

Apical Na⁺/H⁺ antiporter and glycolysis-dependent H⁺-ATPase regulate intracellular pH in the rabbit S3 proximal tubule.

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Research Article

The apical transport processes responsible for proton secretion were studied in the isolated perfused rabbit S3 proximal tubule. Intracellular pH (pHi) was measured with the pH dye, 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein. Steady state pHi in S3 tubules in nominally HCO_3^- -free solutions was 7.08 ± 0.03 . Removal of Na^+ (lumen) caused a decrease in pHi of 0.34 ± 0.06 pH/min. The decrease in pHi was inhibited 62% by 1 mM amiloride (lumen) and was unaffected by 50 μM 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (lumen) and Cl^- removal (lumen, bath). After a brief exposure to 20 mM NH_4Cl , pHi fell by approximately 0.7 and recovered at a rate of 0.89 ± 0.15 pH/min in the nominal absence of Na^+ , HCO_3^- , organic anions, and SO_4^{2-} (lumen, bath). 1 mM N,N'-dicyclohexylcarbodiimide (lumen), 1 mM N-ethylmaleimide (lumen), 0.5 mM colchicine (bath), and 0.5 mM iodoacetic acid (lumen, bath) inhibited the Na^+ -independent pHi recovery rate by 73%, 55%, 77%, and 86%, respectively, whereas 1 mM KCN (lumen, bath) did not inhibit pHi recovery. Reduction of intracellular, but not extracellular chloride, also decreased the Na^+ -independent pHi recovery rate. In conclusion, the S3 proximal tubule has an apical Na^+/H^+ antiporter with a Michaelis constant for Na^+ of 29 mM and a maximum velocity of 0.47 pH/min. S3 tubules also possess a plasma membrane H⁺-ATPase that can regulate pHi , has a requirement for intracellular chloride, [...]

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Apical Na^+/H^+ Antiporter and Glycolysis-dependent H^+ -ATPase Regulate Intracellular pH in the Rabbit S₃ Proximal Tubule

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Abstract

The apical transport processes responsible for proton secretion were studied in the isolated perfused rabbit S₃ proximal tubule. Intracellular pH (pH_i) was measured with the pH dye, 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein. Steady state pH_i in S₃ tubules in nominally HCO_3^- -free solutions was 7.08 ± 0.03 . Removal of Na^+ (lumen) caused a decrease in pH_i of 0.34 ± 0.06 pH/min. The decrease in pH_i was inhibited 62% by 1 mM amiloride (lumen) and was unaffected by 50 μM 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (lumen) and Cl^- removal (lumen, bath). After a brief exposure to 20 mM NH_4Cl , pH_i fell by ~ 0.7 and recovered at a rate of 0.89 ± 0.15 pH/min in the nominal absence of Na^+ , HCO_3^- , organic anions, and SO_4^{2-} (lumen, bath). 1 mM *N,N'*-dicyclohexylcarbodiimide (lumen), 1 mM *N*-ethylmaleimide (lumen), 0.5 mM colchicine (bath), and 0.5 mM iodoacetic acid (lumen, bath) inhibited the Na^+ -independent pH_i recovery rate by 73%, 55%, 77%, and 86%, respectively, whereas 1 mM KCN (lumen, bath) did not inhibit pH_i recovery. Reduction of intracellular, but not extracellular chloride, also decreased the Na^+ -independent pH_i recovery rate. In conclusion, the S₃ proximal tubule has an apical Na^+/H^+ antiporter with a Michaelis constant for Na^+ of 29 mM and a maximum velocity of 0.47 pH/min. S₃ tubules also possess a plasma membrane H^+ -ATPase that can regulate pH_i , has a requirement for intracellular chloride, and utilizes ATP derived primarily from glycolysis.

Introduction

The renal proximal convoluted tubule and superficial proximal straight tubule possess an apical Na^+/H^+ antiporter that contributes to the absorption of luminal bicarbonate and to intracellular pH (pH_i)¹ regulation (1–5). In addition to the apical Na^+/H^+ antiporter, there is evidence that the proximal tubule possesses a Na^+ -independent H^+ -ATPase that may also

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1. Abbreviations used in this paper: BCECF, 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein; BCECF-AM, BCECF-acetoxymethyl ester; DCCD, *N,N'*-dicyclohexylcarbodiimide; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; GTP, guanosine 5'-triphosphate; NEM, *N*-ethylmaleimide; pH_i , intracellular pH.

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function to absorb luminal bicarbonate and regulate pH_i (6–12).

We have recently demonstrated that the rabbit outer medullary proximal straight tubule (S₃ segment) generates a spontaneous luminal acidic disequilibrium pH of -0.46 (13). This finding indicated that the S₃ segment lacks functional luminal membrane-bound carbonic anhydrase and that the luminal fluid is acidified by proton secretion. The previous study did not address the transport mechanism(s) responsible for apical proton secretion in this segment. In the present study, we utilized newly developed fluorescent methodology for monitoring pH_i in isolated perfused tubules to determine directly the apical transport processes responsible for proton secretion in the rabbit S₃ proximal tubule.

Methods

Procedures. Tubules were dissected from male New Zealand white rabbits. The rabbits were killed by cervical dislocation and one kidney was removed and sliced into coronal sections. Superficial proximal straight tubules (S₂ segment) were dissected from the superficial medullary rays. The outer medullary proximal straight tubules (S₃ segment) were dissected by locating the beginning of a descending thin limb of Henle in the outer medulla and dissecting towards the cortex. No attempt was made to distinguish between the S₃ segment of superficial and juxtamedullary nephrons. The mean lengths of the dissected segments were 0.98 ± 0.03 mm for S₂ tubules and 0.94 ± 0.01 mm for S₃ tubules.

The tubules were transferred to a specially designed perfusion chamber (total volume 170 μl) that was blackened to minimize scattering of the excitation and emission light by the tubule and bathing solution. The tubules were mounted on concentric glass pipets, as described (14), except that a guard pipet was not used on the perfusion or collection end. The bathing solution was preheated to 37°C in a water-jacketed chamber and flowed by gravity through Saran tubing into the perfusion chamber at a rate of ~ 2 ml/min. Using this system, the bathing solution was continually flowing during an experiment with minimal tubule movement and without the formation of bubbles in the perfusion chamber. The bathing solution could be completely exchanged with a different solution in ~ 2 s. In all experiments, the tubules were perfused rapidly at a rate of ~ 50 nl/min per ml. Perfusion solutions could be completely changed in ~ 5 s.

