Inhibition of Na⁺-independent H⁺ Pump by Na⁺-induced Changes in Cell Ca²⁺

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ABSTRACT Apical membrane H⁺ extrusion in the renal outer medullary collecting duct, inner stripe, is mediated by a Na⁺-independent H⁺ pump. To examine the regulation of this transporter, cell pH and cell Ca²⁺ were measured microfluorometrically in in vitro perfused tubules using 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein and fura-2, respectively. Apical membrane H⁺ pump activity, assayed as cell pH recovery from a series of acid loads (NH₃/NH₄⁺ prepulse) in the total absence of ambient Na⁺, initially occurred at a slow rate (0.06 ± 0.02 pH units/min), which was not sufficient to account for physiologic rates of H⁺ extrusion. Over 15–20 min after the initial acid load, the rate of Na⁺-independent cell pH recovery increased to 0.63 ± 0.09 pH units/min, associated with a steady-state cell pH greater than the initial pre-acid load cell pH. This pattern suggested an initial suppression followed by a delayed activation of the apical membrane H⁺ pump. Replacement of peritubular Na⁺ with choline or N-methyl-D-glucosamine resulted in an initial spike increase in cell Ca²⁺ followed by a sustained increase in cell Ca²⁺. The initial rate of Na⁺-independent cell pH recovery could be increased by elimination of the Na⁺ removal-induced sustained cell Ca²⁺ elevation by: (a) performing studies in the presence of 135 mM peritubular Na⁺ (1 mM peritubular amiloride used to inhibit basolateral membrane Na⁺/H⁺ antiport); (b) clamping cell Ca²⁺ low with dimethyl-BAPTA, an intracellular Ca²⁺ chelating agent; or (c) removal of extracellular Ca²⁺. Cell acidification induced a spike increase in cell Ca²⁺. The late acceleration of Na⁺-independent cell pH recovery was independent of Na⁺ removal and of the method used to acidify the cell, but was eliminated by prevention of the cell Ca²⁺ spike and markedly delayed by the microfilament-disrupting agent, cytochalasin B. This study demonstrates that peritubular Na⁺ with choline or N-methyl-D-glucosamine resulted in an initial spike increase in cell Ca²⁺ followed by a sustained increase in cell Ca²⁺. The initial rate of Na⁺-independent cell pH recovery could be increased by elimination of the Na⁺ removal-induced sustained cell Ca²⁺ elevation by: (a) performing studies in the presence of 135 mM peritubular Na⁺ (1 mM peritubular amiloride used to inhibit basolateral membrane Na⁺/H⁺ antiport); (b) clamping cell Ca²⁺ low with dimethyl-BAPTA, an intracellular Ca²⁺ chelating agent; or (c) removal of extracellular Ca²⁺. Cell acidification induced a spike increase in cell Ca²⁺. The late acceleration of Na⁺-independent cell pH recovery was independent of Na⁺ removal and of the method used to acidify the cell, but was eliminated by prevention of the cell Ca²⁺ spike and markedly delayed by the microfilament-disrupting agent, cytochalasin B. This study demonstrates that peritubular Na⁺ removal results in a sustained elevation in cell Ca²⁺, which inhibits the apical membrane H⁺ pump. In addition, rapid cell acidification associated with a spike increase in cell Ca²⁺ leads to a delayed activation of the H⁺ pump. Thus, cell Ca²⁺ per se, or a Ca²⁺-activated pathway, can modulate H⁺ pump activity.

INTRODUCTION

In the inner stripe of the outer medullary collecting duct (OMCD), transepithelial H⁺ secretion has been demonstrated to be unaffected by removal of Na⁺ from luminal
and peritubular solutions (Stone, Seldin, Kokko, and Jacobson, 1983). Based on these results, it is believed that apical membrane H⁺ extrusion mediating transepithelial HCO₃⁻ absorption in this segment occurs by a Na⁺-independent mechanism, probably a simple H⁺-ATPase. Using monoclonal antibodies directed against a number of different subunits of a purified H⁺-ATPase, Brown, Hirsch, and Gluck (1988) have demonstrated staining of apical membranes in this segment.

