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Research Article

Proton secretion in the renal medullary collecting duct is thought to occur via a luminal proton-ATPase. In order to determine what mechanism(s) participate in proton transport across medullary collecting duct (MCD) cells membranes, intracellular pH (pHi) regulation and proton extrusion rates were measured in freshly prepared suspensions of rabbit outer MCD cells. Cells were separated by protease digestion and purified by Ficoll gradient centrifugation. pHi was estimated fluorometrically using the entrapped intracytoplasmic pH indicator, 6-carboxyfluorescein. Proton extrusion rates were measured using a pH stat. The resting pHi of MCD cells was 7.19 +/- 0.05 (SE) in a nonbicarbonate medium of pH 7.30. When cells were acidified by exposure to acetate salts or by abrupt withdrawal of ammonium chloride, they exhibited pHi recovery to the resting pHi over a 5-min time-course. Depletion of greater than 95% of cellular ATP content by poisoning with KCN in the absence of glucose inhibited pHi recovery. ATP depletion inhibited proton extrusion from MCD cells. Treatment with N-ethylmaleimide also inhibited pHi recovery. In addition, cellular ATP content was dependent on transmembrane pH gradients, suggesting that proton extrusion stimulated ATP hydrolysis. Neither removal of extracellular sodium nor addition of amiloride inhibited pHi recovery. These results provide direct evidence that a plasma membrane proton-ATPase, but not a Na⁺/H⁺ exchanger, plays a role in proton transport and pHi regulation [...]

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Intracellular pH Regulation and Proton Transport by Rabbit Renal Medullary Collecting Duct Cells

Role of Plasma Membrane Proton Adenosine Triphosphatase

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Abstract

Proton secretion in the renal medullary collecting duct is thought to occur via a luminal proton-ATPase. In order to determine what mechanism(s) participate in proton transport across medullary collecting duct (MCD) cells membranes, intracellular pH (pH_i) regulation and proton extrusion rates were measured in freshly prepared suspensions of rabbit outer MCD cells. Cells were separated by protease digestion and purified by Ficoll gradient centrifugation. pH_i was estimated fluorometrically using the entrapped intracytoplasmic pH indicator, 6-carboxyfluorescein. Proton extrusion rates were measured using a pH stat. The resting pH_i of MCD cells was 7.19 ± 0.05 (SE) in a nonbicarbonate medium of pH 7.30. When cells were acidified by exposure to acetate salts or by abrupt withdrawal of ammonium chloride, they exhibited pH_i recovery to the resting pH_i over a 5-min time-course. Depletion of >95% of cellular ATP content by poisoning with KCN in the absence of glucose inhibited pH_i recovery. ATP depletion inhibited proton extrusion from MCD cells. Treatment with *N*-ethylmaleimide also inhibited pH_i recovery. In addition, cellular ATP content was dependent on transmembrane pH gradients, suggesting that proton extrusion stimulated ATP hydrolysis. Neither removal of extracellular sodium nor addition of amiloride inhibited pH_i recovery. These results provide direct evidence that a plasma membrane proton-ATPase, but not a Na^+ / H^+ exchanger, plays a role in proton transport and pH_i regulation in rabbit MCD.

Introduction

The mammalian medullary collecting duct (MCD)¹ is thought to secrete hydrogen ions via a proton-ATPase on the luminal membrane. Evidence favoring this view is based on the similar properties of proton secretion in the reptilian urinary bladder and the collecting duct. Evidence for a central role of a luminal proton-ATPase in proton secretion in the turtle bladder includes the demonstration that proton secretion in this tissue is electro-

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1. *Abbreviations used in this paper:* MCD; medullary collecting duct; MES, 2-[*N*-morpholino]-ethanesulfonic acid; pH_i , intracellular pH.

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genic (1), tightly coupled to cellular ATP content (2-5), inhibited by nonmitochondrial poisons (5, 6), and isolatable in a microsomal fraction that co-migrates with plasma membrane on sucrose gradients (6). In addition, proton secretion in the turtle bladder requires functional carbonic anhydrase (7) and is stimulated by mineralocorticoids (7, 8). In the MCD proton secretion has also been shown to be electrogenic (9), dependent on carbonic anhydrase (10), and stimulated by mineralocorticoid (11). In addition, a membrane-bound H^+ -ATPase similar to that of the turtle bladder has recently been purified from whole bovine medulla (12); there is no data as to the cellular location of this mammalian proton-ATPase fraction. Finally, the turtle bladder and mammalian medullary collecting tubule are morphologically similar, with both tissues comprised of principal and intercalated cells (7, 13). Metabolic and respiratory acidoses, which stimulate acid secretion in both bladder and MCD, provoke similar morphologic changes in the intercalated cells, but not in the principal cells of both systems (14-16).

The purpose of these experiments was to determine whether a plasma membrane proton ATPase plays a role in transmembrane proton flux and intracellular pH (pH_i) regulation in the mammalian MCD. We have therefore monitored pH_i regulation and proton extrusion rates of cell suspensions isolated from rabbit MCD. In order to obtain a homogeneous cell population, we have isolated collecting duct cells from the inner stripe of the rabbit outer medulla. As will be discussed below, the inner stripe collecting duct consists almost entirely of principal cells and is known to engage in acidification (11, 13, 17).

