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Regulation of Principal Cell pH by Na/H Exchange in Rabbit Cortical Collecting Tubule

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Summary. Changes in intracellular pH (pH_i) were measured using the pH indicator, BCECF, in principal cells from split opened cortical collecting tubules (CCTs) derived from rabbits maintained on a normal diet. This monolayer preparation has the advantage of allowing us to visualize the morphological differences in the two major cell types in this nephron segment under transmitted light. The visual identification of the cell types was verified using emission measurements taken from single principal and intercalated cells in the opened tubule which had been exposed to fluorescein isothiocyanate (FITC)-labeled peanut lectin. We confirmed the existence of an amiloride-sensitive Na/H exchange process activated during intracellular acidosis in principal cells. In addition, the exchanger was active under basal conditions and over a wide range of pH_i. Because the exchanger was active under basal conditions we tested the hypothesis that changes in intracellular Na (Na_i) would alter pH_i in a predictable way. Maneuvers designed to alter Na_i were without significant effects within a 10-min time frame. Specifically, addition of 100 μM ouabain to increase Na_i or exposure of the tubules to 10^{-5} M amiloride to decrease luminal Na entry and reduce Na_i did not have an effect on pH_i. In some experiments we did observe however, after a 30-min exposure to ouabain, a small decrease in pH_i. These results suggest that Na/H exchange is a major regulator of pH_i in principal cells. However, regulation of Na transport by changes in pH_i in principal cells of rabbit CCT via the activity of a Na/H exchanger do not seem to contribute to the feedback control of Na transport.

Key Words pH-sensitive dye · BCECF · ouabain · amiloride · Na transport · feedback regulation

Introduction

It is believed that the Na transport rate in tight epithelia is a function of various intrinsic factors coordinated in such a way as to maintain intracellular Na homeostasis. A factor that has been implicated directly in this process is intracellular pH (pH_i).

There is considerable evidence that apical membrane Na channels in tight epithelia are regulated by pH_i. Transepithelial transport studies on various tissues have demonstrated that acidification of the cytoplasm with CO_2 results in a decrease in the rate of Na transport. Ussing and Zerahn (1951) showed that exposure to CO_2 reduced transepithelial Na transport in frog skin. Palmer (1985) using K-depolarized toad bladder found that acute exposure to CO_2 at the apical side of the bladder at constant extracellular pH resulted in decreased Na transport and apical Na permeability. Subsequently, Harvey, Thomas and Ehrenfeld (1988) showed that acidification of the cytoplasm of frog skin with CO_2 reduced apical Na and basolateral K permeabilities. A direct effect of pH on Na channels has been demonstrated with the patch-clamp technique in inside-out apical patches from rat CCT where alkalinization of the cytoplasmic side of the patch increased channel activity (Palmer & Frindt, 1987*b*).

Because cytoplasmic H ion activity can alter apical Na permeability, the basolateral Na/H exchange mechanism is a potential component of negative feedback because this exchanger provides a means of limiting Na influx by affecting pH_i. Increases or decreases in cell Na (Na_i) will either slow down or speed up the flow of H ions out of the cell via this exchange mechanism. Since an Na/H exchange mechanism exists in the basolateral membrane of CCT (Chaillet, Lopes & Boron, 1985), we hypothesized that if this exchange process were operative under basal conditions then changes in Na_i could lead to predictable changes in pH_i. The development of membrane-permeant fluorescent probes has made it possible to monitor intracellular changes in pH in intact epithelia. With the intracellular indicator 2′7′-bis-(2-carboxyethyl)-5,6-carboxyfluorescein (BCECF) for measuring pH_i we have addressed two questions. First, to what extent does this exchanger contribute to H^+ homeostasis under basal conditions, and second, does this exchanger participate in the negative feedback control of Na transport.

Table 1.

Component	Dissection solution 1	HEPES 5 mM 2	HEPES 25 mM 3	HEPES NH_4^+ 4	HEPES 0 Na 5	CO_2 6% 6	CO_2 6% 7	HEPES 5 mM 8	HEPES 5 mM 9	HEPES 5 mM 10	HEPES 25mM 11
NaCl	140.0	136.5	122.5	126.5	—	114.2	4.0	136.5	136.5	27.0	17.5
NH_4Cl	—	—	—	10.0	—	—	—	—	—	—	—
$NaHCO_3$	—	—	—	—	—	26.0	26.0	—	—	—	—
NMDG HCl	—	—	—	—	136.4	—	110.0	—	—	110.0	110.0
HEPES	—	5.0	25.0	5.0	5.0	—	—	5.0	5.0	5.0	25.0
pH	7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.8	7.0	7.4	7.4

Na-free calibration solutions for intracellular pH

	a	b	c	d	e
KCl	129.0	128.0	126.0	124.0	123.0
HEPES	25.0	25.0	25.0	25.0	25.0
pH	6.5	6.8	7.2	7.5	7.8

The compositions of the above solutions are expressed in mM/liter. In addition to the above components all solutions contained the following (in mM): 2 $CaCl_2$, 1.2 $MgSO_4$, 2.5 K_2HPO_4, 5.5 dextrose, 6 L-alanine, 5 nalactate in all Na-containing solutions and 5 lactic acid in the NMDG-containing solution. NaOH was used in all Na-containing solutions and KOH in Na-free calibration solutions to titrate to the appropriate pH.

Materials and Methods

General

New Zealand white rabbits of either sex (2–3.5 kg body weight) maintained on standard chow were used in this study. CCTs were dissected and split exposing the luminal surface (Palmer & Frindt, 1986). The opened tubule was transferred to a small plastic rectangle prepared with a patch of Cell Tak (Collaborative Research, Bedford, MA) to which the tubule adhered. Mounted preparations were placed luminal side up in a flow-through Lucite chamber fitted on the bottom and top with standard glass coverslips ~0.17 mm thick.

Solutions

The compositions of the solutions used are given in Table 1. HEPES buffer was used in all nonbicarbonate-containing solutions. All chemicals were obtained from Sigma Chemical unless otherwise specified. Nigericin (Molecular Probes, Eugene, OR.) was added to potassium Ringer's solutions (solutions a, b, c, d, and e) from a 10-mM stock (3 parts ethanol: 1 part DMFO) for a final concentration of 10 μM. Individual vials (50 μg) of 2',7'-bis (carboxyethyl)-5,6-carboxyfluorescein acetoxymethyl ester (Molecular Probes, Eugene, OR) BCECF-AM) were stored dry at −20°C and reconstituted in dimethylsulfoxide (10 mM) for each experiment. The final loading concentration of dye was 10 μM in solution 1 for BCECF-AM. Amiloride hydrochloride dihydrate, amiloride 5-(*n*,N-hexamethylene) (HMA) and benzamil were obtained from Research Biochemicals (Natick, MA). Fluorescein isothiocyanate (FITC) labeled peanut lectin was added to solution 1 for a final concentration of 10 μM.

Apparatus

Solutions were gravity fed into a 6-port Hamilton valve. The solution exiting the valve entered a miniature water-jacketed glass coil (Radnoti Glass Technology, Monrovia, CA) for regulating its temperature. From here the warmed solution entered the experimental chamber which was mounted on the stage of an inverted epifluorescence microscope (Zeiss IM35) equipped with quartz interior components and Nomarski differential interference contrast optics. The temperature of the superfusate in the chamber was maintained at 37°C and was verified with a thermistor. The flow rate through the chamber averaged 2.3 ml/min with a chamber volume of 250 μl.