Direct measurement of H⁺ pump activity in cells has been difficult because of the inability to completely remove and rapidly add back required substrates. The most common approach has been to measure cell pH (pHᵢ) recovery from an acid load in the absence of extracellular Na⁺. Na⁺ is removed from the extracellular fluid to prevent any contribution from an Na⁺/H⁺ antiporter or a Na⁺(HCO₃⁻)₂/Cl⁻ exchanger to pHᵢ recovery. This approach assumes that Na⁺ removal has no effect on the activity of the H⁺ pump. However, removal of extracellular Na⁺ is known to increase cell Ca²⁺ (Ca²⁺ᵢ) (Mullins and Requena, 1986; Smith, Dwyer, and Smith, 1989), which could modulate H⁺ pump activity.

In recent studies we used such an approach in the OMCDᵢ to examine the nature of Na⁺-independent H⁺ extrusion (Hays and Alpern, 1990b). We found that after acid loading by NH₃/NH₄⁺ prepulse, Na⁺-independent pHᵢ recovery was inhibited by luminal NEM, unaffected by bath NEM, and unaffected by SCH 28080 (an inhibitor of the gastric H⁺-K⁺-ATPase). These results demonstrated that Na⁺-independent pHᵢ recovery was mediated by an apical membrane NEM-sensitive H⁺-ATPase, probably of the vacuolar type. In these studies, however, the initial rate of cell pH recovery after the acid load was extremely slow and accelerated after 10–15 min. H⁺ pump rate, calculated from the initial rate of pHᵢ recovery, could account for only 5% of transepithelial H⁺ secretion seen in this segment (Hays, Kokko, and Jacobson, 1986). This finding suggests that the approach used to measure H⁺-ATPase activity may have modulated transporter activity. The purpose of this study was to examine the mechanisms responsible for this time course in H⁺ pump activity. The results demonstrate that peritubular Na⁺ removal causes a sustained increase in Ca²⁺ᵢ, which inhibits H⁺ pump activity, causing the initial suppression. This suppression is later superseded by an activation of the H⁺ pump, probably due to cell acidification and a secondary spike Ca²⁺ increase.

METHODS

The technique of in vitro microperfusion of isolated rabbit OMCDᵢ was used as previously described (Hays et al., 1986). Briefly, female New Zealand White rabbits weighing 1.5–2.0 kg were maintained on standard laboratory chow and tap water ad lib. OMCDᵢ segments were identified and dissected free from 1-mm coronal slices at 4°C (pH 7.4, solution 1, Table I). To avoid the outer stripe, perfused segments were dissected from the inner half of the inner stripe. Tubules were transferred into a bath chamber with a volume of ~90 μl, constructed of black lucite to minimize light reflection. The peritubular fluid was continuously exchanged at ~10 ml/min by hydrostatic pressure. With this setup, a complete fluid exchange occurs within 1 s. Tubular lumens were perfused at flow rates of 25–50 nl/min. Bath pH was monitored continuously by placing a commercial flexible pH electrode into the bath (MI-5089; Microelectrodes, Inc., Londonderry, NH). Bath solutions were prewarmed at 37°C, continuously equilibrated with either 94% O₂/6% CO₂ or 100% O₂, and passed to the bath chamber through
CO₂-impermeable tubing (Clarkson Controls and Equipment Co., Detroit, MI). Bath temperature of 37 ± 0.3°C was maintained by a specially designed water-jacketed glass coiled line.

To minimize motion, the distal end of the tubule was sucked gently into a collection pipette. In addition, the average length of the tubule exposed to the bath fluid was limited to ~250–500 μm. The tubules were loaded with 8 μM of the acetoxymethyl derivative of 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM; Molecular Probes, Inc., Eugene, OR) or 5 μM fura-2 (fura-2-AM; Molecular Probes, Inc.) from the bath using solution 1 (Table I). Loading was continued until signal to background fluorescence at 450 nm (BCECF) or 350 nm (fura-2) excitation was ≥ 10:1, usually requiring 10–15 min. Tubules were then washed with solution 1, (Table I) at pH 7.4 for a minimum of 10 min, followed by the control solution of each experiment for at least an additional 5–10 min.

