Intracellular pH Regulation in the Renal Proximal Tubule of the Salamander

Na-H Exchange

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ABSTRACT Using pH-sensitive microelectrodes to measure intracellular pH (pH_i) in isolated, perfused proximal tubules of the tiger salamander *Ambystoma tigrinum*, we have found that when cells are acid-loaded by pretreatment with NH_4^+ in a nominally HCO_3^--free Ringer, pH_i spontaneously recovers with an exponential time course. This pH_i recovery, which is indicative of active (i.e., uphill) transport, is blocked by removal of Na^+ from both the luminal and basolateral (i.e., bath) solutions. Re-addition of Na^+ to either the lumen or the bath results in a full pH_i recovery, but at a lower-than-normal rate; the maximal rate is achieved only with Na^+ in both solutions. The diuretic amiloride reversibly inhibits the pH_i recovery when present on either the luminal or basolateral sides, and has its maximal effect when present in both solutions. The pH_i recovery is insensitive to stilbene derivatives and to Cl^- removal. A transient rise of intracellular Na^+ activity accompanies the pH_i recovery; there is no change of intracellular Cl^- activity. These data suggest that these proximal tubule cells have Na-H exchangers in both the luminal and basolateral membranes.

INTRODUCTION

In 1945 Pitts and Alexander pointed out that renal acid secretion must be a two-step process: (*a*) acid leaves (or alkali enters) the tubule cell across the luminal membrane, and (*b*) acid enters (or alkali leaves) the cell across the basolateral membrane. In their model of the distal tubule, Pitts and Alexander proposed that the luminal step is mediated by Na-H exchange and the basolateral step is mediated by the efflux of HCO_3^-. This scheme was later invoked by Berliner (1952) to account for proximal-tubule acid secretion (i.e., HCO_3^- reabsorption).

A great deal of indirect evidence has since accumulated, linking transepithelial H^+ secretion to Na^+ reabsorption in mammalian proximal tubules (for

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review, see Warnock and Rector, 1979). Experiments at the cellular level have provided somewhat more direct evidence for Na-H exchange. In their experiments on suspensions of isolated rabbit proximal tubules, which were ouabain treated, Bichara et al. (1980) found that pH_i (determined from the distribution of the weak acid DMO) fell in the absence of an out-to-in Na^+ gradient, but recovered in the presence of such a gradient. A clear-cut demonstration of Na-H exchange has been made in brush-border membrane vesicles isolated primarily from proximal tubules (Murer et al., 1976; Kinsella and Aronson, 1980). In addition, Rindler et al. (1979) demonstrated an Na-H exchanger in the MDCK cell line derived from dog kidney. Others have used ion-sensitive microelectrodes to identify Na-H exchangers in nonepithelial cells: mouse soleus muscle (Aickin and Thomas, 1977) and sheep cardiac Purkinje fibers (Deitmer and Ellis, 1980).

The aforementioned data, however, have not provided unambiguous evidence for luminal Na-H exchange in intact renal cells. We now report a direct study of H^+ transport across individual cell membranes of intact cells. Our approach was to use isolated, perfused proximal tubules of the tiger salamander *Ambystoma tigrinum* together with microelectrodes for measuring cell membrane potential and intracellular activities of H^+, Na^+, or Cl^-. The results indicate that there is an amiloride-sensitive Na-H exchanger not only at the luminal membrane, but at the basolateral membrane as well.

A second paper in this series (Boron and Boulpaep, 1983) describes HCO_3^- transport across the basolateral membrane of these cells.

Portions of this work have been reported in preliminary form (Boron and Boulpaep, 1980*a*, *b*, 1982).

METHODS

General

Female tiger salamanders (*Ambystoma tigrinum*; predominantly of the subspecies "nebulosum" and "utahense"), in the neotenic phase, were obtained from Mr. Charles Sullivan (Nashville, TN), and were kept in an aquarium at 4°C and fed small goldfish. Our approach for isolating and perfusing the salamander proximal tubule was the same as that developed by Sackin and Boulpaep (1981). Animals were anesthetized in 0.1% tricaine; the kidneys were removed and cut transversely into several pieces. Single tubules (~100 μm diam), with glomeruli intact, were isolated from just below the ventral surface of the kidney, this dissection being carried out in PVP/HCO_3^- Ringer (solution 8 of Table I) at ~4°C. We dissected free from the rest of the nephron 700- to 1,000-μm lengths of early proximal tubule (immediately distal to the glomerulus and neck segment), teased open the cut ends with fine forceps, and finally transferred the tubule segments to the chamber after drawing them up into a 1-mm-diam glass pipette. The isolated, perfused tubule apparatus was similar to that originally described by Burg and his colleagues (1966). It consists of two assemblies of three concentric pipettes (see Fig. 1). The outermost pipette surrounds the tubule, the middle one cannulates it. Since the space between these two pipettes is airtight, a slight vacuum can be applied to draw up the tubule between the pipettes, causing the tubule to jam at the outer pipette's constriction and to form a seal that is mechanically and electrically tight. Perfusate is introduced into the right-hand pipette assembly via the innermost pipette at the rate of ~1.0 ml/min. The vast majority of the perfusate returns up along the outside of the innermost pipette and escapes through the drain of the middle pipette, where it contacts a calomel half-cell; only a small amount (~20 nl/min) of the perfusate actually enters the tubule lumen. The fluid perfusing the lumen is collected in the left-hand pipette assembly by the innermost pipette, to which suction is applied. In addition to being perfused, tubules were constantly circumfused at ~3 ml/min (i.e., ~10 chamber vol/min). From changes in basolateral membrane potential (V_1) accompanying changes in either basolateral or luminal composition, we estimate that the time constants for solution exchange were ~3 and 5 s, for the bath and the lumen, respectively. Experiments were conducted at ambient temperature (21–25°C). The tubules were visualized with an inverted microscope (Reichert, Vienna, Austria); microelectrode impalements were made at a magnification of 400.

Solutions

The compositions of the Ringer's solutions are given in Table I. For solution 5, [Ca] was increased threefold over standard HEPES Ringer to compensate for Ca, which

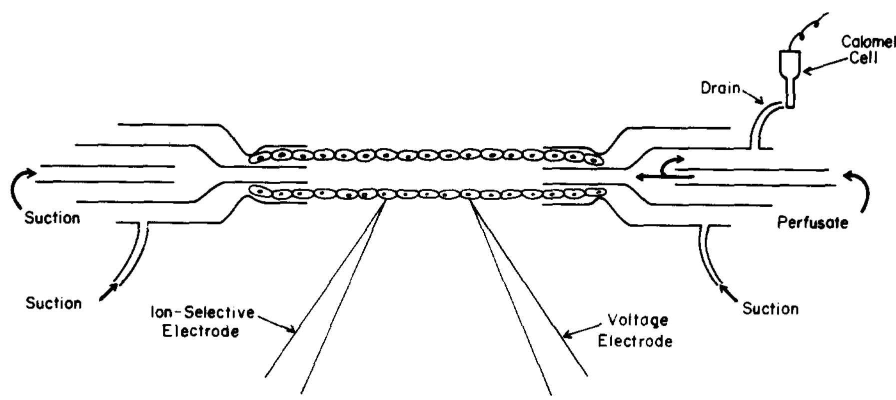

FIGURE 1. Isolated tubule preparation, top view. The tubule (outer diameter ~100 μm) is held at each end by a concentric, three-pipette assembly. Nearby cells are impaled with an ion-sensitive (i.e., pH, Na^+, Cl^-) and a voltage microelectrode.

may have been chelated by the substituting anions. Solution 6 (low-substrate HEPES), in which lactate and amino acids were deleted, was used for basolateral solutions in which SITS was used. Additional solutions, not listed in Table I were also used. For example, a "0-Na/low-substrate" HEPES Ringer was obtained by combining the recipes for solutions 4 and 6. 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate (SITS) was obtained from International Chemical and Nuclear (Cleveland, OH); 4,4′-dinitrostilbene-2,2′-disulfonate (DNDS) was purchased from International Chemical and Nuclear (Plainview, NY); HEPES, NMDG, tetramethylammonium-Cl, and the glucuronate salts were obtained from Sigma Chemical Co. (St. Louis, MO); and BDA-Cl was obtained from Eastman Organic Chemicals (Rochester, NY). The amiloride was a gift of Merck Sharp & Dohme Research Laboratories (West Point, PA).

All solutions were delivered to pipettes or chamber by gravity through CO_2-impermeable Saran tubing (Clarkson Equipment & Controls, Detroit, MI).

Electrodes and Electronics

The pH-sensitive microelectrodes were of the recessed-tip design of Thomas (1974) and were fabricated from 1.0-mm-OD × 0.5-mm-ID pH-sensitive glass tubing (0150; Corning Glassworks, Corning, NY) and 2.2-mm-OD × 1.1-mm-ID aluminosilicate glass tubing (1720; Corning Glassworks). Construction details can be found in Thomas' monograph (1978). The electrodes were filled with 0.1 M HCl and fitted with Ag/AgCl half-cells, which were sealed in place with inlay casting wax. Tip diameters, measured at high magnification under oil, were ≤0.5 μm. The time constants for response to solution changes were 10–20 s. The electrodes were calibrated in pH 4.01 and pH 7.38 buffers, traceable to NBS standards. The average slope was 57.0 ± 0.2 mV/pH unit (n = 78 experiments). Electrode resistances were 10^{11}–10^{12} Ω.