Fluorescent measurement of pH_i . pH_i was monitored using the fluorescent probe 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF) (15) and a microfluorometer coupled to the tubule perfusion apparatus. To load the tubule cells with dye, the tubule was exposed to 13 μM BCECF-acetoxymethyl ester (AM) in the bathing solution for 10 min. (BCECF-AM was stored in dimethyl sulfoxide (DMSO) as a 4-mM stock solution at 0°C). After the loading period, the bath solution was replaced with an identical dye-free solution for at least 5 min before beginning an experiment. The tubule was not exposed to BCECF-AM in the bathing solution during an experiment since (a) some of the stock solution of BCECF-AM in DMSO hydrolyzes over time, forming BCECF that will cause the solution bathing the tubule to fluoresce and interfere with the cell measurements, and (b) the pH_i of the tubule cells immediately after the dye-loading procedure appears to be ~ 0.1 pH units lower than the final steady state pH_i . The acidifica-

tion of pH_i by hydrolyzable esters has been previously reported, e.g., 5(6)-carboxyfluorescein diacetate (16), QUIN-2 acetoxyethyl ester (16), and 5(6)-carboxy-4',5'-dimethylfluorescein diacetate (17).² The tubule was therefore bathed for at least 5 min after the loading procedure in a dye-free solution in order for pH_i to recover.

The excitation spectrum of BCECF has an excitation peak at ~ 500 nm and an isosbestic point at 440 nm. The emission spectrum has a peak at 530 nm (15). The 500/440 nm excitation ratio monitoring the fluorescence emission at 530 nm corresponds to a specific pH_i . A microfluorometer was developed by modifying a microscope (Zeiss IM; Carl Zeiss, Inc., Thornwood, NY) to allow for the monitoring of two excitation intensities simultaneously, which prevented bleaching, dye leakage, and movement artifacts from affecting the measurement (Fig. 1). The tubule cells were excited simultaneously at 440 nm (bandpass filter; Diffract Optics Inc., Signal Technology Corp., Hudson, MA) using a 100-W mercury arc lamp and at 500 nm (bandpass filter; Diffract Optics Inc., Signal Technology Corp.) using a 75-W xenon arc lamp by combining the two beams with a dichroic mirror (Blue reflective filter; Corion Corp., Holliston, MA). The two excitation sources were attenuated with neutral density filters and were each modulated at a different frequency using a five-slot chopper (Stanford Research Systems, Inc., Palo Alto, CA) coupled to the output of each light source. An electronic shutter (Vincent Associates, Rochester, NY) in front of each arc lamp exposed the cells to the excitation light only during data acquisition to minimize bleaching and photodamage. The 530-nm fluorescence emission (bandpass filter; Diffract Optics Inc., Signal Technology Corp.) was detected with a photomultiplier tube (Thorn EMI Gencom, Inc., Fairfield, NJ) and resolved into the 440- and 500-nm components using two lock-in amplifiers (Model SR510; Stanford Research Systems, Inc., Palo Alto, CA). The two fluorescent intensities and the 500/440 nm excitation ratio were recorded on a three-pen strip chart recorder (Model SR6221; Western Graphite Inc., Irvine, CA) and digitized by a computer (model MC-560; Masscomp, Westford, MA) for analysis and storage. In all experiments, the scattered background intensity at 440 and 500 nm were electronically subtracted from the fluorescent intensity at these wavelengths. Data was acquired from the proximal 0.7 mm of the tubule using a 20 \times objective (Carl Zeiss, Inc.). Calibration of intracellular BCECF was performed by monitoring the 500/440-nm excitation ratio at various values of pH_i . pH_i was set approximately equal to the extracellular pH by exposing the cells to a solution containing KCl, 115 mM; NaCl, 20 mM; K₂HPO₄, 2.5 mM; CaCl₂, 1 mM; MgCl₂, 1 mM; glucose, 5 mM; alanine, 5 mM; Hepes, 5 mM; and nigericin, 13.8 μ M that was titrated to a given pH. The calibration curve depicted in Fig. 2 (mean of five determinations) was used to convert all fluorescence data to pH_i . The in vivo calibration curve was identical in S₂ and S₃ tubules. None of the chemicals used in the present study altered the spectral properties or calibration of BCECF.

Solutions. The composition of the perfusate and bathing solutions was as follows: Na⁺, 140 mM; K⁺, 5 mM; Ca²⁺, 1 mM; Mg²⁺, 1 mM; Cl⁻, 144 mM; phosphate, 2.5 mM; glucose, 5 mM; alanine, 5 mM; and Hepes, 5 mM, equilibrated with 100% O₂, pH 7.4. When Na⁺-free solutions were used, NaCl was replaced with tetramethylammonium chloride. In Cl⁻-free experiments, Cl⁻ was replaced with equimolar gluconate and the total Ca²⁺ was increased to 3.5 mM as calcium gluconate. In experiments designed to acutely lower pH_i using the NH₄Cl prepulse technique (18), 20 mM NH₄Cl replaced 20 mM NaCl. When sodium- and chloride-free studies were performed, tetramethylammonium gluconate replaced equimolar NaCl, and total Ca²⁺ increased to 3.5 mM as calcium gluconate. Tetramethylammonium glu-

2. Since BCECF-AM has two more esterified carboxyl groups than 5(6)-carboxyfluorescein diacetate or 5(6)-carboxy-4',5'-dimethylfluorescein diacetate, it would be expected that at identical intracellular concentrations, BCECF-AM would release approximately twice as many protons upon hydrolysis. Whether this release of protons intracellularly fully accounts for the acidification of pH_i is not known.