The microscope was interfaced to an alternating wavelength illumination system (PTI Delta Scan, Photon Technology Int., New Brunswick, NJ) equipped with a 75-watt xenon lamp. Once the excitation beam (490 and 440 nm) from the fluorimeter entered the microscope it was reflected off a dichroic mirror up through the objective (40 × Zeiss, n.a. 0.75). Fluorescence emitted from cells passed back through the objective and encountered an emission filter (520 nm) before entering the photometer. The output of the photometer was stored in a computer which also controlled the illumination system. The area of the tubule illuminated was controlled by a diaphragm located between the light source and microscope. The area from which emitted light was gathered was controlled by a sliding view finder between the objective and photometer. This area averaged between two to four principal cells depending on the width of the opened tubule. In our estimate the intercalated cells make up between 35 and 40% of the total number of cells. In the BCECF-loaded tubules the intercalated cells appeared to load better than the principal cells but we were able to limit our measurements to areas comprised of principal cells only. In all experiments autofluorescence was measured on

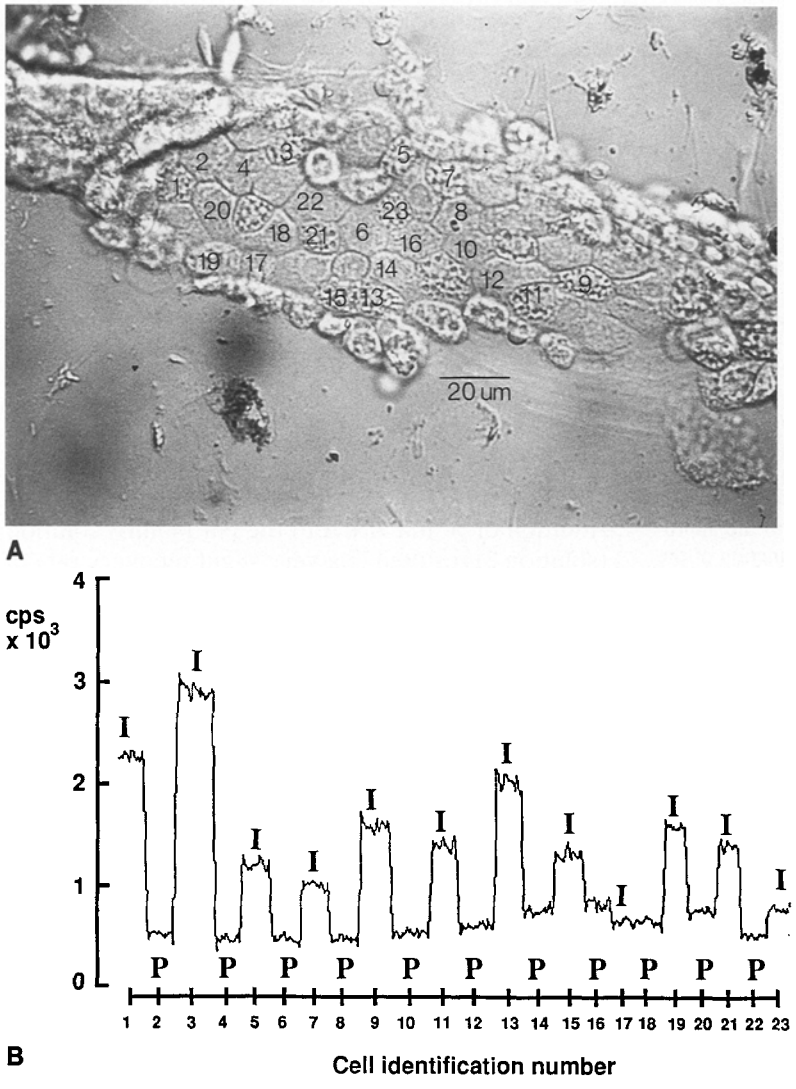

Fig. 1. (*A*) Photomicrograph of opened-tubule preparation loaded with dye and observed with Nomarski optics under transmitted light. Using this opened-tubule preparation we can readily distinguish principal cells (*P*) from intercalated cells (*I*). (*B*) Single-cell fluorescence measurements in counts per second (cps) of FITC-labeled peanut lectin excited at 490 nm in the opened-tubule preparation shown in *A*. Each consecutive measurement corresponds to the cell number as labeled in *A*, beginning with an intercalated cell (*1*) and alternating with a principal cell (*2*) and ending with an intercalated cell (*23*). Autofluorescence on the unloaded tubule is around 450 cps and was not subtracted from the trace. *P* is principal cell and *I* is intercalated cell.

the predetermined experimental area prior to loading the tubule with dye and was automatically subtracted from all traces.

Principal and intercalated cells were distinguished with differential interference contrast optics (Fig. 1*A*). Identification of the two major cell types was further verified using FITC-labeled peanut lectin in this tubule (Lehir et al., 1982; Schwartz, Barasch & Al-Awqati, 1985). Individual cells identified under transmitted light were aligned in the viewfinder between the objective and the photometer, and the fluorescence was measured. We found that more than 90% of the cells identified on a morphological basis as intercalated cells (Fig. 1*A*) stained with the label, confirming that they were B-type intercalated cells (Fig. 1*B*).

The tubule was loaded with BCECF at room temperature for 60 min after which it was superfused with the appropriate solution at 37°C. The loaded tubule was superfused for at least 15 min before beginning an experiment, and the predetermined experimental area was realigned in the viewfinder leading to the photometer. Intracellular calibration of the dye was performed at the end of each experiment on each individual tubule studied. Extracellular pH was varied from 6.5 to 7.8 (solutions a, b, c, d, and e) in the presence of the K/H exchanger, nigericin (10 μM), in 145 mM K according to the method of Thomas et al. (1979). This pH range is in the linear portion of the calibration curve for this dye. The experimentally determined fluorescence ratios are then transformed to pH by calculating the slope and *y* intercept of the calibration curve as shown in Fig. 2. Results are presented as means ± SEM.