TABLE I

SOLUTIONS*

Component	1 Standard HEPES	2 NH_4^+ HEPES	3 pH 6.8 HEPES	4 O-Na HEPES	5 0-Cl HEPES	6 Low substrate HEPES	7 Standard HCO_3^-	8 PVP HCO_3^-
Na^+	97.65	77.65	93.5	0.05	94.05	97.6	100.95	100.95
M^+	0	20.0	0	97.1	0	0	0	0
K^+	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Mg^{++}	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1 0
Ca^{++}	1.8	1.8	1.8	1.8	5.4	1.8	1.8	1.8
Lys^+	0.2	0.2	0.2	0.2	0.2	0	0.2	0.2
meq (+):	105.95	105.95	101.8	105.45	109.55	105.7	109.25	109.25
Cl^-	94.7	94.7	94.7	94.2	0.2	98.1	94.7	94.7
X^-	0	0	0	0	98.1	0	0	0
HCO_3^-	0	0	0	0	0	0	10.0	10.0
$H_2PO_4^-$	0.1	0.1	0.25	0.1	0.1	0.1	0.1	0.1
$HPO_4^=$	0.4	0.4	0.25	0.4	0.4	0.4	0.4	0.4
$Lactate^-$	3.6	3.6	3.6	3.6	3.6	0	3.6	3.6
Glu^-	0.05	0.05	0.05	0.05	0.05	0	0.05	0.05
$HEPES^-$	6.7	6.7	2.7	6.7	6.7	6.7	0	0
meq (−):	105.95	105.95	101.8	105.45	109.55	105.7	109.25	109.25
Glucose	2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.2
Gln	0.5	0.5	0.5	0.5	0.5	0	0.5	0.5
Ala	0.5	0.5	0.5	0.5	0.5	0	0.5	0.5
HEPES	6.7	6.7	10.7	6.7	6.7	6.7	0	0
Sucrose	0	0	0	9.0	0	0	0	0
PVP (g/l)	0	0	0	0	0	0	0	15
pH	7.5	7.5	6.8	7.5	7.5	7.5	7.5	7.5
CO_2 (%)	0	0	0	0	0	0	1.5	1.5
O_2 (%)	100	100	100	100	100	100	98.5	98.5

* Compositions are given in millimolar unless otherwise noted. M^+ is a monovalent cation: in solution 2, NH_4^+; in solution 4, either *bis* (2-hydroxyethyl) dimethylammonium (BDA^+), tetramethylammonium (TMA^+), or *N*-methyl-D-glucammonium ($NMDG^+$). X^- is a monovalent anion, either cyclamate or glucuronate. PVP is polyvinyl pyrrolidone (average molecular weight, 40,000).

The Na-sensitive microelectrodes, also of the recessed-tip design of Thomas (1970), were fabricated from 1.0-mm-OD × 0.5-mm-ID Na-sensitive glass tubing (NAS 11-18; Corning Glassworks) and the aforementioned aluminosilicate glass. Construction details are in Thomas' monograph (1978). The electrodes were filled with NaCl-saturated dry methanol and were fitted with permanently mounted Ag/AgCl half-cells, held in place with Pyseal wax. These electrodes were constructed on the day of the experiment and were calibrated in solutions of 100 mM NaCl, 10 mM NaCl, and 100 mM KCl. Slopes ranged from 56 to 60 mV/10-fold change in activity, selectivities of Na^+ over K^+ ranged from 100 to 450, and resistances ranged from 10^{10} to 10^{11} Ω.

The Cl-sensitive microelectrodes were of the liquid-ion-exchanger type. Aluminosilicate micropipettes, the same as those used for pH- and Na-sensitive microelectrodes, were silanized as follows: the pipettes were dried within a closed vessel (~300 ml) at 200°C for 2 h, after which 10 μl of tri-*n*-butyl-chlorosilane was introduced into the container. After 2 min, the silane fumes were vented from the container, but the pipettes were maintained at 200°C for an additional 30 min. Finally, the pipettes were allowed to cool in an evacuated dessicator over P_2O_5. Cl exchanger (477315; Corning Glassworks) was introduced into the tip of the pipette with a 31-gauge needle, and bubbles were removed with rabbit's whiskers or thin wires. The pipettes were backfilled with 100 mM KCl and fitted with Ag/AgCl half-cells, which were secured with inlay casting wax. The finished electrodes were calibrated in 100 mM KCl, 10 mM NaCl, and 100 mM $NaHCO_3$. They had resistances of 10^9–10^{10} Ω, slopes of 57–58.5 mV/10-fold change in activity, and selectivities for Cl^- over HCO_3^- ranging from 9 to 12.

Ling-Gerard microelectrodes were pulled from 1-mm-OD borosilicate fiber capillaries (Omega Dot) obtained from Frederick Haer (Brunswick, ME) and filled with 3 M KCl. They had resistances of 30–60 MΩ and tip potentials <5 mV.

An ion-sensitive microelectrode and a calomel half-cell in the bath were each connected to one of the dual inputs of an electrometer with 10^{15} Ω input impedance (model 223; W-P Instruments, Inc., Hamden CT). The Ling-Gerard microelectrode and the calomel cell in the drain of the perfusion-side assembly were each connected to one of the dual inputs of an electrometer with 10^{11} Ω input impedance (model 750; W-P Instruments, Inc.). The bath was grounded through a platinum wire. The difference between the potentials of the ion-sensitive and the Ling-Gerard microelectrodes (i.e., that voltage, V_x, due solely to intracellular ion activity) was obtained electronically, filtered (time constant: 0.2 or 1.0 s), and plotted on one channel of a four-channel strip chart recorder (Brush 2300; Gould, Cleveland, OH). The difference between the potential of the Ling-Gerard microelectrode and that of the bath's calomel half-cell (i.e., the basolateral membrane potential, V_1) was similarly obtained, filtered, and plotted on a second channel. The voltage difference between the calomel half-cell of the perfusion pipette's drain and the calomel half-cell of the bath (i.e., the transepithelial potential difference, V_3), as well as the voltage difference between the ion-sensitive microelectrode and the bath's calomel half-cell (i.e., the algebraic sum of V_x and V_1) were likewise plotted on a third and fourth channel.

Impaling Cells with Microelectrodes

During experiments, the perfused tubule rested on a thin layer of hardened Sylgard 184 (Dow Corning, Midland, MI) overlying the glass coverslip that formed the bottom of the chamber. Since the tubule tends to stick to the Sylgard, this arrangement ensures that the tubule offers sufficient resistance to the advancing microelectrode to greatly increase the chances of a successful impalement (Sackin and Boulpaep, 1981). Each microelectrode was rapidly advanced into the cells with a piezo-electric device (see Boron and Boulpaep, 1982, for construction details). Voltage-electrode impalements were considered acceptable if the apparent V_1 suddenly decreased to a stable value. Often there was a further increase in negativity, which we attributed to resealing. On the rare occasion when cell swelling occurred, the voltage electrode was discarded. The success rate for voltage-electrode impalements was generally 80–90%. The success rate for ion-selective-electrode impalements was considerably less. These impalements were considered acceptable if the electrode's voltage achieved a stable value over the course of a few minutes. The pattern of unsuccessful impalements was usually a transient shift in voltage of the proper direction, followed by a return to the initial value. Regardless of electrode types, successful impalements yielded voltages that varied by only a few millivolts from cell to cell.

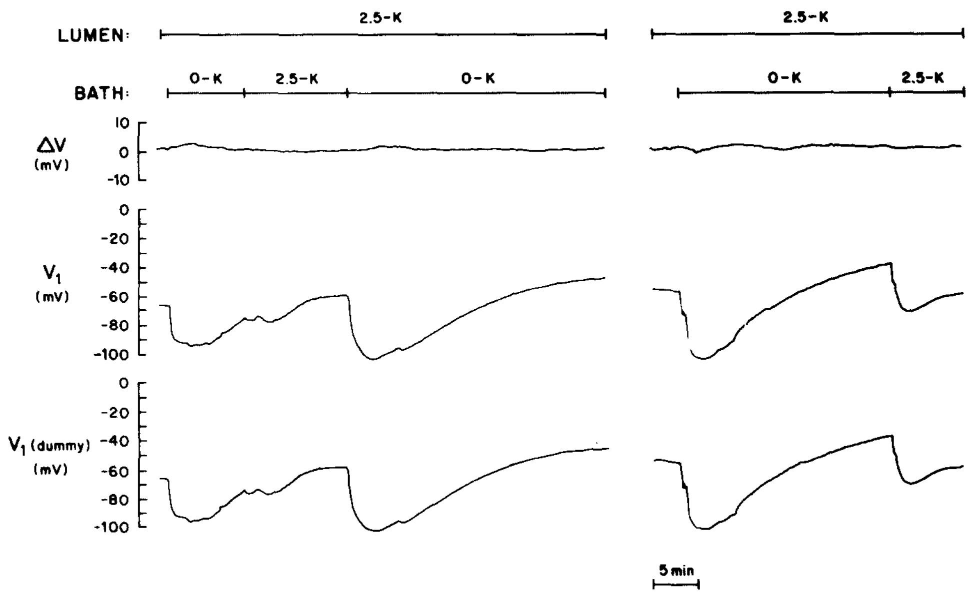

FIGURE 2. Measurements of basolateral membrane potential (V_1) with a "dummy" ion-sensitive electrode. An aluminosilicate pipette, identical to the ones used in making the ion-sensitive microelectrodes, was filled with 3 M KCl and used to measure V_1 (dummy). V_1 was simultaneously measured with a conventional Ling-Gerard microelectrode. ΔV is V_1 (dummy) − V_1. The Ringer was pH 7.5 HCO_3^- (solution 7). This is one of two similar experiments.