Methods

Isolation of medullary collecting duct cells. Collecting duct cells were isolated from the inner stripe of the outer medulla of male New Zealand white rabbits by a modification of the technique first reported by Eveloff et al. (18) for isolation of thick ascending limb cells. Male New Zealand rabbits were killed by cervical dislocation and exsanguinated via the carotid artery. The kidneys were perfused free of blood via the renal artery with 30 ml ice-cold Joklik's minimal essential medium containing 10% fetal bovine serum. They were then perfused with 5 ml of 0.2% collagenase and 0.25% hyaluronidase dissolved in the same medium. The inner stripes were excised from each kidney and finely minced. The minced outer medulla was incubated for 1 h in 0.2% collagenase and 0.25% hyaluronidase in Joklik's minimal essential medium at 37°C under 95% O_2 /5% CO_2 . The resulting digest was centrifuged twice at 75 g for 3 min to harvest the tubular segments. All centrifugations were performed in a Damon/IEC DPR 6000 swinging bucket centrifuge. The tubules were incubated for 20 min in 200 ml of a 0.25% trypsin solution in Joklik's medium with 10% fetal bovine serum at room temperature under a 95% O_2 /5% CO_2 gas phase. The tubule suspension was then centrifuged for 20 s at 700 g and the pellet containing undigested tubules returned to fresh trypsin solution. The supernatant was then centrifuged for 5 min at 300 g to collect the cells, which were then resuspended in 10 ml of iced Joklik's medium with 10% fetal bovine serum. The trypsin diges-

tion of the tubules was repeated seven more times and the cell harvests pooled. The pooled cells were washed again at 300 g for 5 min, resuspended in Joklik's medium, and filtered through a 30-gauge teflon mesh. The resulting cell suspension was then layered over 60-ml continuous Ficoll gradients (5–46%) and centrifuged for 45 min at 2,300 g. The Ficoll was dissolved in a buffer of the following composition: 137 mM NaCl, 5 mM KCl, 0.33 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 1 mM CaCl₂, 2 mM MgCl₂, 0.8 mM MgSO₄, and 20 mM Tris-HCl, pH 7.4. 5 ml of cell suspension were placed on each gradient. The gradients were harvested in 4-ml fractions for a total of 10 fractions. The cells were then washed free of Ficoll by using a 10-fold excess of Joklik's medium containing fetal bovine serum at 300 g for 10 min. As described (18, 19), the MCD cells were located in the upper two fractions, the thick ascending limb cells in the lowest fraction. The MCD fraction contains cells that closely resemble MCD cells in situ by both light and electron microscopy (19). In addition, the MCD fraction was purified for both carbonic anhydrase and ADH-stimulated adenylate cyclase, two enzymes known to be present at high activity in medullary collecting duct (19). Finally, MCD cells exhibited oxidative metabolism distinct from that of the thick ascending limb cells (19).

Measurement of pH_i . The medium used for all experiments was a nominally bicarbonate-free Ringer's composed of NaCl (130 mM), KCl (5 mM), Tris-Hepes (10 mM, pH 7.30), MgSO₄ (0.4 mM), CaCl₂ (2 mM), Na₂HPO₄ (2 mM), and unless specified, glucose (5 mM). Cells were resuspended in nonbicarbonate Ringer's and incubated for 15–20 min in 10 μ M 6-carboxyfluorescein diacetate at 25°C and an extracellular pH titrated to 6.5 by addition of concentrated 2-[*N*-morpholino]-ethanesulfonic acid (MES). The diacetate form of this dye rapidly enters the cells, where it is converted to the optically active 6-carboxyfluorescein by intracellular esterases (20–22). Once loaded with 6-carboxyfluorescein, cells were washed thoroughly to remove MES and extracellular dye. The fluorescence of the dye-loaded cells was monitored using a Perkin-Elmer model MPF44A fluorescence spectrophotometer equipped with temperature-controlled water jacket and a mechanical stirrer. Excitation wavelengths were 492 and 450 nm (slit 4 nm) and emission wavelength was 530 nm (slit 10 nm). Approximately one-million cells were diluted into 3 ml of nonbicarbonate Ringer's and stirred in the fluorimeter at 37°C. At the end of each run the cells were pelleted and the fluorescence of the supernatant assayed directly. By subtracting this extracellular signal from the total signal, the signal generated by the intracellular dye could be calculated.

Standard curves relating pH_i and fluorescence excitation ratio were obtained by placing the cells in medium containing 137 mM KCl and adding the K/H ionophore, nigericin (20, 22). Under these conditions, intracellular and extracellular pH were assumed to be equal; addition of small amounts of concentrated MES to the medium produced incremental acidification thus generating the curve. Fig. 1 shows a typical standard curve relating pH_i (abscissa) and the fluorescence excitation ratio (I_{492/450}; emission 530) for intracellular 6-carboxyfluorescein. It

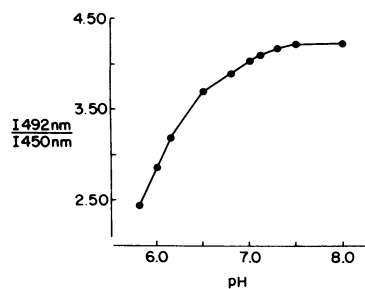


Figure 1. Calibration curve relating fluorescence excitation to pH_i . Approximately 10⁶ MCD cells preloaded with 6-carboxyfluorescein were placed in 3 ml of high potassium medium ([KCl] = 137 mM) and exposed to 6 μ M nigericin. The external and thus the internal pH was then acidified by

stepwise addition of 330 mM MES (final concentration of MES at pH 6.0 = 30 mM). At each pH, the ratio of emission intensities obtained at excitation wavelengths 492 and 450 nm were measured. As described in Methods, the extracellular dye ratio was measured and subtracted from the total signal to give the signal generated by intracellular dye.

is evident from the figure that the emission intensity generated by this excitation ratio increased considerably over the pH range, 6.0–7.5, allowing sensitive estimation of pH_i in this range. Such standard curves were prepared daily; the fluorescence excitation ratios obtained from each run were compared with these standard curves to estimate the pH_i .