Results

Recovery of pH_i from an Acid Load

NH_4 Pulse

In order to determine the suitability of the split-tubule preparation for these studies we acid loaded the cells and monitored the recovery of pH_i. Other investigators have shown that the Na/H exchange

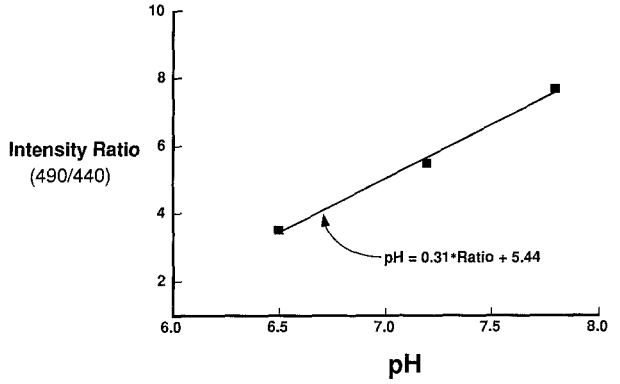

Fig. 2. A representative intracellular calibration curve for BCECF (490/440 nm) as a function of pH. Intracellular and extracellular pH were equilibrated in the presence of 10 μM nigericin added to potassium solutions titrated to the appropriate pH. Experimentally determined ratios were transformed to the appropriate pH using the variables defining the linear function of the calibration curve.

mechanism of the principal cells is present on the basolateral membrane (Wang & Kurtz, 1990; Weiner & Hamm, 1990). In our preparation the basolateral side of the tissue lies flush against the Cell-Tak coated plastic piece, and we wanted to be sure that the superfusate came in contact with this side of the tissue. Figure 3 illustrates an experiment in principal cells which were twice acid loaded by a short exposure to 10 mM NH_4Cl (solution 4). The pH_i recovery phase was measured in the absence and presence of 30 μM amiloride 5-(*n*,N-hexamethylene) (HMA), an analog of amiloride that specifically inhibits Na/H exchange (Simchowitz & Cragoe, 1986; Kleyman & Cragoe, 1988). The ordinate represents pH_i as determined from the fluorescence ratio and the intracellular calibration on this tissue. As shown in Fig. 3*A* removal of external NH_4^+ and Na resulted in a rapid fall in pH_i to a value about 0.5 pH units below the starting pH_i. In the absence of Na_o (NMDG substitution, solution 5) no recovery of pH_i was detectable. Reintroducing Na_o (solution 2) resulted in recovery of pH_i at a rate of 0.13 pH U/min. In a total of 11 tubules acid loaded with NH_4Cl, pH_i recovered fully in the presence of external Na with an average recovery rate of 0.19 ± 0.03 pH U/min (Table 2). These results demonstrate the Na dependence of the rapid recovery from this acid load. In order to confirm that the recovery is due to Na/H exchange we examined the recovery in the presence of 30 μM HMA. Figure 3*B* is a continuation of the above trace. After acid loading the tubule for the second time, removal of external NH_4Cl in a 0 Na solution (solution 5) resulted in a decrease in pH_i with no recovery.

Table 2. Intracellular pH measurements in tubules twice acid loaded with 10 mM NH_4Cl[a]

A. Control Initial	Minimum	Recovery	ΔpH/min
7.43 ± 0.05	6.98 ± 0.05	7.48 ± 0.05	0.19 ± 0.03
B. +HMA Initial	**Minimum**	**Recovery**	***ΔpH/min**
7.48 ± 0.05	7.04 ± 0.04	7.20 ± 0.05	0.02 ± 0.01

[a] *A:* Recovery from the acid load in the presence of Na. *B:* Recovery from the acidosis in the presence of Na + 30 μM HMA. Values are means ± SE. n = 11 except for *n = 9.

Addition of 30 μM HMA to the Na 145 mM solution (solution 2) resulted in a very slight recovery rate of 0.03 pH U/min. With HMA in the Na 145 mM solution the minimum pH_i achieved upon removal of NH_4Cl was similar to that observed after the initial acid load (7.04 ± 0.04 *versus* 6.98 ± 0.05) but the final pH_i was lower (7.20 ± 0.05 *versus* 7.48 ± 0.05) than in the absence of the inhibitor. Also the initial rate of acid extrusion ΔpH/min was much lower than in the control (0.02 ± 0.01 *versus* 0.19 ± 0.03) (Table 2). These results indicate that Na/H exchange contributes significantly to pH_i recovery during this type of acidosis.

These findings demonstrate the presence of the Na/H exchanger in the split tubule, and that this preparation behaves in a similar fashion to the isolated perfused tubule after exposure to an acute acid load. Our results are in agreement with the findings of Chaillet et al. (1985), Wang and Kurtz (1990), and Weiner and Hamm (1990).

Exposure to Acute CO_2

Figure 4*A* is a representative trace of pH_i in principal cells during acute elevation of PCO_2. The ordinate is the pH_i corresponding to the nigericin calibration performed at the end of the experiment. The trace illustrates the effect on pH_i of changing the perfusion solution from NaHEPES pH 7.4 (solution 2) to 6% CO_2/26 mM HCO_3 pH 7.4 (solution 6). The initial switch from nonbicarbonate-buffered solution to CO_2 bicarbonate-buffered solution resulted in a rapid intracellular acidification as expected from the assumption that CO_2 will diffuse into the cytoplasm more rapidly than will HCO_3. There followed a spontaneous recovery of pH_i from 7.1 to a steady-state value of 7.4 at a rate of 0.04 pH U/min. This value was 0.2 pH units lower than the initial pH_i.

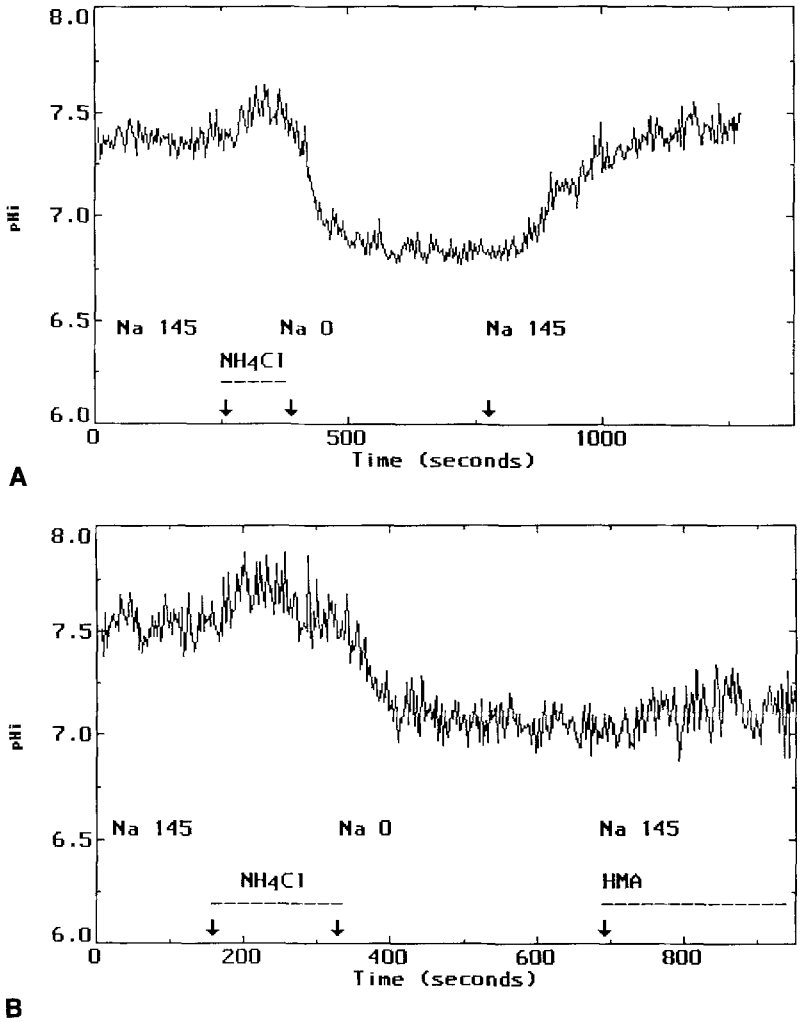

Fig. 3. (*A*) Effect of an NH_4Cl pulse on pH_i. The ordinate represents the pH_i derived from the intracellular calibration of this tissue. The opened tubule was initially superfused with 145 mM Na (solution 2). Acute exposure to 10 mM NH_4Cl (solution 4) (dashed line) resulted in slight alkalinization followed by acidification after removal of the NH_4Cl. Recovery was absent in the presence of 0 Na (solution 5) but returned to the initial value upon reintroduction of Na (solution 2). (*B*) Effect of HMA on pH_i recovery. This is a continuation of the above trace. Another pulse of NH_4Cl (dashed line) resulted in similar changes in pH_i. Exposure to 145 mM Na (solution 2) + HMA (30 μM) (dashed line) prevented recovery over the time scale of this experiment.