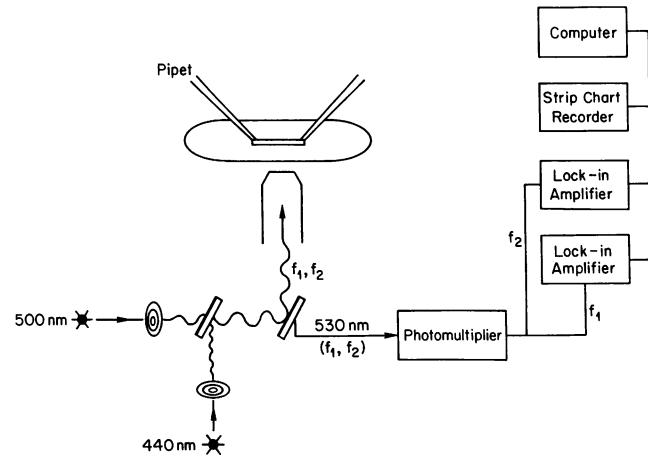


Figure 1. Microfluorometer designed to measure pH_i in isolated peritubular cells. Tubule cells were excited simultaneously at 440 and 500 nm using two excitation sources each chopped at a separate frequency. The 530 nm fluorescence emission was detected with a photomultiplier tube and resolved into the 440 and 500 nm components using two lock-in amplifiers. The data was recorded on a strip chart recorder and was digitized for storage and analysis on a computer (model MC-560; Masscomp).

conate was made by reacting tetramethylammonium hydroxide with equimolar D-gluconic acid lactone. In some experiments, ammonium gluconate was made by reacting ammonium hydroxide with equimolar D-gluconic acid lactone.

Materials. 4,4'-diisothiocyanostilbene-2,2'-disulphonic acid (DIDS) (Sigma Chemical Co., St. Louis, MO). (A stock solution of 50 mM DIDS [dissolved in DMSO] was made on the day of study and kept in the dark). BCECF-AM (Molecular Probes Inc., Junction City, OR), tetramethylammonium chloride (Sigma Chemical Co.), calcium gluconate (Sigma Chemical Co.), magnesium gluconate (Sigma Chemical Co.), sodium gluconate (Sigma Chemical Co.), amiloride hydrochloride dihydrate (Merck Sharp & Dohme Div., West Point, PA), nigericin (Sigma Chemical Co.), iodoacetic acid (Sigma Chemical Co.), potassium cyanide (Aldrich Chemical Co., Milwaukee, WI), ATP (Sigma Chemical Co.), AMP (Sigma Chemical Co.), guanosine 5'-triphosphate (GTP) (Sigma Chemical Co.), tetramethylammonium hydroxide (Sigma Chemical Co.), colchicine (Sigma Chemical Co.), D-gluconic acid lactone (Sigma Chemical Co.), N,N'-dicyclohexylcarbodiimide (DCCD) (Sigma Chemical Co.), and N-ethylmaleimide (NEM) (Sigma Chemical Co.) were used in this study.

Statistics. Results are reported as mean \pm SEM. Unpaired student's *t* test was used to compare group means. Linear regression analysis was used as required.

Results

Apical Na⁺/H⁺ antiporter. The following series of experiments were performed to examine whether the S₃ tubule possesses an

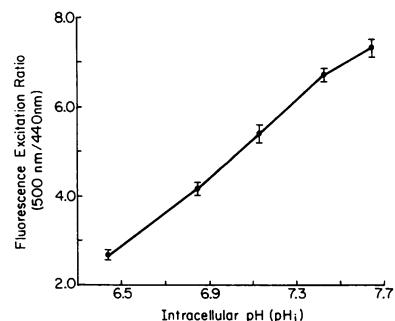


Figure 2. In vivo calibration curve of BCECF in S₃ tubules. Each point represents the mean \pm SEM of five determinations.

apical Na^+/H^+ antiporter. Steady state pH_i in tubules bathed and perfused in Hepes-buffered solutions in the absence of organic anions, SO_4^{2-} , pH 7.4, was 7.08 ± 0.03 ($n = 45$). When luminal Na^+ was decreased from 140 to 0 mM (Fig. 3, Table I), pH_i fell at a rate of 0.34 ± 0.06 pH/min ($n = 9$). Readdition of 140 mM Na^+ to the lumen resulted in the recovery of pH_i . In the presence of 10^{-3} M luminal amiloride (Fig. 3, Table I), the rate of change of pH_i upon luminal Na^+ removal was significantly decreased to 0.13 ± 0.03 pH/min ($n = 6$), $P < 0.05$. In the presence of 50 μM luminal DIDS, the decrease in pH_i upon luminal Na^+ removal was not different from control tubules, 0.31 ± 0.04 pH/min ($n = 8$), and readdition of 140 mM Na^+ to the lumen resulted in the recovery of pH_i (Fig. 3, Table I). When S_3 tubules were perfused and bathed in Cl^- -free solutions, removal of luminal Na^+ resulted in a decrease in pH_i of 0.32 ± 0.05 pH/min ($n = 4$), which was not significantly different from the control rate (Fig. 3, Table I). These results suggest that the S_3 tubule possesses a luminal Na^+/H^+ antiporter.

The kinetics of the apical Na^+/H^+ antiporter were determined by measuring the rate of increase in pH_i upon the readdition of varying concentrations of Na^+ to the lumen after luminal Na^+ removal (Fig. 4, *a* and *b*). A Hanes-Woolf analysis of the data indicates that the Michaelis constant (K_m) for luminal Na^+ is 29 mM with a maximum velocity (V_{max}) of 0.47 pH/min.

Plasma membrane H^+ -ATPase. Additional experiments were performed to determine whether the S_3 proximal tubule possesses a plasma membrane H^+ -ATPase and if it has a role in regulating pH_i . In these experiments, the tubules were acutely acidified using the NH_4Cl prepulse technique (18). S_3 tubules were bathed and perfused for 15 min with 20 mM NH_4Cl and Hepes-buffered solutions, pH 7.4, in the nominal absence of HCO_3^- , organic anions, and SO_4^{2-} . When NH_4Cl was removed from the lumen and bath (Fig. 5 *a*), pH_i fell due to the rapid cellular efflux of NH_3 and then recovered at a rate of 0.39 ± 0.07 pH/min ($n = 6$). When similar experiments were performed in the absence of luminal and basolateral Na^+ , pH_i recovered at a rate of 0.89 ± 0.15 pH/min ($n = 11$), $P < 0.05$ (Figs. 5 [*b*] and 6). These results indicate the presence of a Na^+ -independent pH_i regulatory process in the S_3 segment.