Measurement of proton extrusion. Proton efflux from MCD cells was measured using a Radiometer-Copenhagen pH stat equipped with a Model II titrator, an ABU 12 autoburet, a Model 27 pH meter, and an SBR 3 Titrigraph chart recorder mechanically coupled to the titrator. The titrant was 0.1 mM NaOH. For pH stat experiments the Tris-Hepes and phosphate were omitted from the nonbicarbonate Ringer's. Two-million cells were placed in 3 ml of unbuffered nonbicarbonate Ringer's in a temperature-controlled chamber (Yellow Springs Instruments, Yellow Springs, OH) equipped with a magnetic stirrer and pH electrode at 37°C. The chamber was sealed from the atmosphere by a closely fitting plastic plunger equipped with a small port for additions of cells and solutions. The cells were titrated to the appropriate starting pH with dilute NaOH or HCl. Generally, the cells were acid loaded by incubating them at extracellular pH of 6.5 for 5 min. The extracellular pH was then abruptly raised to 7.40 by adding NaOH. The rate of acid extrusion was measured as the rate of base equivalent addition required to maintain the extracellular pH at 7.40 as protons exited the cells. When cells were pretreated with cyanide, 2 mM KCN was also added to the unbuffered Ringer's, and the solution titrated to the desired pH.

ATP measurements. ATP was measured using the firefly luciferase assay as described by Kimmich et al. (23). ATP was extracted by boiling the sample then pelleting the protein by centrifugation in a Sorvall RC-5B centrifuge at 42,000 g for 20 min. Cells were counted using standard proton counters. For experiments relating ATP levels to transmembrane proton gradients, MCD cells were washed free of glucose and resuspended in 1 ml nonbicarbonate Ringer's titrated to pH 6.50 with MES, to which was added 2 mM KCN and 0.5 mM ouabain. After a 5-min incubation at pH 6.50 at 37°C, 0.3-ml aliquots of cells were resuspended in 5 ml of nonbicarbonate Ringer's with KCN and ouabain at pH 6.50 or 7.50. Aliquots of these new suspensions were taken immediately and after 5 min at 37°C and extracted for ATP content.

Materials. Collagenase was obtained from Worthington Biochemicals, (Cooper Biomedical), Malvern, PA; hyaluronidase was obtained from Boehringer-Mannheim Biochemicals, Indianapolis, IN. Joklik's minimal essential medium was obtained from Gibco, Grand Island, NY; Ficoll was obtained from Pharmacia Fine Chemicals, Piscataway, NJ; and 6-carboxyfluorescein diacetate was from Molecular Probes, Junction City, OR. All other reagents were obtained from Sigma Chemical Co., St. Louis, MO, and were of analytical grade. Firefly lanterns were obtained from Sigma Chemical Co., and homogenized to make a crude luciferase preparation (23). Digitonin was recrystallized three times and prepared as a 3-mM stock in dimethylsulfoxide.

Statistics. In all tables, a single *n* represents the mean of duplicate or triplicate determinations of pH_i ; recovery on a given cell preparation unless specified. Experimental results are given for the mean \pm SEM for at least three separate cell preparations, or are shown in a representative experiment. Groups were compared using unpaired *t* tests; *P* values <0.05 were regarded as significant.

Results

Acid-loading MCD cells. Fig. 2 shows the response of pH_i to exposure of the cells to sodium acetate. The base-line resting pH_i of MCD cells in bicarbonate-free Ringer's was 7.19 ± 0.05 at an extracellular pH of 7.30 (*n* = 38). At time zero, acetate was added. Since the uncharged protonated form of this acid enters the cells far more rapidly than the acetate anion, the cells promptly acidified to $pH_i = 6.50$ despite maintenance of a constant extracellular pH. Over the next 7 min pH_i recovered, eventually reaching its resting value. To allow quantitative comparison of experimental groups, the first 3 min of the timecourse of pH_i recovery was fitted to a linear equation of the form: pH_i

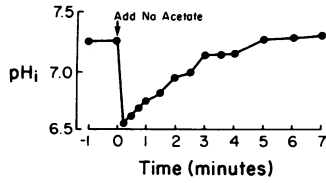


Figure 2. Time course of pH_i after addition of acetate salt. 10^6 MCD cells preloaded with 6-carboxyfluorescein were placed in 3 ml of nonbicarbonate Ringer's and the resting pH_i measured. At time zero, 40

mM Na-acetate was added and the pH_i monitored over the succeeding 7 min. At each point, the fluorescence excitation ratio (492:450) was measured at emission wavelength 530 nm and the results compared with a standard curve similar to that shown in Fig. 1 to obtain estimates of pH_i .

$= k(t) + \text{pH}_{i(t=0)}$, where k is the fitted constant describing the initial rate of pH_i recovery, t is the time after acid loading, and $\text{pH}_{i(t=0)}$ is the pH_i at time zero. Correlation coefficients from these linear fits averaged 0.96. Fitting the pH_i recovery curves to a linear function relating pH_i to time requires no assumptions regarding the mechanism(s) of pH_i recovery, but merely introduces a straightforward means of quantitatively comparing experimental groups. A similar linearization was applied to proton efflux measurements of Ehrlich Ascites cells by Bowen and Levinson (24).