In 10 experiments where we changed from Na-HEPES solution to 6% CO_2/26 mM HCO_3 the measured pH_i stabilized at a level below the initial pH_i measured with NaHEPES solution (7.44 ± 0.03 *versus* 7.62 ± 0.03). This pH_i recovery value was very similar to the pH_i values measured in other experiments obtained in separate groups of tubules, where the only solutions used were 6% CO_2/26 mM HCO_3-buffered solutions (7.38 ± 0.04, n = 18). In tubules bathed with HEPES-buffered solutions the pH_i averaged 7.55 ± 0.03 (n = 37), which was 0.17 pH units higher than the mean pH_i in CO_2/HCO_3-buffered solutions.

Figure 4*B* is a continuation of the above trace where we switched from HEPES Ringer solution to CO_2-HCO_3 but in the presence of HMA. We found that pH_i recovery was inhibited by the addition of HMA. In three tubules tested with this protocol we found that compared to the control tubules (no blocker) recovery was virtually absent (0.01 ± 0.01 *versus* 0.072 ± 0.001 pH U/min, n = 10). These results suggest that Na/H exchange is involved in regulation of intracellular pH under these conditions and is the main process responsible for the restoration of pH_i after intracellular acidification by CO_2.

Changes in Extracellular pH (pH_o)

HEPES-buffered solutions were used in another series of experiments to study the effect of varying extracellular pH (pH_o) on pH_i. As shown in the representative trace in Fig. 5*A*, changing pH_o from 7.4 to 7.0 (solutions 2 and 9) resulted in a decrease in pH_i by 0.31 pH units which was largely reversible upon returning to pH 7.4. Alternatively, increasing

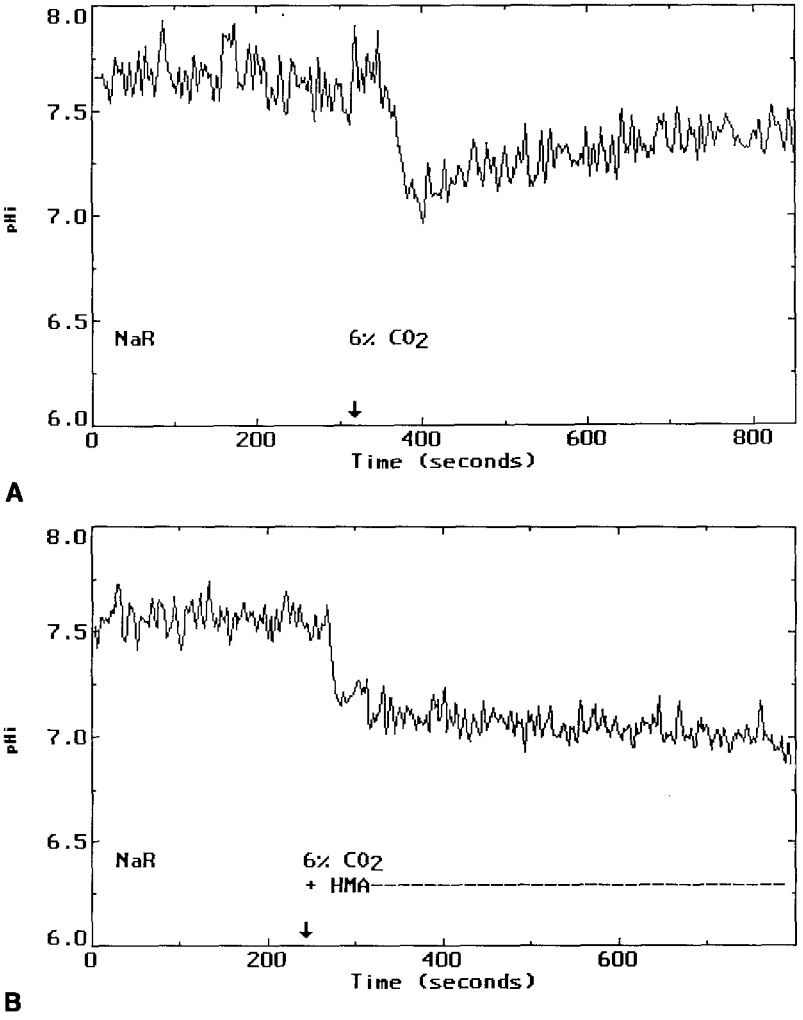

Fig. 4. (*A*) Cell acidification with CO_2. The ordinate corresponds to the pH_i based on the intracellular calibration of the dye in this tissue. The tubule was initially superfused with HEPES solution pH 7.4 (solution 2) and at the first arrow switched to 6% CO_2 bicarbonate-buffered solution (pH 7.4) (solution 6). (*B*) Effect of HMA on pH_i recovery. This is a continuation of the above trace. The tubule was switched from HEPES solution pH 7.4 to 6% CO_2 in the presence of 30 μM HMA (dashed line) which prevented recovery of pH_i over the time scale of this experiment.

pH_o from 7.4 to 7.8 (solutions 2 and 8) yielded a similar result in that pH_i increased by 0.18 pH units. Results of eight similar experiments are summarized in Fig. 5B. These observations show that changes in pH_o lead to changes in pH_i which are in the same direction as the pH_o change but smaller in magnitude. Furthermore, in four of these tubules addition of HMA to the pH 7.4 solution resulted in a decrease of 0.26 ± 0.06 pH units as shown in the bottom curve of Fig. 5*B*. A similar fall in pH_i was observed in a separate series of experiments using CO_2-bicarbonate-buffered solutions after the addition of 10^{-3} M amiloride (Table 3), a concentration known to block the Na/H exchanger (Kleyman & Cragoe, 1988). Therefore, inhibition of Na/H exchanger at normal pH_o caused cytoplasmic acidification, indicating that the exchanger is not quiescent under basal conditions. The changes observed in the pH_i as a result of varying pH_o in the presence of HMA were of the same magnitude as those observed in the absence of the inhibitor (Fig. 5*B*).

Table 3. Two experiments using different concentrations of amiloride performed on principal cells in the opened rabbit CCT using 6% CO_2 solutions[a]

Control	$+10^{-3}$ M amiloride	ΔpH_i
7.41 ± 0.06	7.27 ± 0.05	−0.16 ± 0.04
Control	$+10^{-5}$M amiloride	ΔpH_i
7.34 ± 0.06	7.31 ± 0.05	−0.03 ± 0.02

[a] Values are presented as the mean pH_i ± SE. n = 5 for both groups.

We next looked at the effects of varying extracellular Na (Na_o) on pH_i and the activity of the exchanger.