It would be ideal to place both the ion-sensitive electrode and its reference, the Ling-Gerard microelectrode, in the same cell, thereby ensuring that there be no potential drop between the two electrodes. However, since such electrode placement is impractical, we chose to simultaneously impale two cells separated by ~100 μm. The cells are ~25 μm in width; the intracellular length constant in *Necturus* proximal tubules is ~200 μm (Windhager et al., 1967). It would thus appear that the cells of the amphibian proximal tubule are sufficiently well coupled so that there is little voltage drop between the two impaled cells. Note, however, that this approach would even be valid for cells that were not tightly coupled electrically; cells need only have nearly identical values of V_1. A discrepancy of 1 mV in this regard would produce an error of ~0.017 in pH measurements and one of ~4% in ion activities. To test the validity of this two-cell approach, we simultaneously impaled two cells of a tubule, one with a Ling-Gerard electrode, and a second with a "dummy" ion-sensitive microelectrode (resistance, 5–25 MΩ). The latter consisted of the same aluminosilicate pipettes used with the pH-, Na-, and Cl-sensitive electrodes, but filled with 3 M KCl. In 27 pairs of cells, the mean difference between the V_1 values simultaneously obtained with the Ling-Gerard and dummy electrodes was 0.8 ± 3.7 mV (NS). Fig. 2 illustrates an experiment in which V_1 was altered by varying basolateral $[K^+]$. As can be seen, the V_1 measured by the two electrodes was very similar throughout. More importantly, the electronically obtained difference ($\Delta V = V_1$ [dummy] $- V_1$) remained approximately zero throughout the various manipulations.

Curve-fitting Procedure

Rate constants of exponential pH_i recoveries were obtained as follows: coordinates of 5–12 points (spanning 2–3 time constants) were obtained by hand from a plot of pH_i vs. time, care being taken to exclude from the analysis the initial portion of the curve, which represents a transition from one external solution to another. An iterative, least-

TABLE II

NORMAL VALUES*

Ringer	pH_i	V_1	V_3	a_i^{Na}	a_i^{Cl}
		mV	*mV*	*mM*	*mM*
HEPES	7.43±0.02	−56.7±1.7	−3.8±0.3	26.9±2.8	18.4±2.4
	(n = 49)	(n = 49)	(n = 49)	(n = 6)	(n = 9)
HEPES	7.46±0.04	−61.2±2.9	−4.0±0.5	—	19.2±3.5
to	(n = 10)	(n = 10)	(n = 10)		(n = 5)
HCO_3^-	7.29±0.05	−54.8±2.4	−3.6±0.5	—	10.9±1.6
HCO_3^-	7.30±0.02	−56.7±1.1	−3.3±0.3	24.7±1.5	16.8±1.4
	(n = 39)	(n = 39)	(n = 39)	(n = 10)	(n = 17)

* pH_i, V_1, and V_3 were obtained on same tubules; a_i^{Cl} data were obtained on separate tubules, as were a_i^{Na} data. External pH was 7.5. Means are given ± SE.

squares curve-fitting procedure was used to fit the data to an equation of the form $pH_i = A - B\exp(-kt)$, where k is the rate constant and t is the time.

All mean values are given ± standard error.

RESULTS

Normal Values

The initial values of pH_i, basolateral membrane potential (V_1), and transepithelial potential (V_3) are given in Table II, line 1, for all tubules bathed in standard HEPES Ringer (21–25°C). In a total of 10 of these tubules, measurements were obtained as the HEPES Ringer was replaced with standard HCO_3^- Ringer (or vice versa) at the same external pH (see Table II, lines 2 and 3). The mean pH_i difference in the new steady state (attained after ~5 min) was −0.17 ± 0.02, which is statistically significant ($P = 0.00002$; paired t test). Similarly, the mean difference of V_1 was 6.4 ± 2.0, which is statistically significant ($P = 0.004$), whereas the mean difference in V_3 was 0.4 ± 0.3 mV, which is not statistically significant ($P = 0.065$). Table II, line 4, lists the pH_i, V_1, and V_3 data for all 39 tubules which were incubated in standard HCO_3^- Ringer. Our pH_i of 7.30 in HCO_3^- Ringer is similar to the value of 7.44 derived by Khuri et al. (1974) from their microelectrode measurement of $[HCO_3^-]_i$ in *Necturus* proximal-tubule cells, and to the value of 7.49 obtained by Matsumura et al. (1980) using antimony microelectrodes on the bullfrog proximal tubule. It is also close to the pH_i values of 7.32–7.51 obtained by several investigators (Struyvenberg et al., 1968; Bichara et al., 1980; Kleinman et al., 1980) using the DMO technique with mammalian proximal tubules (though under different conditions).

Intracellular Na^+ activity (a_i^{Na}) is given in Table II, lines 1 and 4, for tubules in HEPES and HCO_3^- Ringer, respectively. The difference in a_i^{Na} values is not statistically significant ($P = 0.54$, unpaired t test). No a_i^{Na} measurements were made during the transition from HEPES to HCO_3^- Ringer. However, based on the properties of the basolateral Na/HCO_3 transport system described in the second paper of this series (Boron and Boulpaep, 1983), we would predict that a_i^{Na} should be lower in HCO_3^--containing than in HCO_3^--free Ringer.

The mean intracellular Cl^- activity (a_i^{Cl}) is given in Table II, lines 1 and 4, for tubules bathed in HEPES-Ringer and HCO_3^- Ringer, respectively. In five tubules in which the transition from HEPES to HCO_3^- Ringer was monitored (Table II, lines 2 and 3), a_i^{Cl} declined by a mean value of 8.3 ± 2.8 mM, which is statistically significant ($P = 0.021$; paired t test).

Acid Loading with NH_4^+

pH_i CHANGES The pH_i of nerve and muscle cells is regulated by ion transport mechanisms, located in the cell membrane, which respond to abrupt intracellular acid loads by extruding acid from the cell and thereby returning pH_i toward normal (Roos and Boron, 1981). Such an abrupt acid load can be imposed by briefly treating the cell with NH_4^+, as originally described for squid axons (Boron and De Weer, 1976*a*, *b*). Fig. 3 illustrates two experiments in which isolated tubules were perfused and circumfused in HCO_3^--free Ringer at pH 7.5 ("standard HEPES," solution 1). Such nominally HCO_3^--free solutions were used in all experiments in the remainder of this paper in order to minimize the effect of a possible basolateral HCO_3^- transporter on pH_i. At the indicated time the tubule cells are exposed, bath and lumen, to Ringer containing 20 mM NH_4^+ at a constant pH (solution 2). This exposure leads to the entry of both NH_4^+ and NH_3, which is present at low concentration. At first (interval *a–b*, Fig. 3), pH_i rapidly increases by ~0.2, because the influx and protonation of the NH_3 exceeds the influx of NH_4^+. In the second phase (*b–c*), pH_i falls slowly as NH_4^+ entry continues and NH_3 now leaves the cell. When the NH_4^+ is removed from the external solution, pH_i falls (*c–d*) far below its initial level as virtually all intracellular NH_4^+ gives up its H^+ and leaves as NH_3. However, the cells recover from this acid load, returning pH_i to normal with an exponential time course (*d–e*).[1] The pH_i recovery cannot be accounted for by a passive event; except for the first several seconds of *d–e*, when pH_i is very low, the recovery of pH_i occurs against the electrochemical gradient for H^+. For example, in Fig. 3A the driving force [$\Delta G_{net} = F(V_1 - E_H)$] favoring