### Table I

| Composition of Solutions | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 |
|--------------------------|----|----|----|----|----|----|----|----|----|----|----|----|
| Na⁺                     | 145| —  | —  | 135| —  | —  | —  | 135| 125| 135| 135| 135|
| K⁺                      | 5  | 5  | 5  | 5  | 5  | 5  | 5  | 5  | 5  | 5  | 5  | 5  |
| Mg²⁺                    | 1.0| 1.0| 1.0| 1.0| 1.0| 1.0| 1.0| 1.0| 1.0| 1.0| 1.0| 1.0|
| Ca²⁺                    | 1.6| 1.6| 1.6| 1.6| 1.6| 1.6| 1.6| 1.6| 1.6| 1.6| 2.8| 2.0|
| Choline                 | —  | 120| 110| —  | —  | —  | —  | 120| 110| —  | —  | —  |
| NH₄⁺                    | —  | 10 | —  | —  | 10 | —  | 10 | —  | 10 | —  | —  | —  |
| N-Methyl d-glucosamine  | —  | 15 | 15 | —  | 135| 125| 15 | 15 | —  | —  | —  | —  |
| Cl⁻                     | 125.2| 125.2| 125.2| 125.2| 125.2| 100.2| 100.2| 100.2| 125.2| 87.6| 86.0|
| HCO₃⁻                   | 2.5| 2.5| 2.5| 2.5| 2.5| 2.5| 2.5| 2.5| 2.5| 2.5| 2.5| 2.5|
| Gluconate               | —  | —  | —  | —  | —  | —  | —  | —  | 40 | —  | —  | —  |
| Propionate              | —  | —  | —  | —  | —  | —  | —  | —  | —  | —  | —  | 40 |
| Glucose                 | 8.3| 8.3| 8.3| 8.3| 8.3| 8.3| 8.3| 8.3| 8.3| 8.3| 8.3| 8.3|
| Alanine                 | 5  | 5  | 5  | 5  | 5  | 5  | 5  | 5  | 5  | 5  | 5  | 5  |
| HEPES                   | —  | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 |

All units are millimolar. All solutions were titrated to 290 mosM with addition of raffinose. Solutions 2, 3, 5, 6, 7, and 8 were titrated to pH 7.40 with 1 N N-methyl-d-glucosamine hydroxide. Solutions 4, 9, 10, 11, and 12 were titrated to pH 7.40 with 1 N NaOH. All HCO₃⁻-containing solutions were equilibrated with 94% O₂/6% CO₂; other solutions were equilibrated with 100% O₂.

Luminal and peritubular solutions used in these studies are listed in Table I. To decrease any contribution of Cl⁻/HCO₃⁻ exchange to the results, CO₂/HCO₃⁻ was removed from all solutions except solutions 1, 7, 8, and 9 (Table I; Hays and Alpern, 1990a). Cl⁻ was not removed from the solutions because of the known Cl⁻ dependence of the H⁺ pump (Kaunitz, Gunther, and Sachs, 1985; Kurtz, 1987; Xie, Crider, and Stone, 1989). In addition, Cl⁻/HCO₃⁻ exchange would not be expected to contribute to pH recovery at the low pH values obtained after an acid load (6.2–6.8), pH values at which the Cl⁻/HCO₃⁻ exchanger is inactivated in a variety of mammalian cell lines (Olnes, Tonnessen, Lüdt, and Sandvig, 1987), mesangial cells (Boyarsky, Ganz, Sterzel, and Boron, 1988), gastric parietal cells (Wenzl and Machen, 1989), and neutrophils (Sinchowitz and Davis, 1991). CO₂/HCO₃⁻-free solutions were bubbled with 100% oxygen passed through a 3 N KOH trap. Nigericin, amiloride hydrochloride, colchicine, cytochalasin B, and all solution salts were purchased from Sigma Chemical Co. (St. Louis, MO). The acetoxymethyl ester form of 1,2-bis(o-aminophenoxy)ethane-(5,5')-(dimethyl)-N,N,N',N'-tetraacetic acid (dimethyl-BAPTA) was purchased from Molecular Probes, Inc.
Cell pH Measurement

BCECF fluorescent emission was measured at 530 nm with an inverted epifluorescent microscope (Nikon Diaphot; Nikon, Inc., Garden City, NY) attached to a dual excitation microspectrofluorimeter (SPEX CM-1; Spex Industries, Edison, NJ) which allows rapid alternation between two excitation wavelengths (500 and 450 nm), as previously described (Hays and Alpern, 1990a). Fluorescence was measured using a 20x objective on an area of the tubule ~125 μm in length that included the entire width of the tubule. No attempts were made to make measurements on single cells. Ultrastructural (Ridderstrale, Kashgarian, Koeppen, Giebisch, Stetson, Ardito, and Stanton, 1988), morphologic (Madsen, Verlander, Linser, and Tischer, 1989), and electrophysiologic (Koeppen, 1985; Muto, Yasoshima, Yoshitomi, Imai, and Asano, 1990) studies all support one cell type in the inner half of the OMCD, where the present studies were performed. Wiener and Hamm (1990) were able to divide OMCD cells into two groups based on ease of BCECF loading, but found that both cell types possessed the same H+/HCO₃⁻ transporters. Generally, the measured segment started ~50–100 μm from the perfusion pipette. Background fluorescence at each of the excitation wavelengths was measured on the tubule before loading with BCECF, and the results were subtracted from the measured fluorescence during the experiment. A fluorescence ratio was then calculated as fluorescence with 500 nm excitation divided by that with 450 nm excitation. The initial rate of change in the fluorescence excitation ratio was defined by the slope of a line drawn tangent to the initial deflection (d[F₅₀₀/F₄₅₀]/dt).