An additional method of acid-loading cells involves preincubation in weak bases such as ammonium salts followed by dilution into a medium that does not contain the weak base (25). Fig. 3 shows such a protocol involving exposure of MCD cells to NH_4Cl . In the top panel of this figure, NH_4Cl was added to cells at time zero. Since the uncharged NH_3 permeated the plasma membrane far more rapidly than the charged acidic form, NH_4^+ , the cells rapidly alkalinized. As first described by Boron and DeWeer (26), abrupt dilution of cells into ammonium-free medium resulted in the efflux of the more permeant NH_3 , leaving behind the more acidic NH_4^+ . Thus, as shown in the bottom panel of this figure, pH_i fell rapidly upon ammonium withdrawal and then recovered gradually. As shown in the upper curve of the lower panel, the presence of extracellular ammonium prevented both the acidification and the recovery.

ATP depletion. To determine whether a plasma membrane proton-ATPase participates in the pH_i recovery from both acid-loading protocols described above, MCD cells were depleted of ATP. In glucose-containing nonbicarbonate Ringer's, at pH 7.30, MCD cells contained basal levels of ATP of 1.5 ± 0.16 pm/cell, a level comparable to that measured by Kimmich et al. (23) in intestinal epithelial cells using the luciferase method. As shown in Fig. 4, addition of 2 mM potassium cyanide (KCN) to MCD cells in the presence of extracellular glucose resulted in only

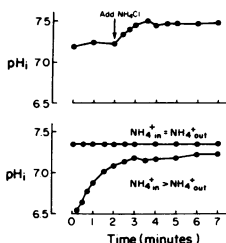


Figure 3. Time course of pH_i after exposure to and withdrawal from NH_4Cl solutions. *Upper panel:* 10^6 MCD cells preloaded with 6-carboxyfluorescein were placed in nonbicarbonate Ringer's. After measurement of resting pH_i , 40 mM NH_4Cl was added to the suspension and the pH_i monitored. *Lower panel:* 10^6 MCD cells preloaded with 6-carboxyfluorescein were incubated for 15 min with

40 mM HN_4Cl and then injected into 3 ml of nonbicarbonate Ringer's in the fluorimeter containing no NH_4Cl ($\text{NH}_{4in}^+ > \text{NH}_{4out}^+$, lower curve) or 40 mM NH_4Cl ($\text{NH}_{4in}^+ = \text{NH}_{4out}^+$, upper curve).

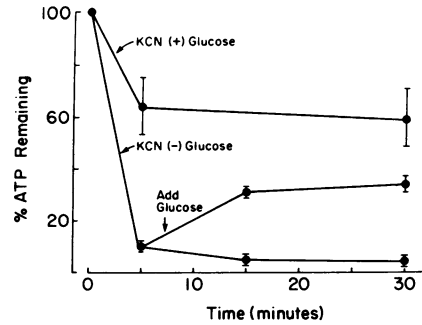


Figure 4. Effect of KCN in the presence and absence of glucose on cellular ATP content. MCD cells were exposed to 2 mM potassium cyanide in the presence (*upper curve*) or absence (*lower curve*) of 5 mM glucose at 37°C . Immediately after addition of cyanide, aliquots of the cells were boiled and extracted for ATP (time zero). After 5 min of exposure to cyanide in the absence of glucose, some MCD cells were resuspended in 5 mM glucose. Data represent mean \pm SE for triplicates performed on three separate preparations.

partial (30–40%) depletion of cellular ATP content. In the absence of glucose, $>90\%$ of ATP was rapidly depleted. Early addition of glucose to ATP-depleted cells restored a significant portion of cellular ATP. These results confirmed those of earlier studies demonstrating a high glycolytic capacity in medullary collecting duct (27), and provided a protocol for depleting these cells of ATP.

Effect of ATP depletion on pH_i recovery rate. Fig. 5 shows in a representative experiment the effect of ATP depletion on pH_i recovery after acetate acid loading. In the top two panels, the pH_i recovery rates of unpoisoned cells (control) and of cells exposed to KCN in the presence of glucose (KCN (+) glucose) are shown to be similar. By contrast, pH_i recovery in cells depleted of ATP (KCN (-) glucose) was markedly inhibited. Some

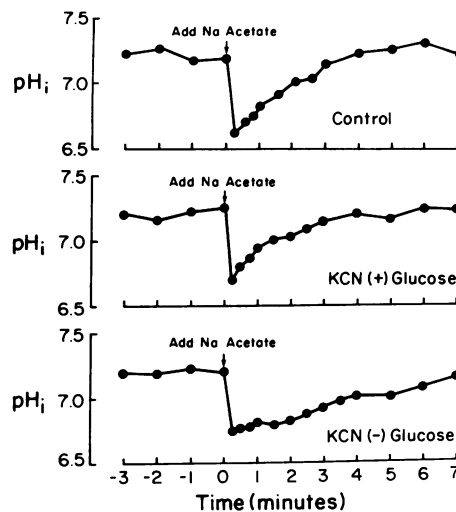


Figure 5. Effect of ATP depletion on pH_i recovery after acetate acid loading. Protocol was as described in Fig. 2. *Top:* MCD cells in 5 mM glucose, no cyanide. *Middle:* MCD cells preincubated 20 min in 2 mM KCN in the presence of 5 mM glucose; both KCN and glucose were present in the nonbicarbonate Ringer's used for the fluorimetry. *Bottom:* MCD cells preincubated 20 min in 2 mM KCN in the absence of glucose—medium in cuvette contains KCN but no glucose.

of the more gradual recovery may have been due to slow uptake of acetate anions by these cells. In Table I, the results of all such experiments are given. The top row of this table shows that pH_i recovery rate constant was similar for the unpoisoned cells and for cells poisoned in the presence of glucose (2.5×10^{-3} pH U/s for control vs 2.25×10^{-3} for KCN (+) glucose), but markedly diminished in cells poisoned in the absence of glucose (1.10×10^{-3}). Since the resting pH_i shown in the middle row was similar for all three experimental groups, the differences in pH_i recovery rates could not have been due to initial pH differences. In addition, the fact that an identical acetate acid load provoked similar pH_i drops as shown in the bottom row indicate that large differences in intracellular buffer capacity could not account for the observed differences in pH_i recovery rates.