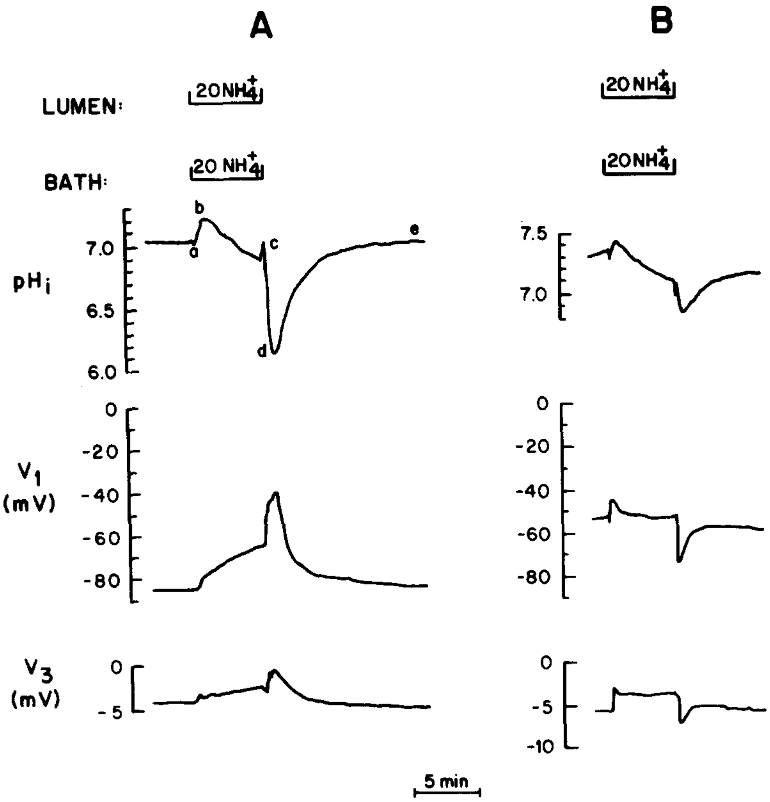

FIGURE 3. pH_i recovery from NH_4^+ acid load. Two tubules were exposed to 20 mM NH_4^+ Ringer in both the lumen and bath for ~5 min. pH_i, basolateral membrane potential (V_1), and transepithelial potential (V_3) are given on the ordinate. Nominally HCO_3^--free, pH 7.5 HEPES Ringer (solutions 1 or 2) was used throughout. A total of 32 similar experiments were performed on 10 tubules.

[1] Note that the time course of the pH_i recovery is somewhat complicated by the continuing exit of NH_3 from the cells during the first portion of interval *d–e* (Fig. 3). From the pH_i time course in experiments in which the recovery of pH_i was blocked by removal of Na^+ (see Figs. 4 and 5 below), we estimate that complete washout of NH_3 from the cells requires ~2.5 min. Under normal circumstances, however, interval *d–e* lasts only ~1 min. Thus, the initial portion of the pH_i recovery curve (*d–e*) is contaminated by NH_3 efflux, which tends to lower pH_i, and therefore to slow the recovery. In our curve-fitting procedure (see Methods), we disregarded points within ~2 min of the NH_4^+ removal, so that the calculated rate constants probably underestimate the true rate constant of the pH_i regulating mechanism by only a small amount.

the entry of H^+ across the basolateral membrane steadily rises to +5.2 kJ/mol as pH_i reaches point *e*. A similar conclusion can be reached regarding luminal H^+ driving forces. Thus, if anything, H^+ would tend to enter the cell passively across both cell membranes. The acid extrusion reflected by the observed pH_i recovery, therefore, must be due to a primary or secondary active transport process.

VOLTAGE CHANGES The application of NH_4^+ characteristically causes a triphasic change in membrane potential, as is the case in squid axons (Boron and De Weer, 1976*a*), though any one of the phases may be absent: (*a*) an abrupt basolateral depolarization, which probably reflects NH_4^+ permeability, (*b*) a slower and smaller hyperpolarization, which may reflect an increase in $[NH_4^+]_i$ or a change in a pH_i-dependent conductance, and (*c*) a slow, further depolarization which may be the result of cell swelling or a change in a pH_i-dependent conductance. In Fig. 3A, phases *a* and *c* are clearly evident; in Fig. 3B, phases *a* and *b* are present. Withdrawal of external NH_4^+ can produce one of two different biphasic patterns of V_1 changes. For the first pattern (Fig. 3A), the rapid depolarization and slower hyperpolarization approximately coincide with the changes in pH_i and may be due to a pH_i-dependent conductance, such as the basolateral K^+ conductance (Steels and Boulpaep, 1976). For the second pattern (Fig. 3B), an instantaneous hyperpolarization is followed by a slower depolarization. Both phases are probably due to parallel changes in the NH_4^+ diffusion potential. Similar, though smaller, changes are seen in V_3. These may have as their primary origin the changes in V_1, as expected for a leaky epithelium.

Involvement of Na^+

Studies on nerve and muscle cells (see Roos and Boron, 1981) have shown that pH_i regulation in these preparations is mediated by either a transport system that exchanges external Na^+ for internal H^+, or by one that exchanges external Na^+ and HCO_3^- for internal Cl^- (and possibly H^+). Since both mechanisms require external Na^+, we tested the Na^+ dependence of the pH_i recovery in tubule cells, as illustrated in Figs. 4 and 5. In the experiment of Fig. 4, we first replace all Na^+ in the bath and lumen with BDA^+ (solution 4). This causes a slow fall in pH_i (not shown), presumably because of continued metabolic production of acid in the absence of acid extrusion. A brief application of NH_4^+, superimposed on the Na-free condition (a combination of recipes for solutions 2 and 4), produces a sizeable acid load from which the cells fail to recover. However, when 100 mM Na^+ is re-introduced into the lumen, pH_i recovers rapidly, which probably reflects the activity of an acid-extruding ion transport system at the luminal membrane. SITS (0.5 mM), added to the basolateral solution to ensure that basolateral HCO_3^- transport (Boron and Boulpaep, 1983) did not influence the pH_i recovery, was later found to have no effect on pH_i in HCO_3^--free Ringer. Fig. 5 illustrates the converse experiment, in which, after the cells are acid-loaded in Na-free Ringer, Na^+ is restored to the bath rather than to the lumen. Since previous studies had never suggested that net acid extrusion from tubule cells would occur anywhere else but at the luminal membrane, we did not expect the basolateral addition of Na^+ to produce a recovery of pH_i. However, as shown in Fig. 5, when Na^+ is added back to the bath, pH_i recovered at the same or perhaps a slightly higher rate than when Na^+ was added back to the lumen. One explanation for the pH_i recovery upon addition of Na^+ to only the bath is that basolateral Na^+ actually leaked into the lumen. In separate experiments

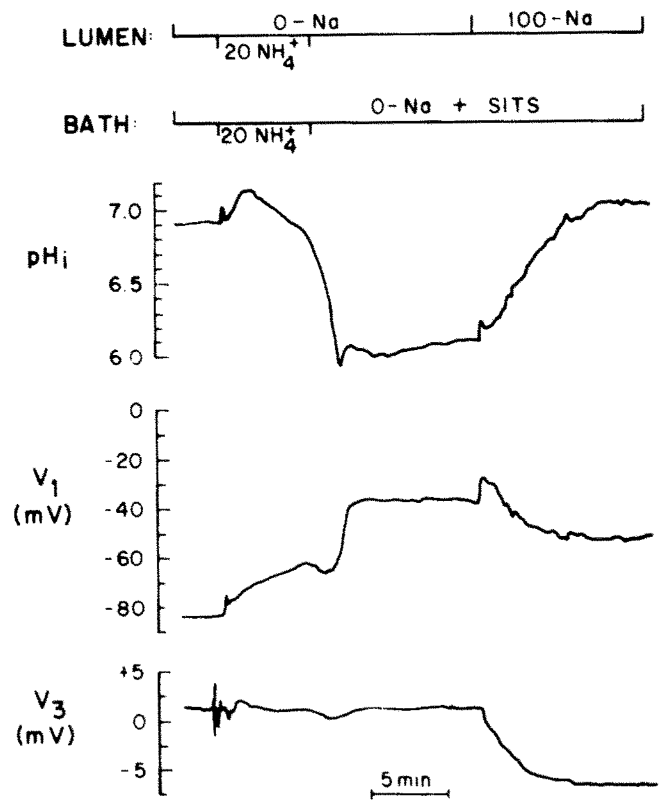

FIGURE 4. pH_i recovery with Na^+ present in lumen only. The tubule cells were exposed to 20 mM NH_4^+ Ringer in bath and lumen while in the continued absence of Na^+ (replaced with BDA^+). After pH_i had fallen to ~6.1, 100 mM Na^+ was added back to lumen only. pH 7.5 HEPES Ringer (solutions 1, 2, or 4) was used throughout. V_1 and V_3 are basolateral membrane potential and transepithelial potential, respectively.

with Na-sensitive microelectrodes, however, we confirmed that the intraluminal Na^+ activity is ~1 mM when the tubule is perfused with Na-free Ringer while being circumfused with 100 mM Na^+ Ringer. Such a low intraluminal Na^+ level is not sufficient to account for the observed pH_i recovery rate, since in other experiments we found that ~5–10 mM Na^+ is required for a half-maximal pH_i recovery rate.

The above data suggest that a Na-dependent acid extrusion mechanism exists at the basolateral as well as the luminal membrane. The basolateral mechanism appears to make a greater contribution to overall acid extrusion than does the luminal system. In 16 tubules, we acid loaded cells in Na-free Ringer and then added 100 mM back to either the bath only, the lumen only, or to the bath and lumen.[2] For five tubules in which Na^+ was present in the lumen only, the average rate constant of pH_i recovery was 0.47 ± 0.07 min^{-1}.