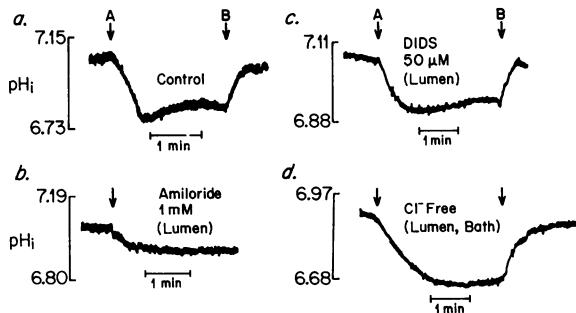


Figure 3. Effect of luminal Na^+ removal and readdition on pH_i in S_3 tubules. (*a*) Control: at *A*, luminal Na^+ was decreased from 140 to 0 mM resulting in a decrease in pH_i ; at *B*, luminal Na^+ was increased to 140 mM, which resulted in the recovery of pH_i . (*b*) Amiloride (10^{-3} M, lumen) significantly decreased change of pH_i upon luminal Na^+ removal. (*c*, *d*) In the presence of 50 μM DIDS (lumen) or Cl^- -free solutions, the rate of change in pH_i during the removal, *A*, and readdition of Na^+ , *B*, to the lumen was not different from the control rate (See Table I for summary of above results).

Table I. Rate of Decrease in pH_i After Luminal Sodium Removal in S_3 Tubules

	Control	Amiloride (lumen) (1 mM)	DIDS (lumen) (50 μM)	Chloride free (lumen, bath)
dpH_i/dt (pH/min)	0.34 ± 0.06	$0.13 \pm 0.03^*$	0.31 ± 0.04	0.32 ± 0.05
<i>n</i>	9	6	8	4

dpH_i/dt was measured in the 1st 16 s after removal of luminal Na^+ .

* $P < 0.05$ vs. control.

When S_2 tubules were acidified after a brief exposure to 20 mM NH_4Cl , like the S_3 segment, pH_i recovered at a rate of 0.55 ± 0.07 pH/min ($n = 10$) (Fig. 5 *c*). Unlike the S_3 segment, when S_2 tubules were acidified in the absence of luminal and basolateral Na^+ , the pH_i recovery rate was significantly decreased to 0.10 ± 0.02 pH/min ($n = 9$) (Figs. 5 [*d*] and 6), $P < 0.001$. Therefore, the Na^+ -independent pH_i regulatory process is quantitatively greater in the S_3 segment.

Further experiments were performed to investigate the Na^+ -independent pH regulatory process in S_3 tubules. In the presence of 1 mM luminal NEM, a H^+ -ATPase inhibitor (exposure time, ~ 15 min), the rate of recovery of pH_i was significantly decreased to 0.40 ± 0.08 pH/min ($n = 7$), $P < 0.02$ (Fig. 7 *a*, Table II). Similarly, 1 mM luminal DCCD, a H^+ -ATPase inhibitor (exposure time, ~ 15 min), significantly decreased the rate of pH_i recovery to 0.24 ± 0.05 pH/min ($n = 9$), $P < 0.01$ (Fig. 7 *b*, Table II).

Since the H^+ -ATPase inhibitors could have altered the recovery of pH_i by inhibiting mitochondrial oxidative phosphorylation, the pH_i recovery rate was measured after an

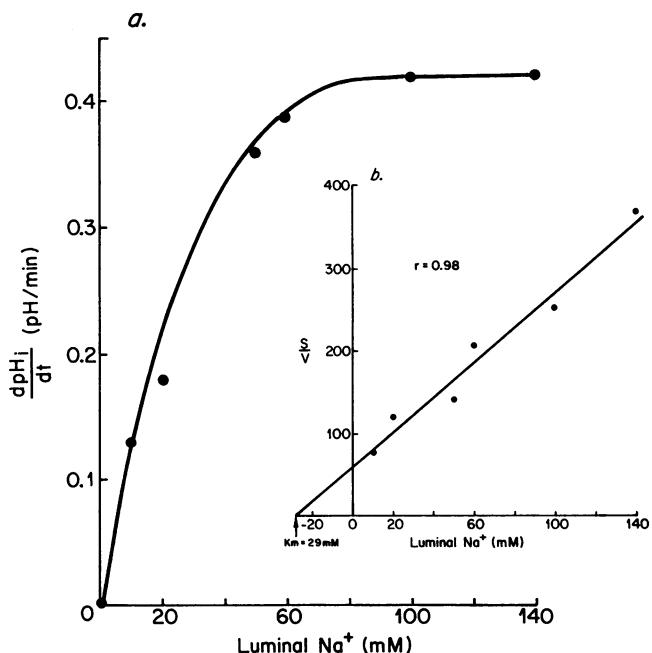


Figure 4. Kinetics of luminal Na^+/H^+ antiporter. (*a*) After removal of luminal Na^+ , varying concentrations of Na^+ (0–140 mM) were readded to the lumen and the rate of increase in pH_i was measured (initial 16 s). Each point represents the mean of at least three determinations. (*b*) A Hanes-Woolf analysis of the data indicates that the K_m is 29 mM for luminal Na^+ ; V_{max} , 0.47 pH/min.

