. A 5-min application and then removal of 10 mM ammonium chloride (solution 5 plus NH_4Cl) was used to acidify principal cells to a mean pH_i of 6.84±0.09 ($n = 10$). After 5 min in a Na^+ free peritubular solution (solution 5) principal cell pH_i increased by only 0.16±0.11 pH U ($n = 6$, NS from 0.00). By contrast, after 5 min in a Na^+ containing peritubular solution (solution 4), pH_i in the same principal cells increased 0.50±0.05 pH U ($n = 6$, $P < 0.05$ vs. Na^+ free). These results show that principal cells possess a Na^+ dependent mechanism for pH_i regulation after an acid load. (In most experiments the peritubular bathing solution was changed first to a Na^+ free solution for 5 min and then to the Na^+ containing peritubular solution; the principal cells were generally at the same, or occasionally slightly higher, initial pH_i on exposure to the Na^+ containing solution. Since the Na^+/H^+ exchanger has been well characterized to be inhibited by an increased pH_i, the increased rate of recovery of pH_i during Na^+ containing basolateral solutions cannot be explained by the difference in the initial pH_i.)

To confirm this Na^+ dependent pH_i recovery as Na^+/H^+ exchange, the inhibitory effect of 1 mM amiloride on pH_i recovery after an acid load was investigated. A typical experiment is shown in Fig. 1. In the presence of peritubular amiloride, the pH_i recovery after 5 min was 0.13±0.05 pH U, while in the absence of amiloride pH_i recovery in the same cells was 0.67±0.08 ($n = 7$, $P < 0.005$ by paired *t* test). These results suggest that a basolateral, amiloride inhibitable, Na^+-dependent mechanism, i.e., Na^+/H^+ exchange, is a major mechanism for principal cell recovery from an acid load.

To determine if Na^+/H^+ exchange is active at baseline pH_i, the effect of Na^+ removal on principal cell pH_i was investigated. Removal of Na^+ from the peritubular solution (changing to solution 5) resulted in a slow acidification of 0.10±0.04 pH U ($n = 8$, $P < 0.05$) over a 5-min period.

Cl^- and principal cell pH_i. Cl^- dependent pH_i regulatory processes appear to be present in most cell types (28); the presence of these was investigated next. Removal of Cl^- from

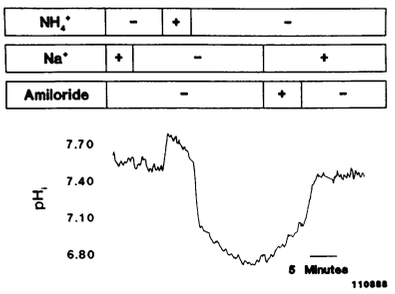

Figure 1. Effect of peritubular Na^+ and amiloride on principal cell pH_i recovery after an acid load. The ammonium chloride pulse technique is used to acid load the cells. In the absence of peritubular Na^+ almost no recovery from intracellular acidification takes place, in fact there is a slow continued acidification in this experiment. When the peritubular solution is changed to one containing 1 mM amiloride and ~ 145 mM Na^+ there is some recovery of pH_i. However, removal of amiloride results in a marked increase in rate of pH_i recovery and a rapid return of pH_i to baseline. Not well demonstrated in this experiment is a slow acidification during removal of peritubular Na^+ before the ammonium chloride pulse. In general, Na^+ free and amiloride containing protocols were not performed on the same principal cell (as performed in this experiment).