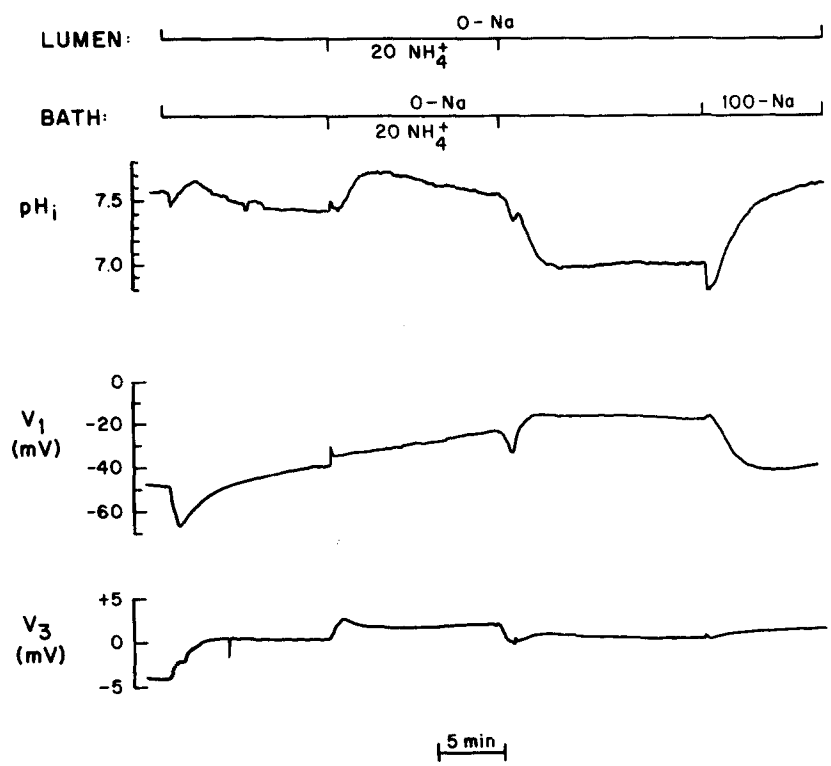

FIGURE 5. pH_i recovery with Na^+ present in bath only. Na^+ was removed from bath and lumen (replaced with BDA^+), and then cells were exposed to 20 mM NH_4^+ Ringer. After pH_i had fallen to ~7.0, 100 mM Na^+ was added to bath only. pH 7.5 HEPES Ringer (solutions 1, 2, or 4) was used throughout. V_1 and V_3 are basolateral membrane potential and transepithelial potential, respectively.

For eight different tubules in which Na^+ was present in the bath only, the mean rate constant was 0.96 ± 0.20 min^{-1}. The difference between these two values is statistically significant (unpaired t test; $P = 0.042$). For three other tubules in which Na^+ was simultaneously returned to the bath and lumen,

[2] In these experiments, all NH_3 was washed out of the cells before Na^+ was added and the pH_i recovery was allowed to begin. Therefore, these rate constants are not contaminated by NH_3 washout.

the mean rate constant was 0.82 ± 0.20 min^{-1} (not statistically significant from either of the previous two averages). A precise comparison of these rate constants is impossible because of the low number of experiments and the variability of the pH_i recovery rates from tubule to tubule.

A Na^+-dependent acid-extruding mechanism existing at both the luminal and basolateral membranes could be accounted for by either Na/HCO_3-Cl/H exchange, or by Na-H exchange. In both cases, recovery of pH_i from an

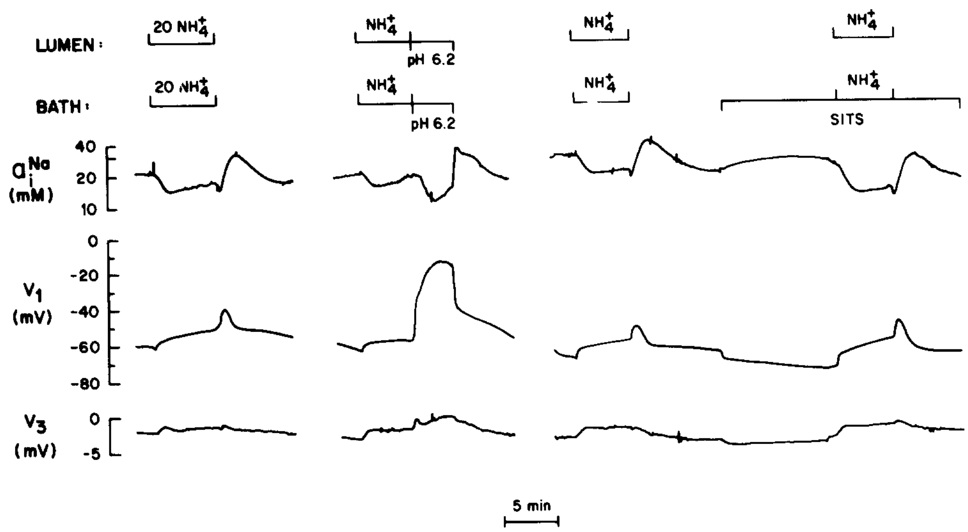

FIGURE 6. Intracellular Na^+ activity during NH_4^+ acid load. In the first segment, cells were exposed, lumen and bath, to 20 mM NH_4^+ Ringer for ~5 min. After NH_4^+ removal, during the time pH_i recovers from acid load, a_i^{Na} overshoots its initial value. In the second segment, the NH_4^+ washout was made in pH 6.2 Ringer to block Na-H exchange. The sharp rise of a_i^{Na} did not occur until pH 7.5 Ringer was returned. In the final segment, the cells were twice more pulsed with NH_4^+, once in the absence and once in the presence of 0.5 mM SITS in the bath. The first gap in the record represents an interval of 16 min; the second gap represents an interval of 4 min. pH 7.5 (or pH 6.2, as indicated) HEPES Ringer was used throughout. V_1 and V_3 are basolateral membrane potential and transepithelial potential, respectively. The overshoot of a_i^{Na} was observed in 18 NH_4^+ pulses on 7 tubules. The inhibition of this overshoot by low pH was demonstrated in eight NH_4^+ pulses on five tubules.

NH_4^+-induced acid load should be accompanied by a transient rise of intracellular Na^+ activity (a_i^{Na}). Fig. 6 illustrates one of five experiments in which a_i^{Na} was monitored with a Na-sensitive glass microelectrode. The application of NH_4^+ causes an initial fall in a_i^{Na} followed by a partial recovery, a pattern also observed in mouse skeletal muscle (Aickin and Thomas, 1977). The initial fall may be due to stimulation of the Na-K pump by NH_4^+, which may substitute for external K^+ (Aickin and Thomas, 1977), whereas the slower rise in a_i^{Na} could be due to a decreased Na-K pump rate or increased Na^+ influx, each secondary to changes in pH_i and/or V_1. The most significant feature of this experiment is the transient overshoot of a_i^{Na} which occurs when external NH_4^+ is removed. This overshoot, which extends ~10 mM above the control a_i^{Na} level, occurs at a time when pH_i is rapidly recovering from the acid load, and thus is probably due to Na^+ entry in exchange for H^+. The subsequent return of a_i^{Na} to its initial value probably reflects activity of the Na-K pump. A second application of NH_4^+ produces similar changes in a_i^{Na}. Now, however, the washout of NH_4^+ is accompanied by a reduction of both luminal and basolateral pH (pH_l and pH_b, respectively) to 6.2. In other experiments (not shown), we have shown that such a low pH almost completely blocks the recovery of pH_i from an NH_4^+-induced acid load. With Na-H exchange thus inhibited during the period of NH_4^+ washout, a_i^{Na} does not promptly overshoot, but instead falls and then slowly rises. When Na-H exchange is suddenly initiated by returning pH_l and pH_b to 7.5, a_i^{Na} abruptly rises. Thus, the magnitude and rate of the rise in a_i^{Na} appear to be correlated with the rate of acid extrusion. It might be noted that the second period of acid loading is accompanied by a sharp depolarization of V_1. This is probably due to the reduction of pH_l and pH_b, and has been seen during such reductions by us and by others (Steels and Boulpaep, 1976), even in the absence of an intracellular acid load. The third NH_4^+ acid load confirms that the normal a_i^{Na} time course is unaffected by the previous treatment at low pH. Although these experiments indicate that Na^+ is involved in the pH_i recovery, they do not distinguish between two possible mechanisms: Na-H exchange and Na/HCO_3-Cl/H exchange. Inasmuch as the latter process is blocked by SITS (Thomas, 1976; Russell and Boron, 1976), we pretreated the basolateral surface of the tubule with this stilbene derivative and applied NH_4^+ for a fourth time. There was no substantial difference between the time course of a_i^{Na} during the pH_i recovery in the two controls and the SITS treatment. This implies that Na/HCO_3-Cl/H is not responsible for the observed changes in pH_i and a_i^{Na}. We note, however, that the cells are not totally insensitive to SITS, as evidenced by the SITS-induced rise in the baseline a_i^{Na} as well as the hyperpolarization of the basolateral membrane.