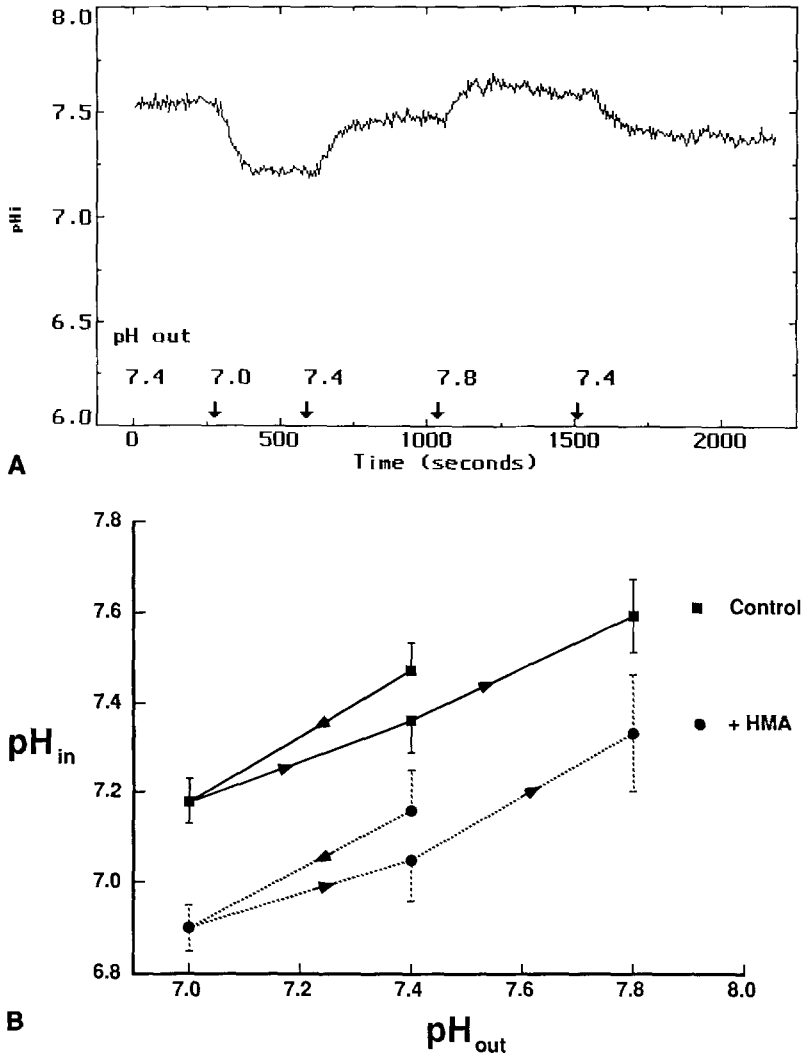

Fig. 5. (*A*) Effect of (pH_o) on (pH_i). The ordinate corresponds to the pH_i based on the intracellular calibration of this tissue. The tubule was initially superfused with HEPES-buffered solution pH 7.4 and switched to 7.0 and back to 7.4. Then the solution was changed again to HEPES-buffered solution pH 7.8 and back to 7.4. (*B*) Effect of pH_o on pH_i. Changing pH_o from 7.4 to 7.0 (solutions 2 and 12) (filled squares) resulted in a change in pH_i from 7.47 ± 0.06 to 7.18 ± 0.05 (n = 8). Alkalinizing the external solution from 7.4 to 7.8 (solutions 2 and 11) resulted in a change in pH_i from 7.36 ± 0.07 to 7.59 ± 0.06 (n = 5). Addition of HMA to the pH_o 7.4 solution caused an initial decrease in pH_i from 7.42 ± 0.08 to 7.16 ± 0.09 (n = 4). Changing pH_o from 7.4 to 7.0 in the presence of HMA resulted in a decrease in pH_i of 0.27 ± 0.05 pH units. Returning to pH_o 7.4 + HMA changed pH_i from 6.89 ± 0.05 to 7.05 ± 0.09 pH units. A change to pH_o 7.8 + HMA increased pH_i to 7.32 ± 0.13 pH units.

Varying Extracellular Na (Na_o)

Figure 6*A* illustrates an experiment in which Na_o was reduced from 145 mM (solution 2) to 35 mM (solution 10) to 0 (solution 5) and back. Lowering Na_o caused an intracellular acidification which was reversible upon returning Na_o to normal. A decrease in pH_i was also observed when Na_o was lowered from 145 to 35 mM in tubules superfused with 25 mM HEPES-buffered solutions (solutions 3 and 11) and bicarbonate-buffered solutions (solutions 6 and 7). The mean change in pH_i observed with lowering Na_o from 145 to 35 mM was −0.12 ± 0.02 (n = 13) (CO_2/HCO_3^- solutions), −0.19 ± 0.04 (n = 9) (25 mM HEPES solutions) and −0.28 ± 0.05 (n = 6) (5 mM HEPES solutions). The variability in the ΔpH_i from series to series may be due to differences in initial pH_i as mentioned above.

In order to assess the role of Na/H exchange on pH_i during changes in Na_o we repeated the above protocol in the presence of 30 μM HMA as shown in Fig. 6*B*. Upon addition of the inhibitor to the Na 145 mM solution pH_i began to decrease slowly. Reducing Na_o did not produce marked changes in pH_i as observed in Fig. 6*A*. Rather there was a gradual decrease in intracellular pH which reached a minimum value of 6.9 in the presence of 0 Na + HMA. The overall change in pH_i at 0 Na + HMA was comparable to the change observed with 0 Na as seen in Fig. 6*A*. In a total of six tubules studied with this protocol the ΔpH_i observed was −0.55 ± 0.10 for 0 Na and −0.51 ± 0.11 for 0 Na + HMA.

As shown in Fig. 6*B* returning Na back to 35 or to 145 mM in the superfusate in the presence of HMA did not reverse the intracellular acidification as observed in Fig. 6*A*. In a total of six experiments we