CO_2/HCO_3^- containing luminal solutions (changing the perfusate from solution 1 to solution 3) for 5–10 min resulted in no significant change in principal cell pH_i (difference −0.02±0.03, P = NS, $n = 4$). Similarly, return of Cl^- to the luminal fluid (change back to solution 1) resulted in no significant change in pH_i (difference −0.01±0.04, P = NS, $n = 4$). Evidence for a basolateral Cl^- linked pH_i regulatory mechanism was then studied. A 5-min removal and then return of Cl^- to the peritubular solution (change from solution 1 to solution 3 and then return to solution 1) resulted in no significant changes in principal cell pH_i (differences −0.02±0.03 and −0.06±0.03, for removal and return of peritubular Cl^-, respectively, $n = 5$, P = NS for both). However, since intracellular Cl^- concentrations in principal cells are quite low, ~ 7–14 mmol/kg dry wt in the rat (29, 30) and principal cells possess a basolateral Cl^- channel (31), removal of peritubular Cl^- may result in gradients for Cl^-/HCO_3^- exchange that are relatively small, possibly explaining the relative absence of pH_i changes after 5 min of peritubular Cl^- removal. A prolonged removal of peritubular Cl^- might result in intracellular Cl^- depletion and an increased gradient for Cl^- entry during return of Cl^- to the peritubular solution. A 30-min incubation in a Cl^- free peritubular solution (solution 3) resulted in a slow increase in pH_i from 7.35±0.08 to a maximum of 7.55±0.10 ($P < 0.005$, $n = 5$). After return of peritubular Cl^- (change back to solution 1) pH_i fell rapidly. 5 min after the change pH_i had fallen by 0.30±0.04 pH U ($P < 0.005$ by paired *t* test, $n = 5$). These results are consistent with the exchange of intracellular HCO_3^- for extracellular Cl^-, i.e., basolateral Cl^-/HCO_3^- exchange activity, during the return of basolateral Cl^- to Cl^- depleted principal cells.

Intercalated cell-baseline pH_i and CO_2/HCO_3^-. β-intercalated cell baseline pH_i was measured in the presence and absence of CO_2/HCO_3^-. In the presence of CO_2/HCO_3^- (luminal and peritubular solution 1) the baseline pH_i was 7.33±0.04 ($n = 32$). In the nominal absence of HCO_3^-/CO_2 (luminal and peritubular solution 4), β-intercalated cell pH_i was 8.05±0.02 ($n = 4$, $P < 0.001$ vs. pH_i in the presence of CO_2/HCO_3^- by unpaired *t* test). However, BCECF may not reliably reflect true pH_i at such extremes of pH. Consistent with this is the finding

that in the nominal absence of CO_2 and HCO_3^- no change was seen in β-intercalated cell apparent pH_i with either addition or removal of basolateral NH_4^+ (data not shown). Therefore, all subsequent intercalated cells experiments were performed with CO_2 present in the both the peritubular and luminal solutions.

Intercalated cells, identification of subtype by Cl^- removal. Basolateral Cl^- removal resulted in two distinct patterns of pH_i response in intercalated cells. Occasional cells underwent a rapid alkalinization from pH_i 7.17±0.07 to 7.64±0.05 ($n = 5$, $P < 0.001$) after basolateral Cl^- removal. This was completely reversible with return of Cl^- to the peritubular solution, consistent with the presence of a basolateral Cl^-/HCO_3^- exchanger. A representative experiment is shown in Fig. 2. These cells were infrequently encountered and further data will not be presented.

The majority of intercalated cells underwent a rapid intracellular acidification after basolateral Cl^- removal, functionally identifying it as the β-intercalated cell. The response of intercalated cells to basolateral Cl^- removal allows for a functional differentiation of the two types of intercalated cells. In all experiments on β-intercalated cells, sub-type was confirmed by intracellular acidosis in response to basolateral Cl^- removal.

In β-intercalated cells with basolateral Cl^- removal, the average decrease in pH_i was −0.70±0.04 pH U in 3.9±0.04 min ($n = 28$, $P < 0.001$). Return of basolateral Cl^- resulted in the return of pH_i to baseline. This has been reported by others and hypothesized to result from acceleration of apical Cl^-/HCO_3^- exchange (32). To examine this hypothesis the effects of luminal Cl^- and HCO_3^- on pH_i was examined. Nominal removal of HCO_3^- from the luminal solution (change to solution 6) resulted in a decrease of 0.41±0.05 pH U ($P < 0.005$, $n = 8$). Luminal Cl^- removal (change to solution 3) caused pH_i to increase 0.45±0.07 pH U ($n = 7$, $P < 0.005$). Furthermore, the removal of luminal Cl^- blocked the effect of basolateral Cl^- removal on intracellular pH (see Fig. 3). Removal of basolateral Cl^- (by changing to solution 3) in the absence of luminal Cl^- resulted in no significant change in pH_i (−0.11±0.07, $n = 6$, P = NS). The subsequent return of luminal Cl^- in the absence of basolateral Cl^- resulted in a marked acidification ($\Delta pH_i = -0.73\pm0.18$, $n = 6$, $P < 0.01$). This pH_i is not significantly different than the pH_i present after basolateral Cl^- removal ($P > 0.3$, unpaired t test), consistent with changes in pH_i via identical mechanisms.