Sensitivity to Amiloride

Studies on mouse skeletal muscle (Aickin and Thomas, 1977), cells of the MDCK line (Rindler et al., 1979), sheep cardiac Purkinje fibers (Deitmer and Ellis, 1980), as well as on membrane vesicles isolated from renal brush-border membranes (Kinsella and Aronson, 1980), indicate that the Na-H exchanger in these preparations is sensitive to the diuretic amiloride. We therefore tested the effect of amiloride on pH_i recovery from an intracellular acid load in the proximal tubule.[3] As shown in Fig. 7, the rate constant for pH_i recovery from

[3] In these experiments (and those of Fig. 9), the true rate constants of the pH_i regulatory process have probably been moderately underestimated because of the NH_3 washout (see footnote 1). However, the decrease of the pH_i recovery rate caused by inhibition by amiloride probably reduces the magnitude of this error.

an NH_4^+-induced acid load, under control conditions (100 mM Na^+ present in both bath and lumen), is initially 1.00 min^{-1}. Basolateral application of 2 mM amiloride reduces this to 0.40 min^{-1}, and luminal application, to 0.34 min^{-1}. After amiloride has been washed out for several minutes, the control pH_i recovery has a rate constant of 0.72 min^{-1}, somewhat lower than the initial value, but still substantially higher than with the basolateral or luminal presence of amiloride. Bilateral application of amiloride has the greatest inhibitory effect, reducing the rate constant to 0.19 min^{-1}, substantially lower than that observed after amiloride washout, 0.72 min^{-1}.

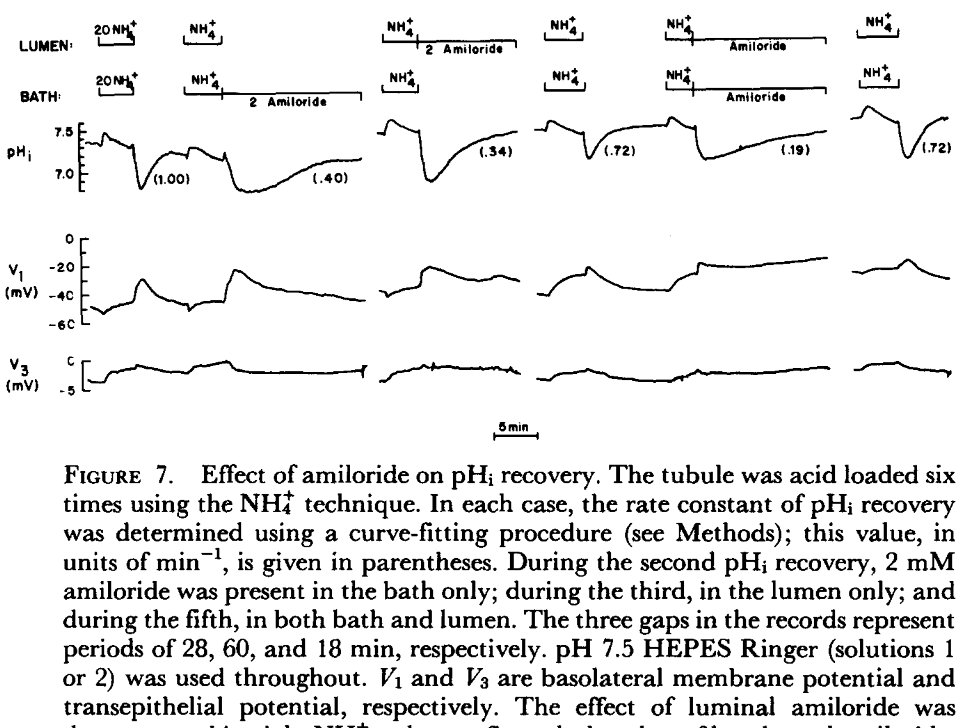

FIGURE 7. Effect of amiloride on pH_i recovery. The tubule was acid loaded six times using the NH_4^+ technique. In each case, the rate constant of pH_i recovery was determined using a curve-fitting procedure (see Methods); this value, in units of min^{-1}, is given in parentheses. During the second pH_i recovery, 2 mM amiloride was present in the bath only; during the third, in the lumen only; and during the fifth, in both bath and lumen. The three gaps in the records represent periods of 28, 60, and 18 min, respectively. pH 7.5 HEPES Ringer (solutions 1 or 2) was used throughout. V_1 and V_3 are basolateral membrane potential and transepithelial potential, respectively. The effect of luminal amiloride was demonstrated in eight NH_4^+ pulses on five tubules; that of basolateral amiloride, in two pulses on two tubules; and that of luminal and basolateral together in three pulses on three tubules.

We have also noted that sensitivity to inhibition by amiloride is substantially increased at low external Na^+ concentrations. For example, 1 mM amiloride reduces the rate of pH_i recovery by 90% or more in the presence of 10 mM Na^+, whereas twice as much of the drug produces an inhibition of only ~75% in the presence of 100 mM Na^+. These data suggest that Na^+ may compete with amiloride for access to the exchanger, consistent with the observations of Kinsella and Aronson (1980). Working with the renal Na-H exchanger of isolated membrane vesicles, they found an apparent K_m for external Na^+ of ~5 mM, and an apparent K_I for amiloride of about 15 μM. Assuming simple competitive inhibition, these K_m and K_I values predict acid extrusion rates which are in rough agreement with our amiloride data.

In separate experiments, we acid-loaded cells in Na-free Ringer and monitored the recovery of pH_i at very low (i.e., 2.5–5 mM) basolateral Na^+ concentrations. Under these conditions, the pH_i recovery is so protracted that basolateral amiloride can be applied and withdrawn two or three times during

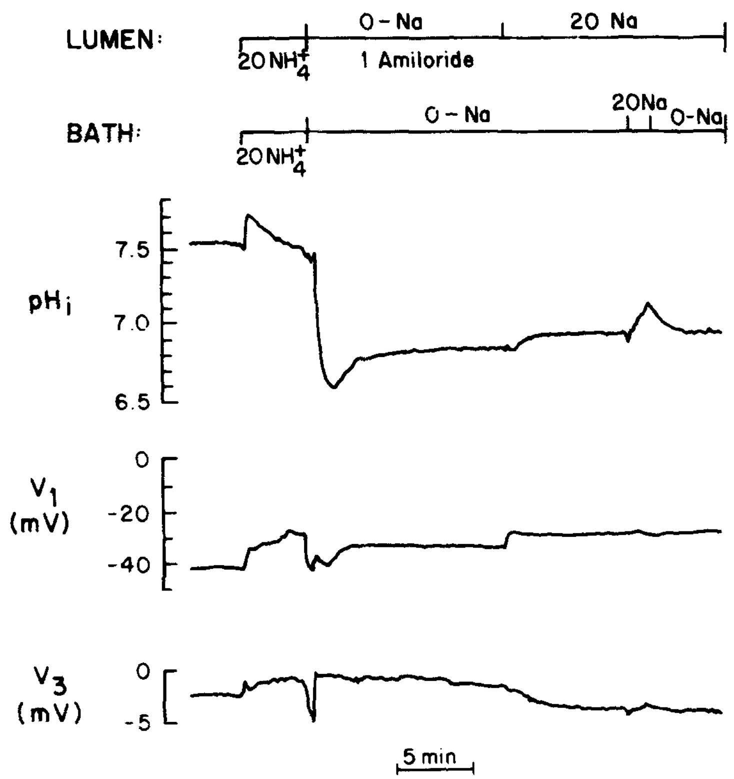

FIGURE 8. pH_i recovery with 20 mM Na^+ in bath, 20 mM Na^+, and 1 mM amiloride in lumen. The cells were acid-loaded with 20 mM NH_4^+ Ringer, after which Na^+ was removed (replaced with BDA^+) and 1 mM amiloride was added to the lumen. Subsequent addition of 20 mM Na^+ to lumen was without effect, but 20 mM Na^+ produced a rapid rise in pH_i when added to the bath. pH 7.5 HEPES Ringer was used throughout. V_1 and V_3 are basolateral membrane potential and transepithelial potential, respectively. This is one of three similar experiments.

a single pH_i recovery. We found that amiloride takes full effect within a few seconds of its application, but requires 2–3 min to wash off.