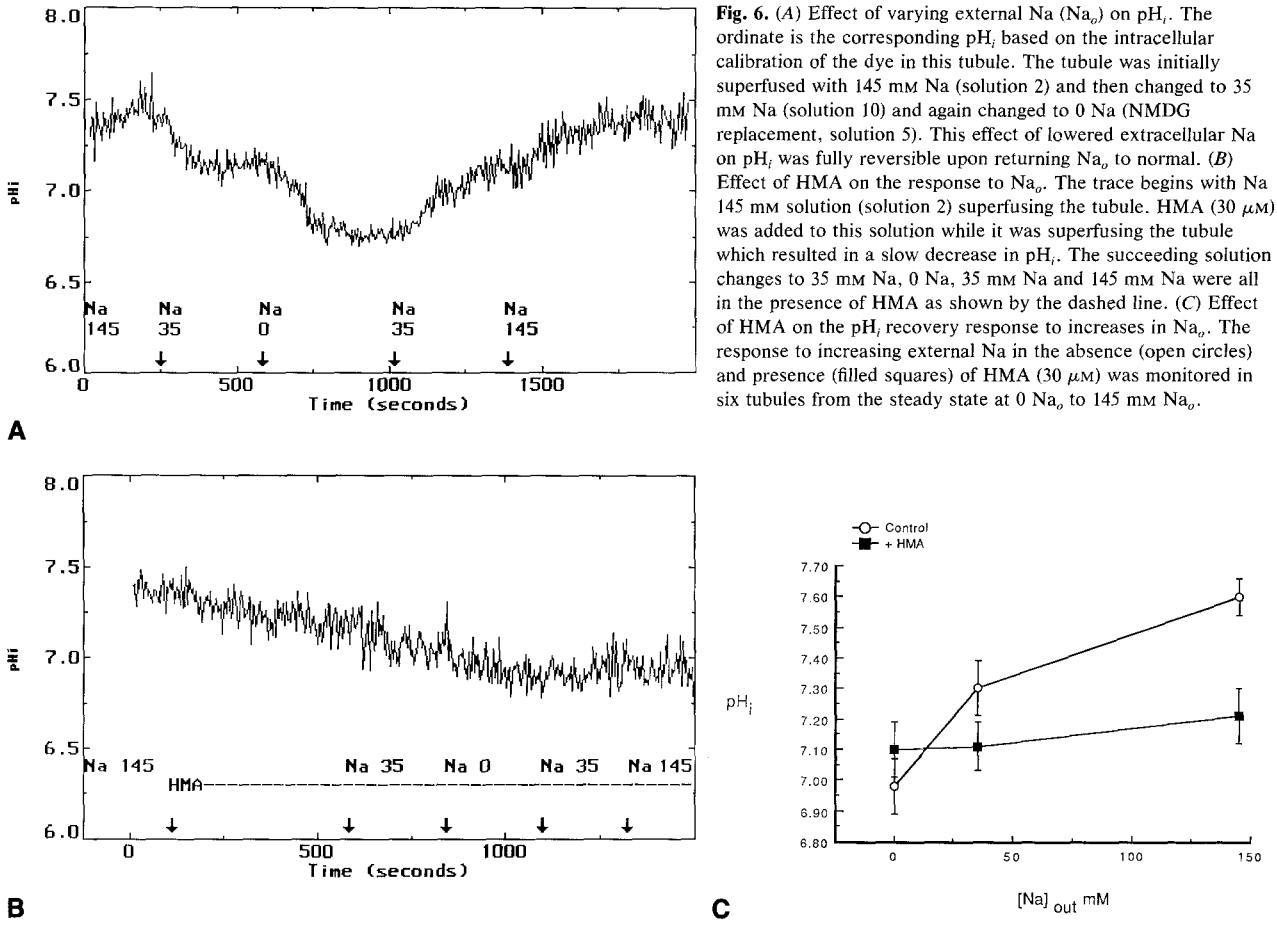

Fig. 6. (*A*) Effect of varying external Na (Na_o) on pH_i. The ordinate is the corresponding pH_i based on the intracellular calibration of the dye in this tubule. The tubule was initially superfused with 145 mM Na (solution 2) and then changed to 35 mM Na (solution 10) and again changed to 0 Na (NMDG replacement, solution 5). This effect of lowered extracellular Na on pH_i was fully reversible upon returning Na_o to normal. (*B*) Effect of HMA on the response to Na_o. The trace begins with Na 145 mM solution (solution 2) superfusing the tubule. HMA (30 μM) was added to this solution while it was superfusing the tubule which resulted in a slow decrease in pH_i. The succeeding solution changes to 35 mM Na, 0 Na, 35 mM Na and 145 mM Na were all in the presence of HMA as shown by the dashed line. (*C*) Effect of HMA on the pH_i recovery response to increases in Na_o. The response to increasing external Na in the absence (open circles) and presence (filled squares) of HMA (30 μM) was monitored in six tubules from the steady state at 0 Na_o to 145 mM Na_o.

compared the recovery of pH_i from the steady state at 0 Na_o to Na_o 35 mM and Na_o 145 mM in the absence and presence of HMA; the overall increase in the pH_i was $+0.13 \pm 0.07$ units compared to a $+0.57 \pm 0.10$ units in the presence and absence of HMA (Fig. 6*C*). This indicates that Na/H exchanger activity is necessary for the recovery of pH_i from the acidification produced by reduction of Na_o.

The results so far have demonstrated that the Na/H exchanger is not only active during recovery from an acid load but is operative under basal conditions, and it is required to extrude acid in the recovery from the acidification produced by lowering of Na_o. Next we wanted to see whether changes in cell Na could lead to changes in pH_i.

Varying Transcellular Transport Rate

Figure 7*A* is a representative trace illustrating the effects of ouabain (100 μM) on pH_i during normal conditions (solution 3, Na_o 145 mM) and low external Na (solution 10, 35 mM Na) in HEPES-buffered solutions. There is a very slight fall in pH_i over a 10-min period, about 0.1 pH unit. In two series of experiments (HEPES- and HCO_3 buffered solutions) the change in pH_i over a time frame of 10 min was not statistically significant compared to timed controls (Fig. 7*B* and *C*). In 2 tubules out of 18 a marked fall in pH_i of 0.3 pH units was observed in response to ouabain. We do not know why these tubules behaved differently from the rest.

Reducing Na_o in the presence of ouabain in HCO_3-buffered solutions (20-min time point, Fig. 7*B*) leads to a decrease in pH_i that was larger than that observed in the corresponding control tubules. This effect was not evident in the series with HEPES buffer (Fig. 7*C*). As shown in the figures, readdition of Na_o 145 mM reversed the acidification. Comparison of experimental and control tubules 30 min after addition of ouabain indicated a slight acidification in the ouabain-treated group relative to controls. In HCO_3-buffered solutions the mean difference in the ouabain-treated tubules was about -0.13 ± 0.05 pH units compared to -0.05 ± 0.06 in the controls, whereas in the HEPES-buffered solutions the mean