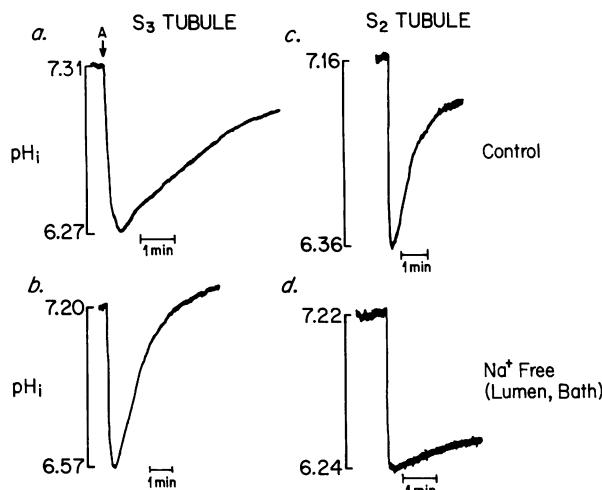


Figure 5. Acute acidification of pH_i after the removal of NH_4Cl in S_2 and S_3 tubules. Tubules were perfused and bathed in 20 mM NH_4Cl for 15 min. After the removal of NH_4Cl (lumen, bath) at A, pH_i decreases due to the rapid cellular efflux of NH_3 . The decrease in pH_i is followed by a recovery of pH_i . S_3 tubule: (a) in the presence of 140 mM Na^+ (lumen, bath) pH_i recovered at a rate of 0.39 ± 0.07 pH/min ($n = 6$); (b) in the absence of Na^+ (lumen, bath) pH_i recovered at a rate of 0.89 ± 0.15 pH/min ($n = 11$), $P < 0.05$. S_2 tubule: (c) in the presence of 140 mM Na^+ (lumen, bath) pH_i recovered at a rate of 0.55 ± 0.07 pH/min ($n = 10$); (d) in the absence of Na^+ (lumen, bath) the pH_i recovery rate was significantly decreased to 0.10 ± 0.02 pH/min ($n = 9$), $P < 0.001$.

~ 30-min exposure of S_3 tubules to 1 mM potassium cyanide (lumen, bath), an inhibitor of mitochondrial ATP production. The pH_i recovery rate of 0.69 ± 0.10 pH/min ($n = 5$) in the presence of 1 mM KCN was not significantly different from the control rate (Fig. 8 a, Table III). These results suggested that if the Na^+ -independent NEM- and DCCD-sensitive pH_i regulatory process was a plasma membrane H^+ -ATPase, the ATP was not being utilized from a mitochondrial source since 1 mM potassium cyanide had no effect on pH_i regulation.

H^+ -ATPase: source of ATP. Previous studies in turtle urinary bladder (19), Ehrlich ascites cells (20), and rabbit inner stripe medullary collecting tubule cells (21) have demonstrated that the H^+ -ATPase in these cells can utilize ATP derived from glycolysis. To determine whether the DCCD- and NEM-sensitive pH_i regulatory process in the S_3 tubule can also utilize ATP derived from glycolysis, S_3 tubules were exposed to the glycolytic inhibitor iodoacetic acid for ~ 30 min (lumen and bath). After exposure to 0.5 mM iodoacetic acid, the rate of

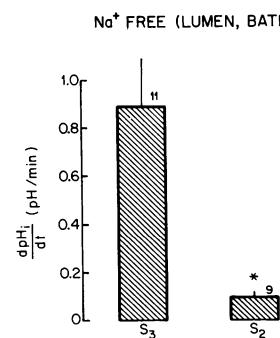


Figure 6. Rate of recovery of pH_i in S_2 and S_3 tubules after acidification with NH_4Cl prepulse in the absence of Na^+ (lumen, bath). The recovery rate of 0.10 ± 0.02 pH/min ($n = 9$) in S_2 tubules was significantly lower than that of 0.89 ± 0.15 pH/min ($n = 11$) S_3 tubules. * $P < 0.001$.

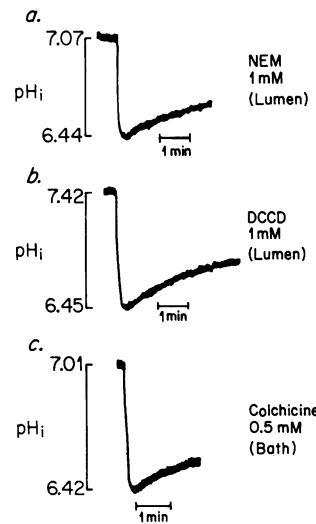


Figure 7. Recovery of pH_i after intracellular acidification in S_3 tubules in the absence of Na^+ : H^+ pump inhibition. (a) NEM, 1 mM (lumen). pH_i recovery rate significantly decreased from 0.89 ± 0.15 ($n = 11$) to 0.40 ± 0.08 pH/min ($n = 7$), $P < 0.02$. (b) DCCD, 1 mM (lumen). pH_i recovery rate significantly decreased to 0.24 ± 0.05 pH/min ($n = 9$), $P < 0.01$. (c) Colchicine, 0.5 mM (bath). pH_i recovery rate significantly decreased to 0.20 ± 0.04 pH/min ($n = 8$), $P < 0.001$.

pH_i recovery was significantly decreased to 0.12 ± 0.04 pH/min ($n = 8$), $P < 0.01$ (Fig. 8 b, Table III).

Exogenous ATP and AMP have recently been reported to increase the ATP content of proximal tubule suspensions via cellular uptake of adenosine, which is converted by adenosine kinase to AMP (22). When S_3 tubules were exposed to 0.5 mM iodoacetic acid in the presence of 0.25 mM ATP or 0.25 mM AMP (lumen, bath), for ~ 30 min, the pH_i recovery rate was normalized (Fig. 8 c and d, Table III). In the presence of 1 mM KCN (lumen, bath) and 0.5 mM iodoacetic acid (lumen, bath), 0.25 mM AMP failed to normalize the pH_i recovery rate (Fig. 8 e, Table III). In S_3 tubules exposed to 0.5 mM iodoacetic acid (lumen, bath) and 0.25 mM GTP (lumen, bath), the pH_i recovery rate significantly decreased to 0.31 ± 0.08 pH/min ($n = 4$), $P < 0.05$ (Fig. 8 f, Table III). These experiments suggest that the S_3 proximal straight tubule possesses a plasma membrane H^+ -ATPase that utilizes ATP derived primarily from glycolysis.