The amiloride sensitivity of the luminal Na-H exchange can be exploited to provide additional evidence for the existence of a basolateral Na-H exchanger. Fig. 8 illustrates an experiment in which the NH_4^+ technique was used to acid-load a cell in the usual fashion. During the period of NH_4^+ washout, Na^+ is absent from both the luminal and basolateral solutions; in addition, 1 mM amiloride is present in the lumen. This treatment causes pH_i to fall to ~6.6, to recover slightly (before Na^+ can be completely washed from the system), and then to level off. When 20 mM Na^+ is introduced to the lumen, pH_i rises only slightly, because of the presence of amiloride in the lumen. In the absence of amiloride, this amount of Na^+ would have caused pH_i to recover rapidly. However, the addition of 20 mM Na^+ to the bath produces a steep rise of pH_i, which is reversed upon removal of basolateral Na^+. (That pH_i falls upon

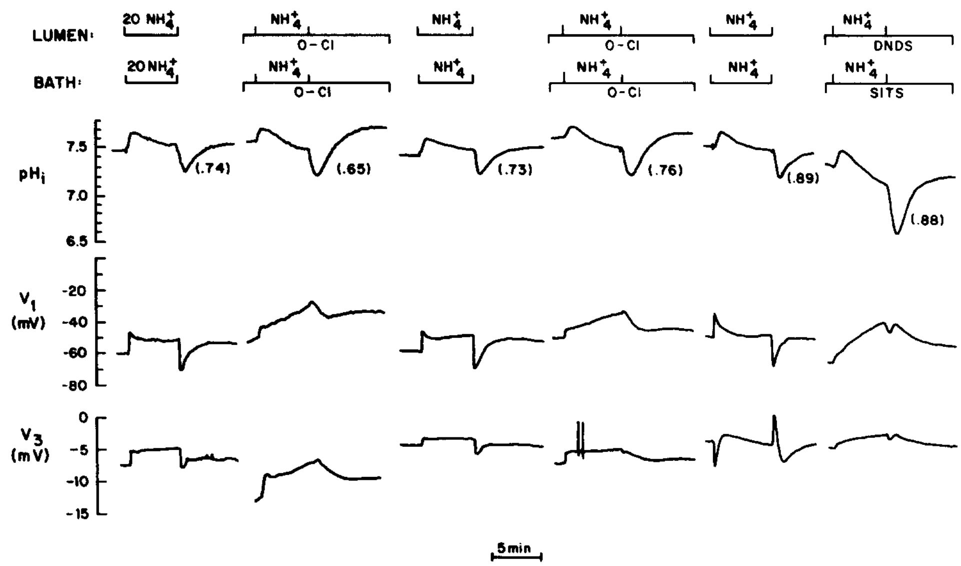

FIGURE 9. Effects of Cl^- removal and of stilbenes on pH_i recovery. The cells were acid loaded six times using the NH_4^+ technique. Cl^- was absent (replaced with glucuronate) during the second and fourth pH_i recoveries; Cl^- had been removed 10 and 17 min, respectively, before the application of NH_4^+ in the two cases. During the sixth pH_i recovery, 2 mM DNDS was present in the lumen and 0.5 mM SITS was present in the bath. The gaps in the records represent intervals of approximately 12, 13, 22, 8, and 20 min, respectively. HEPES Ringer (solutions 1, 2, or 5) was used throughout. V_1 and V_3 are basolateral membrane potential and transepithelial potential, respectively. Several additional experiments in HCO_3^- Ringer (Boron and Boulpaep, 1983) confirm that Cl^- removal is without substantial effect on pH_i recovery.

withdrawal of Na^+ is indicative of underlying acidifying processes.) Since the effect of luminal 20 mM Na^+ is suppressed by amiloride, there can be little doubt that the Na^+ added to the bath also acts at the basolateral membrane and does not elicit the pH_i recovery by diffusing past the tight junctions and acting at the luminal membrane.

Lack of Cl^- Involvement and Insensitivity to Stilbenes

The involvement of Na^+ and the inhibition by amiloride suggest that the pH_i recovery after an acid load is mediated by a Na-H exchanger, whereas the insensitivity of the a_i^{Na} transient to SITS argues against the participation of Na/HCO_3-Cl/H exchange in this (HCO_3^--free) pH_i recovery. To rule out further the possibility of Na/HCO_3-Cl/H exchange, we performed two maneuvers known to block such a transport system, namely, removal of external Cl^- and application of stilbene derivatives. This is illustrated by the experiment of Fig. 9. In standard HEPES-Ringer, pH_i recovers from an NH_4^+ acid load at the usual rate ($k = 0.74\ min^{-1}$). 10 min after replacement of Cl^- with glucuronate (solution 5) in both the luminal and basolateral solutions, NH_4^+ is applied once again. The subsequent recovery of pH_i is only slightly affected ($k = 0.65\ min^{-1}$). Separate experiments (Boron and Boulpaep, 1983) confirm that 10 min in Cl-free Ringer is sufficient to obtain a near-maximal reduction in apparent intracellular Cl^- activity (a_i^{Cl}). These pH_i recoveries, as well as two additional ones in control ($k = 0.73$ and $0.89\ min^{-1}$) and one in Cl-free Ringer ($k = 0.76\ min^{-1}$), indicate that the average inhibition in Cl-free Ringer is 10%. In a final test for the involvement of anion transport, 0.5 mM SITS is applied to the basolateral solution, and 2.0 mM DNDS is applied to the luminal solution. The former compound completely blocks acid extrusion in squid axons at 0.5 mM (Russell and Boron, 1976), whereas the latter produces an 85% inhibition at 1 mM (Russell and Boron, 1979). The use of the dinitro derivative DNDS in the lumen avoids the possible interaction of the amino-reactive agent SITS with amino acids contained in the luminal solution. As can be seen, the stilbenes have no effect on the acid extrusion rate constant ($k = 0.88\ min^{-1}$ vs. $0.89\ min^{-1}$ in the control). Thus, two treatments that block the Na/HCO_3-Cl/H system in other cells fail to prevent pH_i recovery in these proximal tubule cells.

Further evidence for the lack of Cl^- involvement is provided by the experiment of Fig. 10, in which a Cl-sensitive electrode was used to monitor a_i^{Cl} during an NH_4^+ acid load in two tubules. If an Na/HCO_3-Cl/H transporter were responsible for the pH_i recovery in the tubule cells, then the pH_i recovery following withdrawal of NH_4^+ would be accompanied by a fall in a_i^{Cl}. However, Fig. 10 shows that a_i^{Cl} does not decline appreciably following removal of external NH_4^+. The expected decline in a_i^{Cl}, if pH_i were regulated by an Na/HCO_3-Cl/H transporter, can be calculated as follows: the extent of the pH_i recovery after NH_4^+ removal[4] is ~0.7, and the cell's intrinsic buffering power (β_I)[5] is ~36 mM. Taking β_I as 36 mM, the amount of H^+ which must be removed from the cell in order to raise pH_i by 0.7 comes to $(0.7) \cdot 36\ mM \cong 25$

[4] The magnitude of the pH_i recovery cannot be taken from an experiment in which the pH_i-regulating mechanism is operating because the maximum fall in pH_i following NH_4^+ withdrawal would be severely blunted (e.g., see Fig. 9). The true magnitude of the acid load, and thus the amount of acid subsequently extruded, can only be judged in an experiment in which the pH_i-regulating system is blocked, as by removal of external Na^+ (see Figs. 4 and 5). In such experiments, the amplitude of the pH_i decline, as well as its recovery, ranged from 0.6 to 0.8.

[5] The buffering power of the native intracellular buffers can be calculated from the decline in pH_i produced by withdrawal of NH_4^+, providing the pH_i-regulating system is blocked (for details, see Boron, 1977). This condition is met in the experiment of the type shown in Figs. 4 and 5, in which acid extrusion is blocked by removal of external Na^+. The average value for β_I amounts to 35.8 ± 1.5 mM (n = five tubules).

mM. The Na/HCO_3-Cl/H model requires that two equivalents of acid be neutralized in the cell for each equivalent of Cl^- removed from the cell, the stoichiometry actually observed in squid axons (Russell and Boron, 1979, 1982). Thus, for the observed pH_i change, $[Cl^-]_i$ should have fallen by ~12.5 mM in the tubule cells, provided an independent Cl^- transporter did not prevent such shifts. Assuming a reasonable activity coefficient, the predicted fall in a_i^{Cl} would have been ~10 mM, an amount which would have easily been detected.

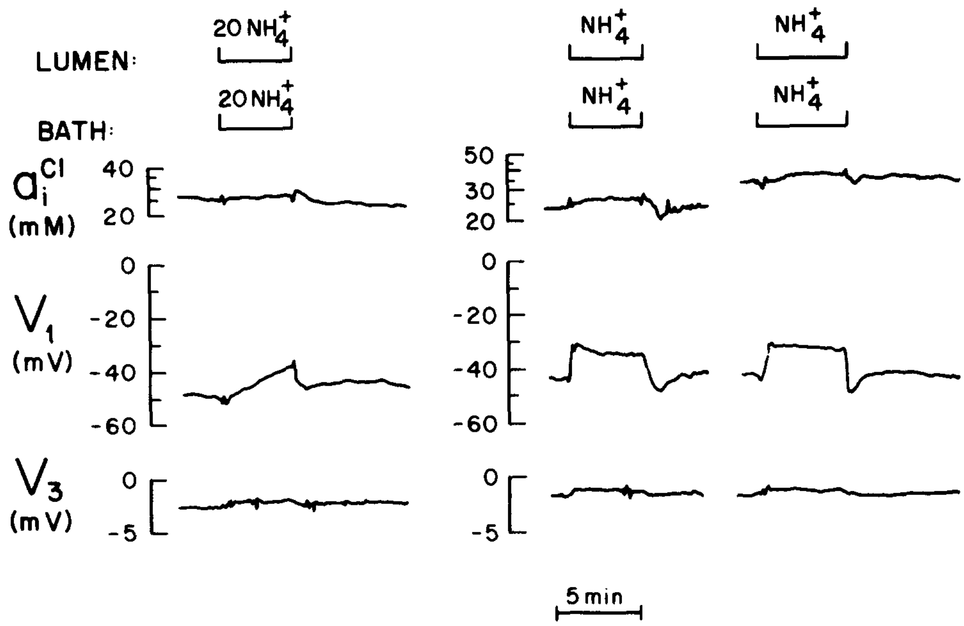

FIGURE 10. Intracellular Cl^- activity during NH_4^+ acid load. Cells were acid loaded with 20 mM NH_4^+ Ringer. The experiment on the right represents a second tubule. The gap in the record for the second tubule represents an interval of 37 min. pH 7.5 HEPES Ringer (solutions 1 or 2) was used throughout. V_1 and V_3 are basolateral membrane potential and transepithelial potential, respectively. These represent the only two experiments of this type in this study.