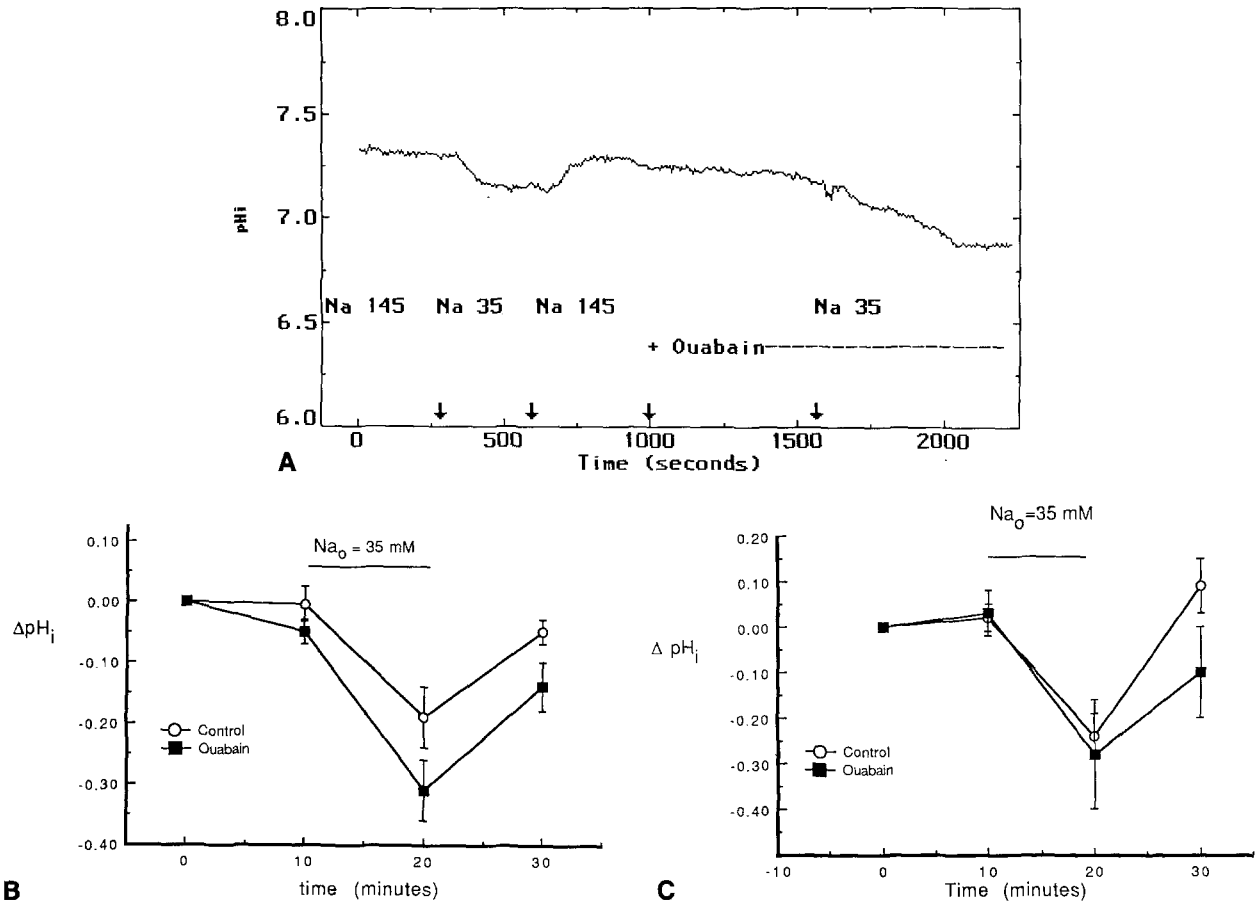

Fig. 7. (*A*) Effect of ouabain on pH_i. The ordinate represents the appropriate pH_i changes based on the calibration of BCECF in the tissue. The tubule was initially superfused with 145 mM Na HEPES solution (solution 3) and switched to 35 mM Na HEPES solution (solution 13) and back. At the third arrow, 100 μM ouabain was added to the 145 mM Na HEPES solution with no discernable effect on the pH_i within this time frame. The solution was then changed to 35 mM Na HEPES solution + ouabain. (*B*) Time course of change in pH_i in control (open circles) and ouabain-treated (filled squares) tubules with HCO_3-buffered solutions. The changes were analyzed from the pH_i in Na_o 145 mM after a change from Na_o 35 mM. The changes were calculated from pH_i 7.35 ± 0.10 (n = 5), ouabain-treated, and pH_i 7.47 ± 0.07 (n = 5), control. (*C*) Time course of changes in pH_i in control (open circles) and ouabain-treated (filled squares) tubules with HEPES-buffered solutions. The changes were analyzed from the pH_i in Na_o 145 mM after a change from Na_o 35 mM. The changes in pH_i were calculated from pH_i 7.42 ± 0.12 (n = 5, except at 30-min point n = 4), ouabain-treated, and pH_i 7.79 ± 0.01, control.

difference was −0.10 ± 0.10 pH units in the experimental and −0.09 ± 0.06 in the controls.

Decreasing luminal Na entry with 10^{-5} M amiloride did not change pH_i (Table 3). Figure 8 is a trace showing the lack of response in pH_i with the ordinate being the pH_i corresponding to the intracellular calibration of the dye. Thus putative changes in intracellular Na with short-term exposure to ouabain and amiloride did not appear to have a large effect on intracellular pH under the conditions of this study.

Discussion

We have used the opened-tubule preparation, previously established in our laboratory for patch-clamp studies (Palmer & Frindt, 1987*a*; Frindt, Sackin & Palmer, 1990), to measure changes in pH_i in principal cells using the pH-sensitive dye BCECF. We demonstrate that the split tubule behaves similarly to the isolated perfused tubule exposed to an acid load which suggests that this preparation can be used to study pH_i regulation. The advantages of using this monolayer preparation are the visualization of the two major cell types in this part of the nephron, ability to control the areas from which fluorescence is measured and the capability of performing parallel experiments using the patch-clamp technique.

Other investigators have demonstrated that a Na/H exchanger exists on the basolateral membrane of cells in the rabbit CCT (Chaillet et al., 1985; Wang & Kurtz, 1990; Weiner & Hamm, 1990). We were also able to show that such a mechanism is present

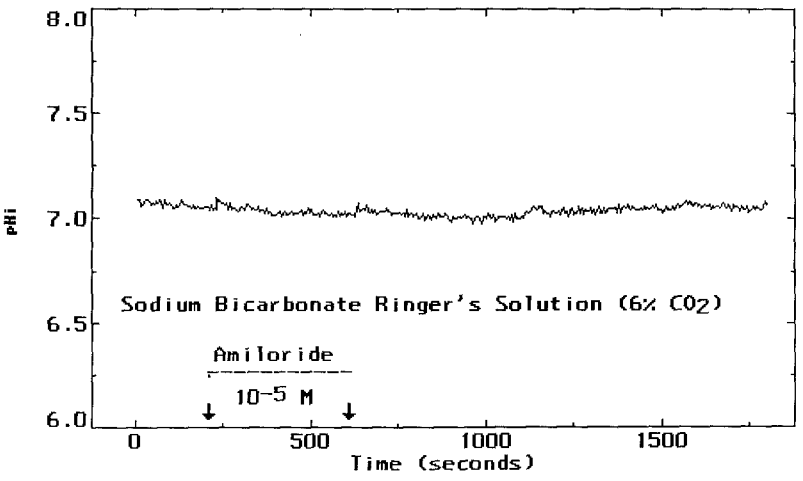

Fig. 8. Lack of effect of amiloride on pH_i. The ordinate represents the corresponding pH_i associated with the intracellular calibration of the dye in this tubule. The tubule was superfused with bicarbonate-buffered solution (solution 6), and at the arrow 10^{-5} M amiloride dihydrate HCl (dashed line) was added to block luminal Na entry. There was no change in pH_i that we could detect. The amiloride was then removed and the pH_i monitored until the end of the trace.

in the principal cells of this opened-tubule preparation in which identifying and recording from individual cell types is relatively straight forward. However, one disadvantage of this superfused preparation is the loss of sidedness: both lumen and blood side are exposed to the same solutions. We did find that the recovery rate from an NH_4Cl-induced acidification measured in our experiments was less than that observed in perfused rabbit CCT (0.2 *versus* 0.8 pH U/min; Chaillet et al., 1985). However, in that study tubules were exposed to a higher concentrations of NH_4Cl (20 mM), leading to a larger acidification. This may account for the faster recovery rate.