One likely explanation for the decrease in pH_i with luminal HCO_3^- removal is the exchange of intracellular HCO_3^- for luminal Cl^- via an apical Cl^-/HCO_3^- exchanger. Therefore the effect of luminal Cl^- on β-intercalated cell response to a decrease in luminal HCO_3^- concentration was evaluated. Gluconate was substituted for the removed HCO_3^- so that luminal Cl^- would remain constant. In the presence of luminal Cl^-, decreasing perfusate HCO_3^- from 25 to 5 meq/liter (changing from solution 1 to 7) caused pH_i to decrease 0.24±0.06 pH U ($P < 0.025$, $n = 4$). In the absence of luminal Cl^- there was no significant change in pH_i (change from solution 3 to 8, $\Delta = +0.05\pm0.03$, P = NS vs. 0.00, $P < 0.01$ vs. changes in the presence of luminal Cl^- by paired t test, $n = 4$). (In additional experiments to test this model, tubules were bathed and perfused with a high K^+ solution containing 5 μM valinomycin in order to voltage clamp both the apical and basolateral membranes. Removal of luminal Cl^- caused pH_i to reversibly increase 0.20±0.05 pH U [$n = 2$]. This is consistent with an apical, electroneutral, Cl^- dependent, base exit process, i.e., Cl^-/HCO_3^- exchange. However, perfusion of CCT with high K^+ solutions results in marked cell swelling [33, 34, and personal observations] and hence this method was not pursued further.)

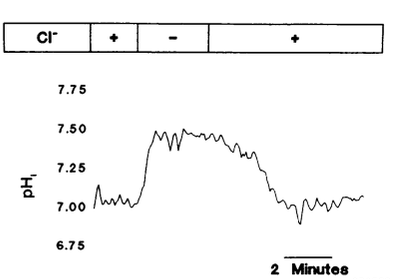

Figure 2. Response of a single α-intercalated cell to peritubular Cl^- removal. A rapid increase in pH_i occurs with removal of peritubular Cl^-, consistent with the presence of a basolateral Cl^-/HCO_3^- exchanger. pH_i returns to baseline with return of peritubular Cl^-.

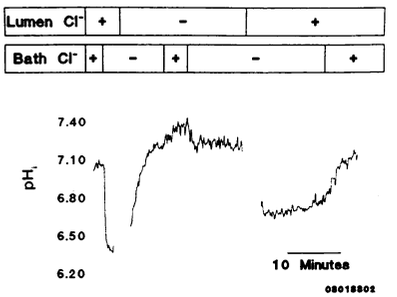

Figure 3. Effect of lumen and bath Cl^- removal on β-intercalated cell pH_i. pH_i rapidly falls to very low levels with removal of peritubular ("bath") Cl^-. Removal of luminal Cl^-, in the continued absence of peritubular Cl^-, reverses the acidification and returns pH_i to slightly above baseline. The return of peritubular Cl^- results in further alkalinization. When peritubular Cl^- is removed a second time, but now in the absence of luminal Cl^-, there is only a very slight acidification, markedly less than the acidification when luminal Cl^- is present, thereby suggesting that the acidification is due to a luminal Cl^- dependent process. This is confirmed by the readdition of luminal Cl^- resulting in cell acidification. Return of peritubular Cl^- then returns pH_i to baseline levels.