We next evaluated whether pH_i recovery from a different acid-loading protocol, ammonium withdrawal, would also show a similar ATP dependence. As shown in Fig. 6, when MCD cells were poisoned with KCN in the presence or absence of glucose and acidified by the ammonium withdrawal protocol, the ATP-depleted cells recovered more slowly than their partially depleted counterparts. In addition, at the endpoint of most time courses, the pH_i of the ATP-depleted cells failed to reach the resting pH_i . This experiment is representative of triplicate pH_i timecourses performed on three separate cell preparations.

Role of extracellular sodium in pH_i regulation. In order to determine whether Na^+/H^+ exchange plays a role in pH_i regulation in MCD cells, we examined pH_i recovery in the absence of extracellular sodium and in the presence of the pyrazine diuretic, amiloride, a known inhibitor of Na^+/H^+ exchange (28). Fig. 7 shows, in a representative experiment, the effect of removing extracellular sodium on the rate of pH_i recovery after an acetate acid load in MCD cells. It is clear that removal of extracellular sodium did not alter pH_i recovery rate in these cells. When sodium was replaced with potassium to a final sodium concentration of 1–2 mM, the rate of pH_i recovery was $2.45 \pm 0.43 \times 10^{-3}$ pH U/s for low sodium, as compared with $2.60 \pm 0.35 \times 10^{-3}$ for normal sodium groups ($n = 4$). In addition,

Table I. Effect of ATP Depletion on pH_i Recovery after Acid Loading

	Control	KCN + Glucose	KCN - Glucose
pH_i recovery rate (pH U/s $\times 10^3$)	2.50 ± 0.52 (SEM)	2.25 ± 0.27	$1.10 \pm 0.13^*$
Resting pH_i	7.14 ± 0.03	7.12 ± 0.05	7.13 ± 0.04
pH_i drop (pH U)	0.46 ± 0.02	0.44 ± 0.05	0.45 ± 0.05
	$n = 4$	$n = 5$	$n = 5$

MCD cells preloaded with 6-carboxyfluorescein were placed in the fluorimeter and the resting pH_i measured. After addition of 40 mM Na^+ -acetate, the pH_i rapidly fell and gradually recovered, as shown in Fig. 2. The pH_i recovery rate was determined as described in Methods. The pH_i drop was measured as the difference in pH_i between the resting pH_i before acetate addition and the most acidic pH_i reached after addition of acetate. Control cells were suspended in nonbicarbonate Ringer's with 5 mM glucose present. KCN cells were poisoned with cyanide in the presence (+ glucose) or absence (- glucose) of glucose. Each n represents duplicate or triplicate measurements of pH_i recovery rate, resting pH_i , and pH_i drop on a single MCD cell preparation. * $P < 0.005$.

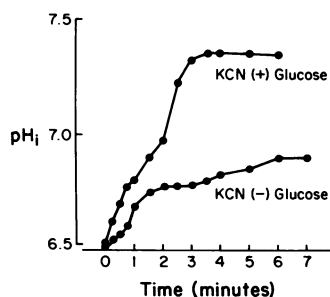


Figure 6. Effect of ATP depletion on pH_i recovery after ammonium withdrawal. Cells were preloaded with 6-carboxyfluorescein and 40 mM NH_4Cl as described in Fig. 3. In addition, cells were poisoned with 2 mM KCN in the presence (upper curve) or absence (lower curve) of 5 mM glucose. At time zero, 40 μ l of concentrated cell suspension was injected into 3 ml nonbicarbonate Ringer's containing KCN with (upper curve) or without (lower curve) glucose and pH_i recovery monitored. This experiment is representative of duplicate or triplicate determinations performed on three different cell preparations.

in the presence of Na^+ , 0.5 mM amiloride did not alter the recovery rate, with control values of $3.31 \pm 0.71 \times 10^{-3}$ pH U/s as compared with $3.32 \pm 0.68 \times 10^{-3}$ for the amiloride-treated cells ($n = 3$). In both the cation replacement and amiloride treatment experiments, the resting pH_i and pH_i drops were comparable between experimental groups. Taken together, these observations indicate that Na^+/H^+ exchange does not play a significant role in pH_i regulation in this preparation.

Inhibitor sensitivity of pH_i recovery. Since Na^+/K^+ -ATPase also requires ATP, the inhibition of pH_i recovery observed in cells depleted of ATP might have been due to inhibition of this enzyme. We therefore monitored pH_i recovery in the presence and absence of ouabain. As shown in Table II, when MCD cells were exposed to 0.5 mM ouabain for time periods similar to those used to poison the cells with KCN, no significant alteration in the rate of pH_i recovery was observed (top row, left panel). These results indicate that the inhibition of pH_i recovery observed in ATP-depleted cells could not have been caused by inhibition of the Na^+/K^+ -ATPase.