DISCUSSION

Our results show that when proximal tubule cells are acid-loaded by exposing them to NH_4^+ in HCO_3^--free solutions, intracellular pH spontaneously recovers. This recovery cannot be accounted for by passive processes and therefore must be due to a primary or secondary active, acid-extruding ion transport system located in the plasma membrane. Four types of acid-extruding mechanisms have been described for the plasma membranes of animal cells: (*a*) an electroneutral exchange of external HCO_3^- and Na^+ for internal Cl^- and possibly H^+ (the Na/HCO_3-Cl/H exchanger), which occurs in squid axons (Russell and Boron, 1979, 1982), snail neurons (Thomas, 1977), and barnacle muscle (Boron et al., 1981); (*b*) an electroneutral exchange of external Na^+ for internal H^+, which occurs in mouse skeletal muscle (Aickin and Thomas,

1977) and sheep cardiac Purkinje fibers (Deitmer and Ellis, 1980); (*c*) an ATP-driven electroneutral H-K pump, which occurs in the stomach (Sachs et al., 1978); and (*d*) an ATP-driven, electrogenic H^+ pump, which exists in the toad bladder (Al-Awqati, 1978) and possibly in the distal nephron. In the first two cases, the energy for acid extrusion is probably provided by the Na^+ gradient. We found that acid extrusion by proximal tubule cells also requires Na^+ and is accompanied by a transient rise in a_i^{Na}. This observation does not allow us to distinguish between Na-H exchange and Na/HCO_3-Cl/H exchange.

Four arguments, however, indicate that the Na/HCO_3-Cl/H system does not make a substantial contribution to pH_i regulation in proximal tubule cells. (*a*) Acid extrusion occurs in nominally HCO_3^--free Ringer. It is unavoidable that metabolic production of CO_2 would lead to the generation of some HCO_3^- in unstirred layers surrounding the cells. In barnacle muscle, which has a highly infolded surface membrane and a diameter of ~1,200 μm, [HCO_3^-] in this unstirred layer was estimated to be ~0.6 mM, sufficient to support the Na/HCO_3-Cl/H system at ~12% of its maximal rate (Boron et al., 1981). Inasmuch as the salamander's proximal tubule cells have a height of only ~20 μm and are not so highly infolded as the barnacle muscle, [HCO_3^-] in the unstirred layer is probably substantially less. (*b*) Acid extrusion is not blocked by the stilbenes SITS and DNDS. (*c*) Acid extrusion is not blocked by the simultaneous removal of Cl^- from lumen and bath. (*d*) Acid extrusion is not accompanied by a fall in a_i^{Cl}.

The primary evidence for a Na-H exchanger is the transport system's sensitivity to amiloride, a diuretic known to inhibit Na-H exchange in several preparations (see Results). Furthermore, our data are consistent with published values for the apparent K_I of amiloride (Rindler et al., 1979; Kinsella and Aronson, 1980), and K_m for Na^+ (Kinsella and Aronson, 1980).

Corroborative evidence for the Na-H exchange hypothesis comes from a rough estimate for the transporter's stoichiometry. As noted in the Results, the net amount of H^+ extruded from the cell ($\Delta[H^+]_i$) during a recovery from an NH_4^+-induced acid load is ~25 mM. The amount of Na^+ entering the cell during the pH_i recovery can be estimated from Fig. 6. For example, in the first NH_4^+ pulse of Fig. 6, a_i^{Na} was 17.8 mM just before the NH_4^+ washout. After the NH_4^+ washout, a_i^{Na} rose (presumably due to Na-H exchange) and then fell (due to the Na-K pump). The level to which a_i^{Na} would have risen in the absence of the Na-K pump can be estimated by extrapolating the time course of the a_i^{Na} decline, on a linear a_i^{Na} scale, back to the time when the NH_4^+ washout began. For the first NH_4^+ pulse of Fig. 6, the extrapolated a_i^{Na} is 41.5 mM. Thus, the Δa_i^{Na} due to Na-H exchange is 23.7 mM, and $\Delta[Na^+]_i$ is 31.6 mM, assuming an activity coefficient of 0.75. For the four NH_4^+ pulses of Fig. 4, the mean $\Delta[Na^+]_i$ is 32 ± 3 mM. Thus, we estimate that $\Delta[H^+]_i/\Delta[Na^+]_i$ is 0.78. If there are no changes in cell volume that independently affect pH_i or a_i^{Na}, then the $\Delta[H^+]_i/\Delta[Na^+]_i$ ratio is the same as the ratio of net fluxes, and is thus in rough agreement with the predicted stoichiometry of 1:1.

For proximal-tubule acid secretion to occur, a Na-H exchanger need be present only at the luminal membrane. A surprising result was that the tubule cells appear to have Na-H exchangers on the basolateral membrane as well. Three lines of evidence support the hypothesis of basolateral Na-H exchangers. First, when cells were acid loaded in the absence of Na^+, acid extrusion could be initiated by adding Na^+ to either the luminal or basolateral solution. Second, acid extrusion was inhibited by amiloride when the drug was added to the basolateral as well as to the luminal solution. It is highly unlikely that, with amiloride added to the bath, enough of this competitive inhibitor could have diffused into the lumen to appreciably diminish luminal Na-H exchange. Finally, luminal amiloride failed to block pH_i recovery from an acid load when Na^+ was added to the basolateral solution (Fig. 8).

Other investigators (Ullrich et al., 1975; Mello-Aires and Malnic, 1979; Chan and Giebisch, 1981) have noted that transepithelial acid secretion by rat proximal tubules is inhibited only 20–65% by reducing external Na^+ to ~5 mM. It has been suggested that the apparently Na-independent portion of acid secretion is not mediated by Na-H exchange. However, our data, as well as those of Kinsella and Aronson (1980), would argue that one should expect little inhibition of Na-H exchange until $[Na^+]_o$ is in the range of ≤5 mM. Because of (*a*) the difficulties of washing out all Na^+ from in vivo kidney preparations and (*b*) the relatively low K_m for Na^+, it may be impossible to test adequately the Na dependence of renal acid secretion except in preparations of isolated perfused tubules, isolated cells, or membrane vesicles. Our data do not rule out the existence of other Na-independent, luminal H^+-secreting, and HCO_3^-- or OH^--reabsorbing mechanisms. When cells are acid-loaded in a Na-free environment (Figs. 4 and 5), pH_i recovers very slowly, if at all. At such low values of pH_i, the acid-loading rate is probably minimal because of (*a*) reductions in metabolism and (*b*) unfavorable gradients for passive H^+ influx and HCO_3^- efflux. We conclude that hypothetical Na^+-independent mechanisms could account for only a small fraction of the maximal acid extrusion rate in salamander proximal tubule cells.

Perhaps the most important attribute of the Na-H exchanger, with respect to both pH_i regulation and renal acid secretion, is the sensitivity of the transporter to changes in pH_i. The rate of pH_i recovery from an acid load is proportional to the net acid transport rate. Inasmuch as the rate of acid loading is probably very small in the absence of basolateral HCO_3^- transport (Boron and Boulpaep, 1983), the pH_i recovery rates in the present study probably reflect acid extrusion (i.e., Na-H exchange) alone. The exponential time course of the pH_i recoveries implies that the net Na-H exchange rate varies linearly with pH_i, assuming that intracellular buffering power is invariant of pH_i. That is, the net Na-H exchange rate is highest at low pH_i values, and gradually falls toward zero as pH_i increases toward a threshold value. The linear dependence of acid extrusion rate on pH_i, and the existence of a pH_i threshold, has previously been demonstrated for the Na/HCO_3-Cl/H pH_i-regulating system of barnacle muscle (Boron, 1980), both when it mediates net acid extrusion (Boron et al., 1979) and Cl-Cl exchange (Boron et al., 1978). It is clear that, for both the Na-H exchanger of the salamander proximal tubule and the Na/HCO_3-Cl/H system of barnacle muscle, the pH_i threshold for the transporter does not correspond to the pH_i at which the system is at thermodynamic equilibrium. For example, at an a_i^{Na} of 24 mM, an a_o^{Na} of 75 mM, and a pH_o of 7.5, the Na^+ gradient is sufficient for a 1:1 Na-H exchanger to drive pH_i nearly 0.6 pH units higher than the normal pH_i value for salamander proximal tubule cells. Thus, the basis for a threshold pH_i value must be kinetic rather than thermodynamic. One explanation for this pH_i dependence is that there is a functional group on the cytoplasmic surface of the transport protein, whose protonation at low pH_i leads to an increase in V_{max} or a decrease in the K_m values for one or more substrates. An alternate explanation is a simple Michaelis-Menten-type dependence on $[H^+]_i$, though this is less likely given the lack of the expected sigmoidal pH_i dependency.

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