These results suggest that Cl^-/base exchange at the apical membrane, in series with a basolateral Cl^- transport mechanism, most likely a Cl^- channel (35), is present in the β-intercalated cell.

β-intercalated cell Na^+/H^+ exchange. β-Intercalated cells were examined for the presence or absence of basolateral Na^+/H^+ exchange activity. The ammonium chloride pulse technique was used to acid load the cells to a mean pH_i of 6.77±0.07 ($n = 16$). A typical experiment is shown in Fig. 4. In a Na^+ free peritubular solution (solution 2) pH_i recovered 0.08±0.04 pH U ($n = 11$) after 5 min. When Na^+ was returned to the peritubular solution pH_i recovery was markedly increased to 0.38±0.07 pH U ($n = 11$, $P < 0.001$ by paired t test).

The inhibitory effect of basolateral amiloride on pH_i recovery of β-intercalated cells was then investigated. A concentration of 1 mM amiloride was used (in solution 1) in order to achieve a high degree of inhibition of the Na^+/H^+ exchange (36). Results are summarized in Fig. 5. After intracellular acid loading, the pH_i recovery at 5 min in the presence of amiloride was 0.05±0.04, while in the absence of amiloride the recovery was 0.37±0.05 pH U ($n = 5$, $P < 0.025$). β-intercalated cell recovery from an acid load is via a basolateral Na^+ dependent, amiloride inhibitable mechanism, i.e., Na^+/H^+ exchange.

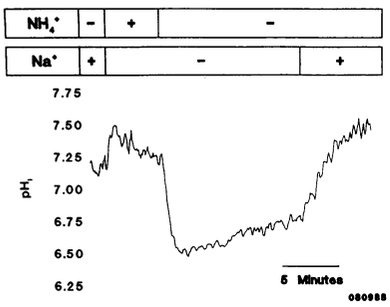

Figure 4. Na^+ dependence of β-intercalated cell pH_i recovery after an acid load. The ammonium chloride pulse technique was used for acid loading and pH_i in a single β-intercalated cell measured (see text for details). In the absence of peritubular Na^+ little recovery of pH_i occurred. When Na^+ was returned to the peritubular solution there was a rapid recovery of pH_i to baseline levels.

Mechanism of pH_i recovery in β-intercalated cells after basolateral Cl^- removal. Basolateral Cl^- removal from β-intercalated cells results in a marked acidification as described above. These intercalated cells possess multiple acid-base transporters (basolateral Na^+/H^+ exchange, luminal Cl^-/HCO_3^- exchange, and basolateral H^+-ATPase; see introduction) which may participate in regulation of pH_i. Yet in the continued absence of basolateral Cl^- for up to 10 min, no significant recovery of pH_i occurred (data not shown), despite the presence of basolateral Na^+. To examine the activity of basolateral Na^+/H^+ during the absence of basolateral Cl^- (and therefore a low pH_i), the effect of amiloride was studied. Peritubular amiloride (1 mM dissolved in solution 3) resulted in a further decrease in pH_i of 0.40±0.13 pH U ($n = 7$, $P < 0.025$) after 5 min. This decrease was reversible with the removal of amiloride, suggesting that β-intercalated cell basolateral Na^+/H^+ exchange is active during the absence of basolateral Cl^-.

Discussion

These studies address the mechanisms of pH_i regulation in the rabbit CCT. The three major cell types (principal cell, α-intercalated cell, and β-intercalated cell) of this segment of the nephron were studied separately utilizing differences in BCECF loading characteristics (25) and response to peritubular Cl^- removal (32). The markedly different responses of the different cell types to peritubular Cl^- removal serve to substantiate our method of studying these cell types separately. The location of Na^+/H^+ exchange and Cl^-/HCO_3^- exchange were studied in particular because of the apparent ubiquity of these transporters in mammalian cells.