The right panel of Table II shows the effect of *N*-ethylmaleimide on pH_i recovery of MCD cells. This nonspecific sulfhydryl reagent, which is known to inhibit proton-ATPases in a number of tissues (12, 29), produced a statistically significant 30–40% inhibition of pH_i recovery in MCD cells. In all experiments shown in this table, both resting pH_i (middle row) and acetate-induced pH_i drop (bottom row) were not different between control and experimental groups.

Effect of ATP depletion on cellular proton extrusion. To determine whether the observed changes in pH_i recovery rate were caused by transmembrane proton flux or by intracellular buff-

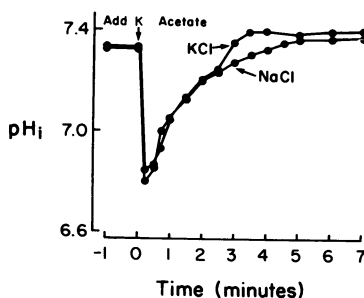


Figure 7. Effect of removal of extracellular sodium on pH_i recovery after acetate acid load. MCD cells were washed and resuspended in KCL medium and then suspended in KCl or standard nonbicarbonate Ringer's (NaCl) media. After measurement of resting pH_i , 40 mM potassium acetate was added and the pH_i recovery monitored. The data are representative of duplicate or triplicate determinations performed with four batches of cells.

Table II. Effect of Inhibitors on pH_i Recovery

	Control	Ouabain (0.5 mM)	Control	<i>N</i> -ethylmaleimide (1 mM)
pH_i recovery rate ($pH\ U/s \times 10^3$)	2.95 ± 0.52 (SEM)	2.68 ± 0.47	3.27 ± 0.71	$2.03 \pm 0.52^*$
Resting pH_i	7.22 ± 0.08	7.27 ± 0.07	7.22 ± 0.05	7.20 ± 0.10
pH_i drop ($pH\ U$)	0.54 ± 0.02	0.52 ± 0.07	0.53 ± 0.05	0.48 ± 0.05
	$n = 4$	$n = 4$	$n = 4$	$n = 4$

pH_i recovery rate, resting pH_i , and pH_i drop were measured as described in Methods and Table I. The control experiments for each inhibitor were performed on the same MCD cell preparations. Cells treated with inhibitors were poisoned for 15–60 min before measurement of pH_i .

* $P < 0.01$.

ering, we measured cellular proton extrusion directly using a pH stat. Fig. 8 shows a representative control experiment designed to demonstrate that the pH stat method measures transmembrane proton flux. The cells were incubated for 5 min at an extracellular pH of 6.60. The medium was then abruptly alkalinized by addition of concentrated NaOH to pH 7.40 and the titrator started (lower curve). The ordinate represents the amount of base equivalents added by the titrator to the extracellular medium to maintain the pH at 7.40. When the rate of base addition (as indicated by the slope of the line) had reached zero, 7 μ M digitonin was added to permeabilize the plasma membrane. The acid release provoked by this detergent confirmed the earlier finding that the resting pH_i of these cells is more acidic than the extracellular pH. Addition of digitonin in the absence of cells had no effect on the extracellular pH. When the medium surrounding these same permeabilized cells was again acidified to pH 6.60, and abruptly alkalinized to pH 7.40 (upper curve), the rate of acid release from the cells to the extracellular solution was far more rapid than before permeabili-

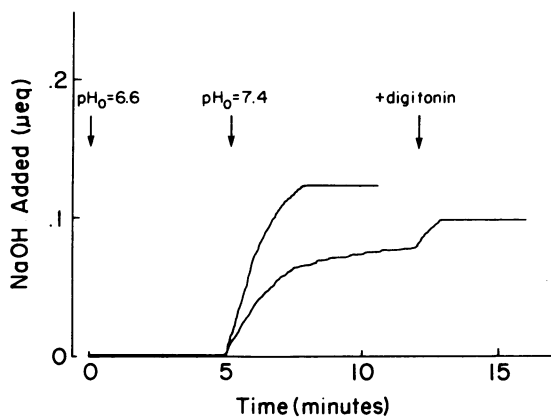


Figure 8. pH stat measurement of proton efflux from MCD cells. Two-million MCD cells were washed and resuspended in unbuffered nonbicarbonate Ringer's, placed in the 3 ml pH stat cell, and titrated to extracellular pH = 6.60. After 5 min at this pH, a small volume of 1 M NaOH was added to abruptly raise the pH of the external solution to 7.40. The titrator was then started and added 0.1 mM NaOH at a rate sufficient to maintain the extracellular pH at 7.40 (lower curve). At $t = 12$ min, 7.5 μ M digitonin was added and the titrator restarted. At $t = 17$ min, the extracellular pH was again adjusted to pH 6.6 and the chart recorder reset to the origin. After 5 min, concentrated NaOH was added to raise the extracellular pH to 7.40 and the titrator again turned on (upper curve). This figure is representative of three similar runs at acidic pH_i 's 6.50–6.70 and alkaline pH_i 's 7.25–7.50 performed on three different cell preparations.

zation. Since permeabilization of the plasma membrane markedly increased the rate of proton flux, the addition of base equivalents by the pH stat must have been a measure of the rate of proton flux across the plasma membrane.