H^+ -ATPase: fusion with plasma membrane. In a recent study, Schwartz et al. demonstrated that acidic endocytic vesicles, possibly containing H^+ pumps, fused with the apical membrane of the proximal straight tubule when pH_i was decreased acutely on exposure to CO_2 in the presence or absence of Na^+ (8). The role of proton pump fusion in pH_i regulation was therefore investigated in S_3 tubules exposed to 0.5 mM colchicine (bath), an inhibitor of microtubule function, for

Table II. Recovery of pH_i After Intracellular Acidification in S_3 Tubules in the Absence of Sodium: H^+ Pump Inhibition

	Control	NEM (1 mM)	DCCD (1 mM)	Colchicine (0.5 mM)
$d\text{pH}_i/dt$ (pH/min)	0.89 ± 0.15	$0.40 \pm 0.08^*$	$0.24 \pm 0.05^\dagger$	$0.20 \pm 0.04^\ddagger$
Minimum pH_i	6.42 ± 0.06	6.38 ± 0.06	6.53 ± 0.06	6.52 ± 0.06
n	11	7	9	8

DCCD and NEM were present in the lumen for ~ 15 min before performing a study.

Colchicine was present in the bath for ~ 15 min before performing a study.

* $P < 0.02$ vs. control.

† $P < 0.01$ vs. control.

‡ $P < 0.001$ vs. control.

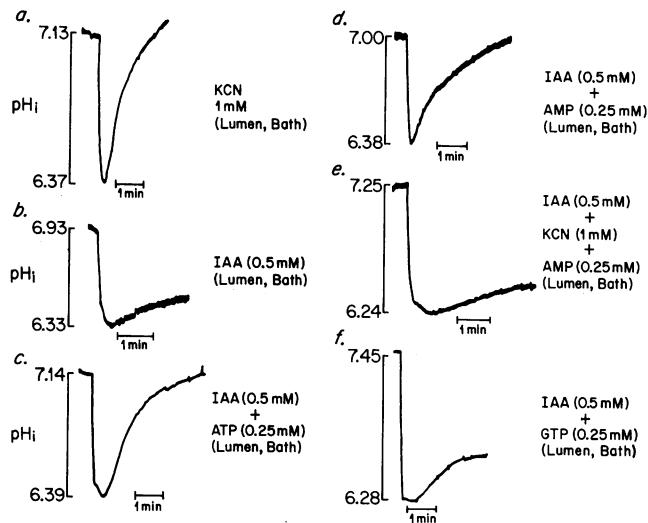


Figure 8. Recovery of pH_i after intracellular acidification in S_3 tubules in the absence of Na^+ : metabolic inhibition. (a) KCN, 1 mM (lumen, bath). pH_i recovery rate was $0.69 \pm 0.10 \text{ pH/min}$ ($n = 5$), which was not significantly different from that of the control. (b) Iodoacetic acid, 0.5 mM (lumen, bath). pH_i recovery rate was significantly decreased to $0.12 \pm 0.04 \text{ pH/min}$ ($n = 8$), $P < 0.01$. (c) Iodoacetic acid, 0.5 mM plus ATP, 0.25 mM (lumen, bath). pH_i recovery rate was $0.69 \pm 0.10 \text{ pH/min}$ ($n = 9$), which was not significantly different from that of the control. (d) Iodoacetic acid, 0.5 mM plus AMP, 0.25 mM (lumen, bath). pH_i recovery rate was $1.13 \pm 0.17 \text{ pH/min}$ ($n = 7$), which was not significantly different from that of the control. (e) Iodoacetic acid, 0.5 mM plus KCN, 1 mM plus AMP, 0.25 mM (lumen, bath). pH_i recovery rate was significantly decreased to $0.11 \pm 0.05 \text{ pH/min}$ ($n = 4$), $P < 0.02$. (f) Iodoacetic acid, 0.5 mM plus GTP, 0.25 mM (lumen, bath). pH_i recovery rate was significantly decreased to $0.31 \pm 0.08 \text{ pH/min}$ ($n = 4$), $P < 0.05$.

~ 15 min in the nominal absence of Na^+ , HCO_3^- , organic anions, and SO_4^{2-} . The pH_i recovery rate after acidification was significantly decreased to $0.20 \pm 0.04 \text{ pH/min}$ ($n = 8$), $P < 0.001$ (Fig. 7 c, Table II). These results complement those of Schwartz and suggest that in S_3 tubules, after intracellular acidification, exocytosis of vesicles containing H^+ pumps contributes to the recovery of pH_i .

H^+ -ATPase: Cl^- dependence. Further experiments were performed to determine the Cl^- dependence of the H^+ -ATPase and the sidedness of the process. S_3 tubules were, as in the previous experiments, perfused and bathed in Hepes-buffered solutions, pH 7.4, in the absence of luminal and basolateral Na^+ , organic anions, and sulphate. When chloride was removed from the lumen and bath solutions just as the tubule was acidified, (by removal of NH_4Cl) the pH_i recovery rate was not different than the control rate (Fig. 9 a). This result indicates that extracellular chloride is not required for pH_i recovery. When S_3 tubules were perfused and bathed in a Cl^- -free solution for 30 min to decrease the intracellular chloride concentration and then acidified by removal of 20 mM ammonium gluconate, pH_i failed to recover normally (Fig. 9 b). These results suggest that intracellular rather than extracellular chloride is required for pH_i recovery. Intracellular Cl^- may have been required both for the H^+ -ATPase and for a plasma membrane Cl^- -base exchanger. However, 50 μM DIDS in the lumen and bath did not affect the pH_i recovery rate (Fig. 9 c), which suggests that reversal of a plasma membrane Cl^- -base

exchanger was not contributing significantly to the recovery of pH_i (Fig. 9 c).³

Discussion

Fluorescent measurement of pH_i . In this study, new fluorescent methodology was developed to measure pH_i in the isolated perfused renal tubule preparation. Several techniques are currently available for measuring pH_i in living cells (18). Of the several methods available, optical techniques have a number of important advantages, notably, rapid response time, excellent pH sensitivity, and a high signal-to-noise ratio. Despite these advantages, several difficulties are encountered when monitoring pH_i in cells with optical probes, which include (i) dye leakage (active, passive), (ii) bleaching, and (iii) photodynamic damage (24). The fluorescent pH probe, BCECF (16), has a maximum in its excitation spectrum at ~ 500 nm and an isosbestic wavelength at ~ 440 nm; therefore, the ratio of the 500 to 440 nm excitation intensities (emission 530 nm) will be independent of dye leakage and bleaching artifacts. In previous studies using BCECF, excitation wavelength switching has been achieved with either filter wheel rotation, motorized monochrometers, or manual sliding of interference filters in front of the excitation source. These methodologies are limited in that the two excitation intensities are not determined simultaneously. Therefore a new technique was developed in the present study to excite the tubule cells at 440 and 500 nm simultaneously. By modulating the 440 and 500 nm fluorescent excitation intensities at two separate frequencies, the two excitation intensities could be measured simultaneously by using two lock-in amplifiers to demodulate the 530-nm fluorescence emission into the 440- and 500-nm components. By measuring the 500/440 nm excitation ratio simultaneously, the inaccuracy in the measurement due to dye loss and bleaching artifacts was minimized, and rapid changes in the fluorescent excitation ratio could be monitored in real time.