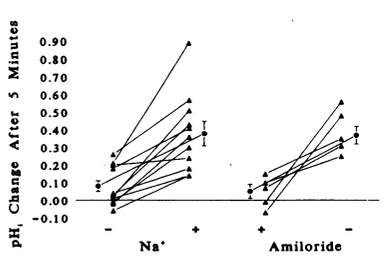

Figure 5. Inhibition of β-intercalated cell pH_i recovery by peritubular Na^+ removal and by amiloride. In the absence of peritubular Na^+, β-intercalated cell pH_i recovery was only 0.08±0.04 pH U after 5 min. In the presence of Na^+, pH_i recovery in the same β-intercalated cells was 0.38±0.07 pH U ($P < 0.001$). Similarly, 1 mM peritubular amiloride decreased β-intercalated cell pH_i recovery from 0.37±0.05 to 0.05±0.04 pH U after 5 min ($P < 0.025$). Lines connect pH_i recovery in the same β-intercalated cell. (▲, individual β-intercalated cell, ●, mean±SEM).

The first major result of these studies is the demonstration that Na^+/H^+ exchange is present at the basolateral membrane of both principal cells and β-intercalated cells. Principal cell pH_i recovery after an acid load was Na^+-dependent and amiloride inhibitable. The use of nominally CO_2/HCO_3^- free solutions minimizes the likelihood that Na^+-dependent HCO_3^- transport was responsible for the observed pH_i changes. The most likely mechanism for an amiloride-sensitive, Na^+-dependent recovery from an acid load is a Na^+/H^+ exchanger. Although not specifically tested, this exchanger is apparently present only on the basolateral membrane since little, if any, pH_i recovery occurred with no peritubular Na^+, even in the presence of luminal Na^+.

A basolateral membrane Na^+/H^+ exchanger was also identified in the β-intercalated cell. In the absence of basolateral Na^+, almost no recovery of pH_i occurred from ammonium chloride pulse-induced intracellular acidosis. Amiloride resulted in a similar inhibition of pH_i recovery after an acid load. Since the studies on β-intercalated cells were performed in CO_2/HCO_3^- containing solutions, it is possible that the recovery was via HCO_3^- transporters. Multiple Na^+-linked HCO_3^- transporters, such as $Na(HCO_3^-)_3^{-2}$ = (37, 38) and Na^+-dependent Cl^-/HCO_3^- exchange (39), are known to exist. However, amiloride has not been shown to inhibit any of these (36). These results therefore provide the first evidence that basolateral Na^+/H^+ exchange is present in the β-intercalated cell.

Na^+/H^+ exchange is a major mechanism of pH_i regulation after acid loads in almost every cell in which it has been studied (40). It also may be involved in growth factor signal transduction (41), cell volume regulation (42), and transepithelial Na^+ transport (as in the proximal tubule, for example [43]). Boron et al. (44) has identified a Na^+/H^+ exchanger on the basolateral membrane of the rabbit CCT. Although intercalated and principal cells could not be differentiated by their techniques, they concluded that Na^+/H^+ exchange was present at the basolateral membrane of principal cells of the rabbit CCT because of the predominance of principal cells and because Na^+/H^+ exchanger activity was seen in all experiments. In addition, they too found no evidence for luminal Na^+/H^+ exchange activity in the CCT. The results of the present study extend their findings by demonstrating the presence of a basolateral Na^+/H^+ exchanger in both principal cells and β-intercalated cells. Breyer et al. (45) and Hays et al. (46) have demonstrated basolateral Na^+/H^+ exchange in the inner stripe of the outer medullary collecting duct, which appears to be functionally equivalent to the α-intercalated cell in the CCT. Na^+/H^+ exchange has also been shown in papillary collecting tubule cells (47, 48). Therefore, all cells of the collecting tubule probably have basolateral Na^+/H^+ exchange.