BCECF fluorescence excitation ratios were calibrated intracellularly using the method of Thomas, Buchbaum, Simnik, and Racker (1979). Tubules were bathed and perfused with well-buffered solutions (25 mM HEPES, 60 mM phosphate, and appropriate [HCO₃⁻]) of varying pH containing 7 μM nigericin (a K⁺/H⁺ antiporter) and 120 mM K⁺ (Hays and Alpern, 1990a). Because of the small amount of variability between tubules, a calibration generated in 10 tubules was used to convert F₅₀₀/F₄₅₀ fluorescent ratios to pH units in all experimental studies.

Cell Calcium (Ca²⁺) Measurement

Fura-2 fluorescence was measured alternately with 380 and 350 nm excitation with 510 nm emission. Fluorescence was measured using a 63x fluorite objective, on an area of the tubule ~25 μm long and 15 μm wide. Generally, the measured segment started 50–100 μm from the perfusion pipette. Background fluorescence at each of the excitation wavelengths was measured on the tubule before loading with fura-2, and the results were subtracted from the measured fluorescence during the experiment.

Fura-2 fluorescence excitation ratios were calibrated intracellularly using the method of Grynkiewicz, Poenie, and Tsien (1985). After tubule loading with fura-2, tubules were bathed and perfused with a zero Ca²⁺, HEPES-buffered solution (pH 7.4) containing 2 mM EGTA without ionomycin for 10 min before changing to the same solution with 4 μM ionomycin. After the fluorescence ratio stabilized, usually within 6–10 min, luminal and peritubular solutions were rapidly changed to a similar solution containing 1.6 mM Ca²⁺ (pH 7.4, solution 6, Table I) without EGTA. Ca²⁺ values were calculated by the formula (Grynkiewicz et al., 1985):

\[ \text{Ca}^{2+} = K_d (R - R_{\text{min}})/(R_{\text{max}} - R) \times (S_\text{R}/S_{\text{I}}) \]  

using a \( K_d \) of 224 nm (Grynkiewicz et al., 1985). \( R \) is the experimental ratio of fluorescence intensity at excitation wavelengths 350 and 380 nm (350/380). \( R_{\text{min}} \) is the ratio measured at zero Ca²⁺ with ionomycin, \( R_{\text{max}} \) is the ratio measured at 1.6 mM Ca²⁺ with ionomycin, and \( S_\text{R}/S_{\text{I}} \) is the ratio of fluorescence measured at 380 nm excitation with zero Ca²⁺ and 1.6 mM Ca²⁺, respectively, both with ionomycin. On six tubules, the calculated \( R_{\text{min}} \) was 1.19 ± 0.03, \( R_{\text{max}} \) was 5.47 ± 0.49, and the \( S_\text{R}/S_{\text{I}} \) was 3.34. Because of the small amount of variability of
baseline fluorescence intensity ratios between tubules (1.84 ± 0.09), a calibration generated in six tubules was used to convert F350/F380 fluorescent ratios to Ca$^{2+}$ in all experimental studies.

Statistics

Results are reported as mean ± SE. The data were analyzed using analysis of variance and the two-tailed Student’s t test for paired and unpaired data, as appropriate.