Under steady-state conditions we found differences in the apparent pH_i when superfusing the tubule with bicarbonate- *versus* nonbicarbonate-buffered solutions (CO_2-HCO_3 solutions pH_i = 7.39 ± 0.04 *versus* HEPES-buffered solutions pH_i = 7.55 ± 0.03). We found no difference in the mean pH_i in principal cells exposed to 5 mM HEPES-buffered solutions and 25 mM HEPES-buffered solutions. In comparison, Weiner and Hamm (1990) working with BCECF-loaded and perfused rabbit CCTs also found differences in pH_i of principal cells (CO_2-HCO_3-buffered solutions pH_i 7.36 ± 0.05 SE *versus* HEPES-buffered solutions pH_i 7.77 ± 0.06 SE). Wang and Kurtz (1990) using BCECF-loaded perfused rabbit CCTs and confocal microscopy found a somewhat lower value of pH_i in principal cells (7.09 ± 0.06) perfused with bicarbonate-buffered solutions.

We demonstrated that acid loading principal cells either by switching from nonbicarbonate- to bicarbonate-containing solutions or by exposure to a pulse of NH_4Cl, resulted in an immediate acidification followed by spontaneous recovery. With CO_2/HCO_3-containing solutions the pH_i recovered to a value which was lower than the initial pH_i measured with the tubule bathed in the HEPES solution. As pointed out in the results section this recovery value was very similar to the pH_i measured in tubules exposed only to bicarbonate-containing solutions (Table 3). A Cl/HCO_3 exchanger has been identified on the basolateral membrane of rabbit CCT so it is possible that the lower pH_i may reflect the exchange of intracellular HCO_3 for extracellular Cl (Wang & Kurtz, 1990; Weiner & Hamm, 1990). The recovery from a CO_2 acid load was blocked by HMA which suggests that Na/H exchange is the primary mechanism involved during recovery. This was also observed during recovery from an NH_4Cl pulse and is in agreement with what others have found using high concentrations of amiloride to block the exchanger (Chaillet et al., 1985; Weiner & Hamm, 1990).

Varying extracellular pH in HEPES-buffered solutions resulted in changes in pH_i in the same direction. Addition of the blocker HMA to the pH 7.4 solution resulted in an intracellular acidification which suggests that the exchanger is active under basal conditions. In addition the intracellular pH changes associated with the changes in extracellular pH were the same in the absence and presence of the blocker.

We also observed that changes in pH_i occur in response to changes in extracellular Na (Fig. 6). To see whether these changes are mediated by a reduction in H^+ extrusion via the Na/H exchanger we varied external Na in the presence of the inhibitor and found that changes in pH_i were blunted. Furthermore, we observed intracellular acidification in the presence of the blocker and normal extracellular Na (Fig. 6*B*). These results suggest that the Na/H exchange rate is not zero in the normal steady state but is working to balance production and/or influx of acid. Thus under basal conditions and during imposed acid-base perturbations pH_i in principal cells

of rabbit CCT is dependent on the activity of the Na/H exchanger.

The addition of ouabain to the 145 mM Na solution was largely without effect on pH_i within a 10-min time frame. However, we did observe an intracellular acidification of 0.1 to 0.2 pH units after 30 min of exposure to the blocker compared to the timed controls (Fig. 7*B* and *C*). It is unlikely that the effect of ouabain is delayed by poor access to the basolateral membrane, since addition of HMA, which also acts at the basolateral surface, had rapid effects on cell pH. We have not measured Na_i under the conditions of our experiments. Natke and Stoner (1982) using helium-glow photometry in single nonperfused rabbit CCT measured a fourfold increase in Na_i (control Na 17 mM) after exposure to 10^{-5} M ouabain for 1 hr. Sauer et al. (1989) using electron microprobe analysis on freeze-dried cryosections of isolated perfused rabbit CCT reported an intracellular Na of 10 mmol/kg wet weight which increased to 114 mmol/kg wet weight after a 10-min exposure to 10^{-4} M ouabain.

In contrast to our results, Harvey and Ehrenfeld (1988) found an acidification of the frog skin epithelial cells by as much as 0.3 pH units after exposure to ouabain. The magnitude of this change would have been well within the resolution of our system. On the other hand, Grinstein, Cohen and Rothstein (1984) found no effect of ouabain treatment on pH_i in lymphocytes.

We also blocked luminal Na entry with 10^{-5} M amiloride and expected that Na_i would decrease and pH_i increase because of the more favorable gradient for Na entry across the exchanger. We were not able to demonstrate any change in pH_i with addition of this concentration of amiloride, which is expected to block Na channels, but not the Na/H exchanger.

The present results are consistent with the idea that pH in principal cells is dependent on the balance of two opposing proton fluxes. One is a proton influx which may be dependent on the electrochemical gradient for this ion across the cell membrane. The metabolic production of acid may also contribute to the H^+ load to be extruded out of the cell. The other proton movement would be via the Na/H exchanger which would extrude protons under basal conditions as well as during acidification of the cytoplasm. This pump-leak concept accounts for the effects of HMA and reduction of Na_o under basal conditions. It is also consistent with the observed changes in pH_i when pH_o is varied.

The absence of a strong intracellular acidification after addition of ouabain was surprising. However, in terms of a pump-leak system this finding could be explained by simultaneous effects on the influx and efflux mechanisms. The presumed intracellular accumulation of Na (and loss of K) would depolarize the cell membrane potential, diminishing the proton leak. This would partially offset the reduction in acid extrusion via the Na/H exchanger due to a diminished Na gradient. Thus the net change in pH_i might be small.

When HEPES buffer was replaced by HCO_3/CO_2 at the same pH_o, pH_i decreased rapidly, presumably due to an increased acid load from the highly permeable CO_2. We observed a partial recovery which was dependent on the Na/H exchanger, but the steady-state pH_i remained lower than that in HEPES (Fig. 4). The difference in steady-state pH_i may reflect an increase in the effective proton permeability in the presence of CO_2/HCO_3. If $[HCO_3^-]$ in the cell is lower than in the outside solution, CO_2 will be pulled into the cell and HCO_3^- and H^+ will be formed. The driving force for this reaction is the $[HCO_3^-]$ gradient, which in turn depends on the membrane potential and a permeability of the membrane to HCO_3^-. However, if HCO_3^- is retained by the cells, the concentration would build up until the cell pH returned to its original level. HCO_3^- could leave the cell either through anion channels or through a Cl/HCO_3 exchanger. Cl channels have been observed in the basolateral membrane of the principal cells of the CCT (Sansom, La & Carosi, 1990), although their permeability to HCO_3^- was not tested. Evidence for a Cl/HCO_3 exchanger has been obtained from measurements of cell pH during Cl removal (Wang & Kurtz, 1990; Weiner & Hamm, 1990).

In conclusion we were able to demonstrate that the Na/H exchanger in the principal cells of the rabbit CCT is active under steady-state conditions at basal levels of cell pH. Cell pH was sensitive to extracellular Na, extracellular CO_2/HCO_3, extracellular pH and amiloride. Changes in pH_i in response to presumed alterations in transcellular Na transport and Na_i were slow and modest when the Na pump was blocked with ouabain and absent when Na entry was inhibited with amiloride. Although a contribution of pH_i to the regulation of Na channels under these circumstances cannot be ruled out, it appears likely that other factors may be more important in feedback inhibition of Na entry in this epithelium.

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