The finding that the major mechanism of pH_i recovery in β-intercalated cells was Na^+/H^+ exchange was initially quite surprising. These cells presumably have basolateral H^+-ATPase (20), and intracellular acidification may stimulate the insertion of additional H^+-ATPase from endosomes (49, 50). The inhibition of pH_i recovery by the absence of basolateral Na^+ or the presence of amiloride suggests that H^+-ATPase is not a major factor in acute pH_i regulation in the β-intercalated cell. Several factors may account for the observation that Na^+ independent processes (i.e., H^+-ATPase) apparently do not contribute significantly to acute pH_i recovery. Membrane H^+-

ATPase activity appears to be regulated by insertion of H^+-ATPase into and removal from the plasma membrane via exocytosis and endocytosis, respectively (49–51). This process might not allow H^+-ATPase to acutely regulate pH_i. To the extent that endocytosis and exocytosis of H^+-ATPase might acutely regulate pH_i, the intracellular alkalinization during the application of ammonium chloride could lead to endocytosis of H^+ from the basolateral membrane (51), thereby relatively decreasing the potential H^+-ATPase-mediated pH_i recovery during the first 5 min of acidosis. Also possible is that this cell, which functions to secrete base into the urine, might turn-off the mechanisms of HCO_3^- secretion, e.g., H^+-ATPase, in response to intracellular acidosis. These considerations and the results of this study suggest that a basolateral Na^+/H^+ exchanger participates in the regulation of β-intercalated cell pH_i; insertion of H^+-ATPase into the basolateral membrane of the β-intercalated cell may be geared toward transepithelial HCO_3^- transport and not acute pH_i regulation.

Does the basolateral Na^+/H^+ exchanger in β-intercalated cells have some role in CCT bicarbonate secretion? Recent studies by Star et al. (52) found that bilateral (luminal and basolateral) Na^+ removal did not affect rabbit CCT HCO_3^- secretion. However, other studies have differed. McKinney and Burg studied HCO_3^- secreting CCT and found that bicarbonate secretion was dependent on peritubular Na^+ (18). Arruda et al. (53) showed in the turtle bladder (a model epithelium for the CCT) that bilateral Na^+ removal decreased HCO_3^- secretion. Further studies are necessary to delineate the role, if any, of Na^+/H^+ exchange in CCT HCO_3^- secretion.

The other major class of acid-base transporters examined in this study was the Cl^-/HCO_3^- exchange process. Principal cell Cl^-/HCO_3^- exchange was carefully examined. Short-term (5 min) removal of either luminal or basolateral Cl^- did not result in a significant change in pH_i. A prolonged removal and then return of basolateral Cl^- was necessary to demonstrate changes in principal cell pH_i, consistent with the presence of a basolateral Cl^-/HCO_3^- exchanger, albeit relatively inactive. This transporter clearly differs from that in the α- and β-intercalated cells. First, pH_i in α- and β-intercalated cells responds rapidly and dramatically to peritubular Cl^- removal (see Figs. 2 and 3). Next, the principal cell Cl^-/HCO_3^- exchanger does not cross-react with antibodies to band 3 protein, as does the Cl^-/HCO_3^- exchanger of the α-intercalated cell (22, 23). Finally, it is located at the opposite membrane from the β-intercalated cell Cl^-/HCO_3^- exchanger. The role of the principal cell Cl^-/HCO_3^- exchanger is not known, but it could function in recovery from intracellular alkalosis, as Cl^-/HCO_3^- exchangers do in many other cell types (28, 45, 54). The minimal (and slow) response to basolateral Cl^- removal in principal cells is consistent with relative inactivity of this transporter at baseline pH_i and confirms that the principal cell is not involved significantly in transepithelial HCO_3^- transport.

In intercalated cells, basolateral Cl^- removal resulted in opposite pH_i responses in two different populations. Schwartz et al. (32) first showed this differential pH_i response to basolateral Cl^- removal. In the present studies, the majority of intercalated cells responded with a rapid intracellular acidification. The less frequent intercalated cell responded to basolateral Cl^- removal with intracellular alkalinization, consistent with the presence of basolateral Cl^-/HCO_3^- exchange. In view of the less frequent occurrence of these intercalated cells and their alkalinization in response to basolateral Cl^- removal, they are functionally identified as α-intercalated cells. Schwartz et al. (55) has recently described a third type of intercalated cell; these cells were infrequently found and appeared to have a basolateral Cl^-/HCO_3^- exchanger. In the present study these cells could have been identified as α-intercalated cells.