Fig. 9 shows the effect of ATP depletion on transmembrane proton flux. In the absence of cellular ATP, protons were extruded far more slowly than when ATP was present. In addition, less net acid was extruded by the ATP-depleted cells. Since glycolysis generates acid, it was possible that the presence of glucose stimulated proton efflux by increasing glycolytic proton generation rather than by increasing cellular ATP levels (upper curve of Fig. 9). The effect of anaerobic glycolysis on proton extrusion at constant extracellular pH is shown in Fig. 10. Addition of glucose to glucose-starved KCN treated cells (curve A) produced a measurable stimulation of acid extrusion, presumably as a result of stimulating glycolysis. The rate of this proton efflux is <20% of that observed when cells were acid loaded and abruptly returned to an alkaline medium (Fig. 9). Basal acid extrusion by cells poisoned with KCN in the presence of glucose (curve B) was barely measurable, even with the expanded ordinate and condensed timescale used in this figure. These results demonstrate that the more rapid rate of cellular proton extrusion observed in the presence of glucose was not caused by increased glycolytic proton generation in these cells.

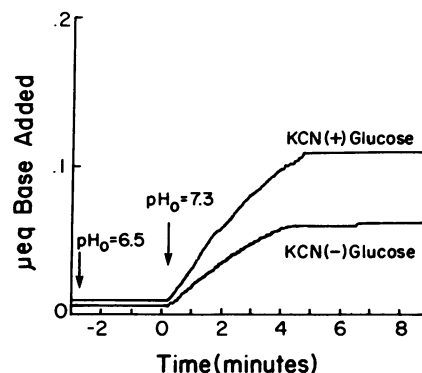


Figure 9. Effect of ATP depletion on proton extrusion after mineral acid loading. pH stat protocol as described in Fig. 8. MCD cells were incubated at pH 6.50 for 5 min, then the extracellular medium alkalinized to pH 7.3 at $t = 0$. Upper curve represents cells pretreated with 2 mM KCN in the presence of 5 mM glucose (5 mM glucose and 2 mM KCN present in the pH stat medium). Lower curve represents cells pretreated with 2 mM KCN in the absence of glucose (2 mM KCN present in the pH-stat medium with no glucose). The figure is representative of duplicate or triplicate determinations performed on four cell preparations.

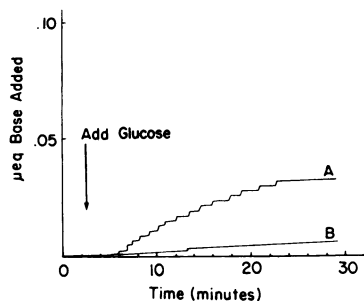


Figure 10. Effect of anaerobic glycolysis on proton extrusion. Curve A: Cells were poisoned with KCN in the absence of glucose and placed in the pH stat. At the arrow, 5 mM glucose was added. Curve B: Cells were poisoned with KCN in the presence of 5 mM glucose and placed in the pH stat to determine the steady

state rate of proton extrusion. Note that the ordinate and abscissa of this figure differ from those of Figs. 8 and 9. The figure is representative of duplicate determinations performed on three cell preparations.

Effect of transmembrane pH gradients on cellular ATP content. In order to determine the effect of transmembrane pH gradients on cellular ATP content, MCD cells were depleted of ATP by poisoning with KCN in the absence of glucose in a solution of pH 6.50. After diluting cells into medium of pH 6.50 or 7.30, aliquots were extracted immediately or after 5 min of incubation at 37°C. The depletion of ATP was larger in acid-loaded cells diluted into alkaline medium than in cells maintained in medium of constant pH. As shown in Fig. 11, cells kept at pH 6.50 retained 24%±0.04 more ATP than cells alkalinized to pH 7.30 ($P < 0.02$, paired *t* test; $n = 4$). The results represent triplicate ATP extractions and assays performed on four different MCD cell preparations. These experiments were performed in the presence of 0.5 mM ouabain, to prevent consumption of ATP by the Na^+/K^+ ATPase. These results are consistent with the presence of an ATP-consuming acid extrusion mechanism such as a proton-ATPase in these cells. It is also possible that alkalinizing the cell interior enhanced ATP degradation by ATPases other than the plasma membrane proton-ATPase.

Discussion

Intracellular pH plays a critical role in volume regulation (30), cell growth (31), the actions of some hormones (33), and the regulation of many metabolic processes (31). Since the pH_i measured in most cells is 6.90–7.50 (25), values considerably higher

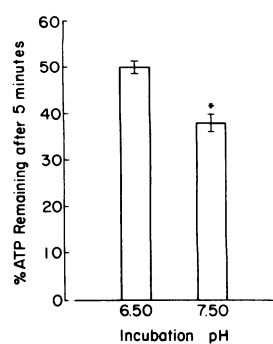


Figure 11. Effect of extracellular pH on cellular ATP content. MCD cells were washed free of glucose and resuspended in nonbicarbonate Ringer's titrated to pH 6.50 with MES, to which was added 2 mM KCN and 0.5 mM ouabain. After 5 min at pH 6.50 at 37°C, aliquots of cells were resuspended in nonbicarbonate Ringer's with KCN and ouabain at pH 6.50 or 7.50. Aliquots of these new suspensions were extracted for ATP content at time zero and after 5 min at the

new pH. ATP contents for the 5-min timepoints are expressed as the percentages of ATP present at time zero remaining at 5 min. $P < 0.02$, paired *t* test; $n = 4$

than the pH_i predicted for electrochemical equilibrium, cells must have proton transport mechanisms capable of extruding protons against their electrochemical gradient. Such proton secretory mechanisms include Na^+/H^+ exchange (proximal tubule, lymphocytes, cultured cell lines) and plasma membrane proton-ATPases (turtle bladder mitochondrion-rich cells, gastric oxyntic cells, bacteria) (25, 30–32). In epithelial cells specialized to transport acid, these proton extruding mechanisms may be used to remove net acid from the organism. We have examined pH_i recovery and acid extrusion by cells of one such acid-secreting epithelium, the MCD, in order to determine which mechanism(s) might account for proton transport in this segment.