RESULTS

pH$_i$ Recovery from an Acid Load

Previously, we found that the time course for Na$^+$-independent pH$_i$ recovery from an acid load (NH$_3$/NH$_4^+$ prepulse) performed in the absence of CO$_2$/HCO$_3^-$ was manifest as a biphasic response (Hays and Alpern, 1990b). Shown in Fig. 1 is a typical tracing. After acid loading, tubule cells alkalinized at a slow rate for 1–2 min and then pH stabilized. 10–15 min later, a gradually increasing rate of cell alkalinization occurred which returned pH$_i$ to a level significantly more alkaline than pre-NH$_3$/NH$_4^+$ pulse values. The relatively slow initial rate of Na$^+$-independent pH$_i$ recovery indicated a H$^+$ pump rate that accounted for 5% of the observed rates of transepithelial H$^+$ secretion (HCO$_3^-$ absorption) found in this segment (see below; Hays et al., 1986). Thus, these results were suggestive of an initial inhibition followed by a subsequent activation of the apical membrane H$^+$ pump.
The next set of studies was designed to confirm this postulate (Fig. 2). Tubules were initially bathed with a control solution containing 145 mM Na⁺ and 25 mM HCO₃⁻ (pH 7.4, solution 1, Table I) and luminally perfused with a Na⁺-free, CO₂/HCO₃⁻-free solution (pH 7.4, solution 2, Table I). The peritubular solution was then changed to a Na⁺-free, CO₂/HCO₃⁻-free solution containing 10 mM NH₄Cl (pH 7.4, solution 3, Table I). After 3–4 min exposure to this solution, the peritubular solution was changed to an identical solution without NH₄Cl (pH 7.4, solution 2, Table I). After varying intervals, acid loads were again induced by short (1 min) NH₃/NH₄⁺ pulses.

After the initial acid load, pHᵢ recovered at a slow rate as seen in our previous studies (Hays and Alpern, 1990b). Subsequent NH₃/NH₄⁺ pulses were associated with gradually increasing rates of pHᵢ recovery. In seven tubules, the initial rate of pHᵢ recovery was 0.06 ± 0.02 pH units/min, while pHᵢ recovery increased maximally to a rate of 0.63 ± 0.09 pH units/min when measured in response to a later acid load (P < 0.001). The initial proton flux (Jₚ) was 0.47 ± 0.13 pmol/mm · min (buffer capacity 13.09 ± 1.11 mmol · liter⁻¹ · pH unit⁻¹ [pHᵢ changed from 7.49 to 6.21]), a

\[ J_p = \frac{dpH_i}{dt} \cdot V/mm \cdot \beta_p \]  

where V/mm is the cellular volume of the tubules per millimeter length and \( \beta_p \) is the buffer capacity. A cell volume of 6.11 × 10⁻¹⁰ liter/mm was used (outer tubular diameter 36.5 μm and inner diameter 23.4 μm). The buffer capacities were determined for each acid load during NH₃/NH₄⁺ withdrawal by the method of Roos and Boron (1981).
rate ~ 5% of the rate of transepithelial H⁺ secretion in this segment (Hays et al., 1986). The $J_H$ increased maximally to $6.59 \pm 0.90$ pmol/mm $\cdot$ min (buffer capacity $16.62 \pm 5.48$ mmol $\cdot$ liter$^{-1} \cdot$ pH unit$^{-1}$ [$pH_i$ changed from 7.85 to 6.66]) when measured 12–15 min after the initial acid load ($P < 0.001$). These results support an initial inhibition and subsequent activation of the H⁺ pump during the recovery from an acid load.

**Effect of Peritubular Na⁺ Removal and NH₃/NH₄⁺ Addition on pHᵢ and Ca$_{2+}$**

The next series was designed to examine the effects of Na⁺ removal and peritubular NH₃/NH₄⁺ addition on pHᵢ and Ca$_{2+}$. Fig. 3 A shows a typical tracing of pHᵢ in these tubules. Peritubular Na⁺ removal resulted in a slow cell acidification, as we previously noted (Hays and Alpern, 1990a). After peritubular NH₃/NH₄⁺ addition and removal, the cells acidified further. Once again, pHᵢ recovery was initially slow ($0.03 \pm 0.01$ pH units/min, $n = 6$) and accelerated later ($0.72 \pm 0.09$ pH units/min, $n = 5$).

Fig. 3 B shows a representative tracing of Ca$_{2+}$ in these tubules. Peritubular Na⁺ removal resulted in a dramatic spike increase in Ca$_{2+}$, which was followed by a sustained increase in Ca$_{2+}$. After peritubular NH₃/NH₄⁺ addition, Ca$_{2+}$ dropped transiently and then gradually drifted upward. Subsequent peritubular withdrawal of
NH₃/NH₄⁺ resulted in a second transient spike increase in Ca²⁺, after which Ca²⁺ returned to its elevated baseline.