Luminal Cl^-/HCO_3^- exchange was clearly present in β-intercalated cells. The pH_i decrease with basolateral Cl^- removal in the β-intercalated cell was completely dependent on the presence of luminal Cl^-, suggesting that the removal of basolateral Cl^- affected a luminal, Cl^- dependent transporter. Basolateral Cl^- removal probably induces intracellular Cl^- exit via a basolateral Cl^- channel (3, 35), thereby resulting in an increased lumen to cell Cl^- gradient. The decrease in β-intercalated cell pH_i with basolateral Cl^- removal is therefore consistent with a luminal Cl^-/HCO_3^- exchanger transporting Cl^- from the lumen into the cell in exchange for HCO_3^-. Similarly, the increase in pH_i with luminal Cl^- removal and the decrease in pH_i with luminal HCO_3^- removal are consistent with a luminal Cl^-/HCO_3^- exchanger. Inhibition of the fall in pH_i in response to a decrease in luminal HCO_3^- by removal of luminal Cl^- shows that an apical Cl^-/HCO_3^- exchanger is present at the apical membrane of the β-intercalated cell. Studies by Schuster and Stokes have suggested the presence of a luminal transporter able to exchange Cl^- for either HCO_3^- or Cl^-(56); this transporter is stimulated by CO_2/HCO_3^- (57). CCT HCO_3^- secretion appears to involve the coupled, 1:1 reabsorption of Cl^-, suggestive of a luminal Cl^-/HCO_3^- exchanger being responsible for HCO_3^- secretion (52). In summary, these results provide direct functional evidence for luminal Cl^-/base exchange, most likely Cl^-/HCO_3^- exchange, in series with basolateral Cl^- transport in the β intercalated cell type of the rabbit CCT.

An interesting finding in the β-intercalated cell is that no pH_i recovery occurred during the intracellular acidosis associated with basolateral Cl^- removal. Yet this was at a pH_i at which Na^+/H^+ exchange activity was clearly demonstrable after NH_4^+ pulse induced acidosis. Inhibition of Na^+/H^+ exchange by amiloride during basolateral Cl^- removal resulted in a reversible decrease in pH_i, confirming that Na^+/H^+ exchange is still active in the absence of basolateral Cl^-. This suggests that Na^+/H^+ exchange activity occurs, but that the maximal rate of H^+ extrusion is inadequate to return pH_i to normal levels and that the luminal Cl^-/HCO_3^- exchanger has a large capacity for extruding HCO_3^-, at least under certain conditions.

In summary, these studies provide the first direct examination of the pH_i regulatory mechanisms of the various cell types of the heterogeneous rabbit CCT. Principal cells are shown to have both a basolateral Na^+/H^+ exchanger and a previously unidentified basolateral Cl^-/HCO_3^- exchanger. The principal cell Cl^-/HCO_3^- exchanger is relatively inactive at baseline pH_i. Studies of single intercalated cells utilized basolateral Cl^- removal to functionally separate α- and β-intercalated cells and allow their individual study. The α-intercalated cell is functionally identified by intracellular alkalosis in response to peritubular Cl^- removal; the β-intercalated cell by intracellular acidosis. In addition to direct functional evidence of β-intercalated cell luminal Cl^-/HCO_3^- exchange, a basolateral Na^+/H^+ exchanger in the β-intercalated is demonstrated. Thus, both of these adjacent cell types have a basolateral Na^+/H^+ exchanger. Cl^-/HCO_3^- exchange activity is found in

all three cell types of the CCT, but the polarity of distribution and basal activity differs, suggesting different functions for the Cl^-/HCO_3^- exchangers in these neighboring cells.

Acknowledgments

The authors thank John Herndon for technical assistance during the performance of these studies. The continued support of Dr. Saulo Klahr is appreciated.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases grants DK-07126, DK-09976, and DK-34394. Dr. Hamm is an Established Investigator of the American Heart Association.

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