Measurement of pH_i has been accomplished by a number of methods including intracellular electrodes, the distribution of weak acids or bases (e.g., dimethylxazolidine-2,4-dione [DMO] and Acridine Orange), or by the use of pH-sensitive intracellular dyes such as 6-carboxyfluorescein (20, 25). Although electrodes measure pH_i most accurately, most mammalian cells are too small for technically satisfactory recordings. The weak acids and bases distribute into all cell compartments, including relatively alkaline mitochondria and acidic lysosomes, and thus do not measure directly the pH adjacent to the plasma membrane (25). In addition, the prolonged period required for equilibration of these indicators in many cell systems (33) precludes their use for the study of rapid changes in pH_i . By contrast, 6-carboxyfluorescein has been shown in a number of cell types to distribute almost exclusively in the compartment of greatest interest in the study of transmembrane proton transport, the cytoplasm (20–22). Since this dye is preloaded into the cells before the experiment, there is no delay in measurement of pH_i while the dye distributes. We have therefore measured the fluorescence of cell suspensions preloaded with 6-carboxyfluorescein in order to estimate resting pH_i and to monitor changes in pH_i . Although in the present study the actual value of pH_i is of less importance than the changes observed during acid-loading and recovery, the value of 7.19 measured for MCD cells in bicarbonate-free medium at external pH of 7.30 is entirely consistent with resting pH_i observed in a variety of cell systems using a variety of methods (25). In addition, the relative acidity of the intracellular space compared with the extracellular medium at external pH 7.25–7.5 was confirmed on the pH stat using digitonin (Fig. 8).

The cell suspensions used in these experiments express morphologic and enzymatic characteristics of the inner stripe of the outer medullary collecting duct. Their viability has been confirmed by measurement of O_2 consumption, ATP levels (19), and, in this study, by the demonstration of pH_i regulation. In the outer medulla, the collecting duct is composed of two cell types, the principal and intercalated cells. In our suspensions, we find only principal cells (19). This result can be explained by the fact that in the rabbit medulla the proportion of intercalated cells diminishes gradually from ~50% at the corticomedullary junction to 0% in the papilla (13). In the outer stripe and in the cortex, collecting duct acid secretion is thought to occur only via the intercalated cells (14, 15). However, the striking morphologic differences between inner stripe principal cells and principal cells found in turtle bladder and cortical collecting duct (13) makes it hazardous to assign functions to the inner stripe principal cell based on direct analogy to these other tissues. There is also direct evidence that inner stripe principal cells participate in acidification. Thus, Dobyan et al. (34) have shown that all of the cells in this segment stain positively for carbonic anhydrase. In addition, Koeppen (17), using intracellular elec-

trodes in isolated perfused rabbit collecting ducts, has found a single cell type in this segment by electrophysiologic criteria; using similar methods, two cell types have been described in outer stripe and cortical collecting ducts (35). Morphologically, principal cells in the inner stripe resemble those found in the papillary collecting duct. In the papilla, acid secretion is believed to occur despite the absence of intercalated cells (36, 37). Thus, the fact that the cells in the present study are principal cells does not preclude their participation in acid secretion in vivo.

Because cells in suspension are oriented randomly, we could not examine directly the apical or basolateral locations of H⁺ transport. Since coupled extrusion of alkali and H⁺ from the cells would have obscured both intra and extracellular pH changes, we acid loaded the cells, thereby stimulating H⁺ transport almost exclusively. In addition, it was anticipated that omission of HCO₃⁻ would further diminish net alkali transport.

The observations that neither removal of extracellular sodium (Fig. 7) nor treatment with amiloride inhibited pHi recovery provide evidence that Na⁺/H⁺ exchange does not participate in pHi regulation in this preparation. Indeed, MCD cells may not have Na⁺/H⁺ exchangers on their plasma membranes in situ. Consistent with this hypothesis, bicarbonate reabsorption in isolated perfused rabbit MCD was not inhibited either by removal of luminal sodium or by luminal amiloride (11). In addition, in this same preparation, neither net sodium reabsorption nor secretion has been detectable (38).

Consistent with the fact that the mammalian medulla functions in a relatively anaerobic environment, the MCD has been shown in this study (Fig. 4) and others (27) to have a high glycolytic capacity. In the present study cells poisoned with KCN in the presence of glucose supported pHi recovery rates indistinguishable from those of unpoisoned cells. These results suggest that MCD cells can regulate pHi, and presumably can secrete protons under anaerobic conditions, energized by glycolysis alone.

As outlined in the introduction, a plasma membrane proton-ATPase has been demonstrated to mediate proton secretion in the turtle bladder. Due to functional and morphological similarities between the two tissues, luminal acidification in the mammalian MCD has also been attributed to a plasma membrane proton-ATPase. Our results using purified suspensions of rabbit MCD cells provide further direct evidence for a role for such a plasma membrane proton-ATPase in transmembrane proton transport and pHi regulation. We have demonstrated that the recovery of pHi from acid loading is inhibited by depletion of cellular ATP (Figs. 2, 4, and 5; Table I) and by exposure of the cells to *N*-ethyl maleimide, a potent but nonspecific inhibitor of proton-ATPases (Table II). In addition, the rate of acid extrusion from the cells is ATP dependent (Fig. 9). Finally, conditions that evoke acid extrusion result in measurable depletion of cellular ATP stores.

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