Apical Na^+/H^+ exchanger. In S_3 tubules, steady state pH_i was 7.08 ± 0.03 ($n = 45$) in Hepes-buffered solutions, pH 7.4, in the absence of organic anions and sulphate. This value is similar to that reported in the rat proximal convoluted tubule (5, 25) and the rabbit S_2 proximal straight tubule (4) in HCO_3^- -buffered media. The results of the present study provide direct evidence for an apical Na^+/H^+ antiporter in rabbit S_3 tubules as has previously been demonstrated in the rabbit and rat proximal convoluted tubule (2, 5), the rabbit S_2 proximal straight tubule (4), and the salamander proximal tubule (3). The antiporter was shown to play an important role in pH_i regulation in the S_3 segment. Kinetic studies revealed a K_m of

3. These results do not rule out the presence of a Cl^- -base exchanger in the S_3 segment. These findings suggest, however, that if a Cl^- -base exchanger is present under the conditions of this study, this transporter does not contribute to the recovery of pH_i after an acute intracellular acid load as recently described in Madin-Darby canine kidney cells (23). Also, since these experiments were performed in the nominal absence of HCO_3^- , the rate of Cl^- -base exchange is likely decreased (23).

4. The kinetics of Na^+ -dependent recovery of pH_i could have been affected by the paracellular backleak of Na^+ from bath to lumen. Assuming a transepithelial Na^+ permeability of $6 \times 10^{-5} \text{ cm/s}$ (26) and a tubule radius of $10 \mu\text{m}$, a backflux of 300 pmol/mm per min would be expected. Since the length of the tubules studied was 0.7 mm, with a

Table III. Recovery of pH_i After Intracellular Acidification in S_3 Tubules in the Absence of Sodium: Metabolic Inhibition

	Control	KCN (1 mM)	Iodoacetic acid (0.5 mM)	Iodoacetic acid (0.5 mM) + AMP (0.25 mM)	Iodoacetic acid (0.5 mM) + ATP (0.25 mM)	Iodoacetic acid (0.5 mM) + KCN (1 mM) + AMP (0.25 mM)	Iodoacetic acid (0.5 mM) + GTP (0.25 mM)
dpH_i/dt (pH/min)	0.89±0.15	0.69±0.10	0.12±0.04*	1.13±0.17	0.69±0.10	0.11±0.05‡	0.31±0.08§
Minimum pH_i	6.42±0.06	6.42±0.03	6.43±0.06	6.41±0.08	6.36±0.04	6.20±0.04	6.42±0.07
n	11	5	8	7	9	4	4

All compounds were present in the lumen and bath for ~ 30 min before performing a study. dpH_i/dt was measured in the initial 16 s of pH_i recovery. * $P < 0.01$ vs. control. ‡ $P < 0.02$ vs. control. § $P < 0.05$ vs. control.

29 mM for luminal Na^+ and a V_{max} of 0.47 pH/min.⁴ The K_m of 29 mM is almost identical to the value of 27.3 mM recently reported by Chaillet et al. for the Na^+/H^+ antiporter in the basolateral membrane of the rabbit cortical collecting duct (28). The V_{max} of 0.47 pH/min in the S_3 segment is lower than previously reported in the cortical collecting tubule (28) and may reflect either a lower number of antiporters or a decreased rate of ion transport per antiporter in the S_3 segment. In a recent study, Kragh-Hansen et al. using the rapid filtration technique failed to find evidence for a Na^+/H^+ exchanger in brush border vesicles isolated from rabbit outer medulla (29). However, in a more recent study, antiport activity was demonstrated using acridine orange (27). The reason for the lack of demonstrable Na^+/H^+ antiport activity in the initial study is not clear, but may be due to the lower V_{max} of the antiporter in the S_3 segment. Recent studies indicate that the V_{max} of the antiporter in the S_2 segment is three to four times the value in the S_3 segment (27, Kurtz, I., unpublished results). These kinetic differences possibly account for the low HCO_3^- absorptive rate in the S_3 segment (< 8 pmol/min per mm) as compared with the S_2 tubule (13).

Plasma membrane H^+ -ATPase. In the S_3 tubule after acute intracellular acidification, pH_i recovered more rapidly in the absence of luminal and basolateral sodium than in the presence of Na^+ . This finding suggests that either (a) a Na^+ -dependent base efflux pathway such as a basolateral Na^+ -base cotransporter diminished the rate of recovery of pH_i in the presence of Na^+ , or (b) the rate of the Na^+ -independent pH_i regulatory process is diminished in the presence of Na^+ . The results of the present study do not distinguish between these possibilities. In the rat proximal tubule a Na^+ -independent DCCD-sensitive transport process accounts for ~ 15% of luminal HCO_3^- absorption (9). The Na^+ -independent pH_i recovery rate in the S_3 tubule was also DCCD sensitive in addition to being NEM sensitive, but was not affected by inhibition of mitochondrial oxidative phosphorylation with KCN. Iodoacetic acid, an inhibitor of glycolysis, significantly decreased the Na^+ -independent pH_i recovery rate despite normal functioning mitochondria. When S_3 tubules were exposed to iodoacetic

acid in addition to either AMP or ATP, the pH_i recovery rate was normalized. GTP, however, failed to normalize the rate of pH_i recovery. These findings suggest that the S_3 tubule possesses a plasma membrane H^+ -ATPase that utilizes ATP derived primarily from glycolysis. The mechanism by which this occurs is unknown, but may involve the physical proximity of membrane-bound glycolytic enzymes and the transport proteins as postulated in erythrocytes, vascular smooth muscle, and cultured cells (30–32). It is also possible that the quantity of ATP produced from glycolysis is greater than mitochondrial ATP production rate in the S_3 segment. The utilization of ATP derived from glycolysis by a transport process in the proximal tubule is surprising given the current dogma that the rate of glycolysis in this part of the nephron is low. A recent preliminary report, however, suggests that the rate of glycolysis in the rabbit proximal straight tubule is greater than that in the proximal convoluted segment (33).

When glycolysis was inhibited with iodoacetic acid, exogenous AMP or ATP normalized the pH_i recovery rate. Recent studies have demonstrated that exogenous adenosine nucleotides increase the cytoplasmic ATP concentration in proximal tubule suspensions via cellular uptake of adenosine, which is converted to AMP by adenosine kinase. The sidedness of the process was not examined in the present study as the nucleotides were added to both the luminal and bathing solutions. The finding in the present study that 1 mM KCN prevented exogenous AMP from normalizing pH_i in the presence of iodoacetic acid suggests that functional mitochondria were required for exogenous AMP to normalize the pH_i recovery rate when glycolysis was inhibited. Therefore, ATP derived from a mitochondrial source under certain circumstances can be utilized.

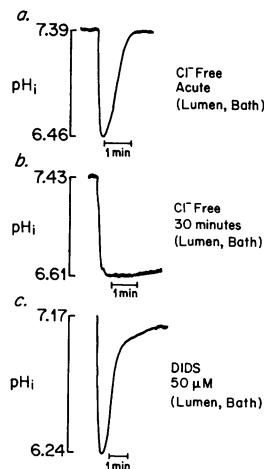


Figure 9. (a) Cl^- was removed from the bath and lumen immediately after acidification of pH_i by NH_4Cl removal. Acute Cl^- removal failed to inhibit the recovery of pH_i . (b) Cl^- was removed from bath and lumen for 30 min to decrease the intracellular Cl^- before acidification of pH_i by ammonium gluconate removal. After chronic Cl^- removal, pH_i failed to recover normally. (c) Recovery of pH_i in the presence of 50 μM DIDS (lumen, bath). 50 μM DIDS failed to inhibit the recovery of pH_i . All experiments in (a–c) were performed in the absence of Na^+ (lumen, bath).

perfusion rate of ~ 50 nl/min per mm, the mean luminal Na^+ concentration would be ~ 2 mM greater than the perfusate Na^+ concentration. Using the corrected luminal Na^+ concentration in the kinetic analysis, the V_{max} for the luminal Na^+ is 0.49 pH/min instead of 0.47 pH/min and the K_m for luminal Na^+ is 34 mM instead of 29 mM. The K_m of 29 mM and the corrected value of 34 mM for luminal Na^+ bracket the K_m of 32 mM recently determined for Na^+ in outer medullary brush border vesicles (27).

lized by the proton pump, which indicates that the coupling to glycolysis is not obligatory.

In contrast to the proton pump utilizing ATP derived primarily from glycolysis in the S₃ tubule, Na⁺ efflux by the basolateral Na⁺-K⁺-ATPase in the proximal tubule is coupled to mitochondrial oxidative phosphorylation (34). The rate of Na⁺-coupled transport is greatest in the earliest portion of the proximal tubule (S₁ segment) and decreases along the length of the proximal tubule (35) as does the number of mitochondria. In the S₁ segment of the proximal tubule, which has the highest rate of fluid and HCO₃⁻ absorption, the mitochondria are large, numerous, and arranged perpendicularly near the basolateral cell membrane (36). In the S₃ segment, which has the lowest rate of fluid and HCO₃⁻ absorption (13), mitochondria are few in number and are scattered throughout the cytoplasm (36).

Recent studies by Schwartz et al. have demonstrated that acidic endocytotic vesicles fuse with the apical membrane of S₂ proximal straight tubules when exposed to CO₂ (8). These vesicles were presumed to contain H⁺ pumps that fused with the apical membrane during acute cytoplasmic acidification. Colchicine inhibited the fusion process. In the present study, colchicine was also found to impair the Na⁺-independent pH_i recovery rate in S₃ tubules, which suggests that fusion of cytoplasmic vesicles containing H⁺ pumps occurred in response to acute acidification of pH_i. In the present study, unlike the S₃ segment, S₂ tubules failed to recover pH_i normally in the absence of luminal and basolateral Na⁺. Although these results do not rule out a pH_i regulatory role for a plasma membrane H⁺ pump in the S₂ segment, the role of the H⁺ pump in regulating pH_i appears to be quantitatively greater in the S₃ segment. Whether the difference is due to a greater number of copies of plasma membrane H⁺-ATPase in the S₃ segment, a greater flux of protons per ATPase, or simply a greater number of fusion events is not presently known. The results of the present study complement the study of Abdelkhalek et al. (12), which localized extramitochondrial microsomal HCO₃⁻-ATPase along the rabbit nephron. In that study, the highest enzyme activity was found in the S₃ proximal straight tubule and the late distal convoluted tubule. Whether this enzyme is the same enzyme involved in H⁺ transport or pH_i regulation remains to be determined.

Recent studies have documented the requirement of the renal H⁺-ATPase for chloride (10, 11, 37). Chloride, in addition to providing a conductance for the electrogenic H⁺ pump, appears to activate the pump at a separate regulatory site. In a recent study, Cl⁻ stimulated proton pump transport in a dose-dependent manner with a K_{0.5} of 7 mM (11) for chloride. The results of the present study also document the requirement of the H⁺ pump for intracellular chloride since the Na⁺-independent pH_i recovery rate was significantly decreased in S₃ tubules depleted of intracellular chloride.

In summary, S₃ proximal tubules possess an apical Na⁺/H⁺ antiporter with a K_m of 29 mM for luminal Na⁺ and a V_{max} of 0.47 pH/min. S₃ tubules also possess a plasma membrane H⁺-ATPase that can regulate pH_i, has a requirement for intracellular chloride, and utilizes ATP derived primarily from glycolysis.

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