The initial Ca²⁺ values in seven tubules bathed in 135 mM Na⁺ was 125 ± 28 nM. After peritubular Na⁺ removal, Ca²⁺ rose to a peak value of 666 ± 217 nM (P < 0.001) and then fell. Peritubular NH₃/NH₄⁺ addition caused a transient Ca²⁺ decrease from 223 ± 31 to 109 ± 16 nM (P < 0.001) and NH₃/NH₄⁺ withdrawal caused a transient Ca²⁺ increase from 165 ± 26 to 262 ± 41 nM (P < 0.005). Ca²⁺ then fell before slowly rising to 254 ± 42 nM over the next 5–10 min, and then slowly fell toward initial control Ca²⁺ values near the end of the experiment (141 ± 29 nM).

![Table of Peritubular [Na⁺] and [NH₃/NH₄⁺]

| Peritubular [Na⁺], mM | Peritubular [NH₃/NH₄⁺], mM |
|------------------------|-----------------------------|
| 135                    | 0                           |
| 0                      | 10                          |
| 0                      | 0                           |

![Graphs of APPARENT pH and APPARENT Ca²⁺ over TIME (min)]

**Figure 4.** (A) Na⁺-independent cell pH recovery after sequential peritubular Na⁺ removal (Na⁺ replaced with N-methyl-D-glucosamine) and acid load. The protocol followed was similar to that described in Fig. 3, except that N-methyl-D-glucosamine replaced choline in all solutions (solutions 5 and 6). (B) Cell Ca²⁺ after sequential peritubular Na⁺ removal (Na⁺ replaced with N-methyl-D-glucosamine) and acid load. The protocol followed was as in A.

at 15 min after NH₃/NH₄⁺ removal). In some experiments, when the tubule was later pulsed with NH₃/NH₄⁺, Ca²⁺ values followed a similar pattern to that seen above; that is, Ca²⁺ fell transiently during exposure to NH₃/NH₄⁺, and increased transiently upon withdrawal of the NH₃/NH₄⁺.

In the above studies, choline was substituted for Na⁺, which may have contributed to the unusual time course of pHᵢ recovery. We next examined pHᵢ recovery from an acid load in an identical manner except that N-methyl-D-glucosamine rather than choline was substituted for Na⁺. Fig. 4 A shows a typical tracing. Once again, pHᵢ,

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recovery was initially slow (0.04 ± 0.01 pH units/min, n = 6) and accelerated at a later time point.

Fig. 4 B shows a representative tracing of Ca\(^{2+}\) in these tubules. Once again, peritubular Na\(^{+}\) removal resulted in a dramatic spike increase in Ca\(^{2+}\), followed by a sustained increase in Ca\(^{2+}\). Later, peritubular NH\(_3\)/NH\(_4\)\(^+\) addition caused a transient Ca\(^{2+}\) decrease and NH\(_3\)/NH\(_4\)\(^+\) withdrawal resulted in a transient increase in Ca\(^{2+}\), similar to the results found in the earlier choline studies. In seven tubules, peritubular Na\(^{+}\) removal caused Ca\(^{2+}\) to increase from 118 ± 16 to 883 ± 127 nM. NH\(_3\)/NH\(_4\)\(^+\) addition caused Ca\(^{2+}\) to transiently fall from 329 ± 39 to 159 ± 21 nM, and NH\(_3\)/NH\(_4\)\(^+\) withdrawal caused a transient increase in Ca\(^{2+}\) from 167 ± 19 to 239 ± 15 nM. Ca\(^{2+}\) then fell before slowly rising to 224 ± 19 nM over the next 5-10 min. Ca\(^{2+}\) values then slowly returned toward initial control Ca\(^{2+}\) values near the end of the experiment (148 ± 24 nM, 15 min after the acid load). Thus, these studies show that Na\(^{+}\) removal causes a spike increase in Ca\(^{2+}\) and then a sustained increase in Ca\(^{2+}\). The effects of NH\(_3\)/NH\(_4\)\(^+\) are probably attributable to an effect of cell alkalinization to transiently decrease Ca\(^{2+}\) and of cell acidification to transiently increase Ca\(^{2+}\).

**Effect of CO\(_2\)/HCO\(_3\)**

In the above studies CO\(_2\)/HCO\(_3\) was absent, which may have contributed to the unusual time course. We next examined pH\(_i\) recovery from an acid load in the presence of CO\(_2\)/HCO\(_3\). The protocol for these studies was the same as in Fig. 2 except that all solutions contained CO\(_2\)/HCO\(_3\) (Fig. 5). In these studies, the initial pH\(_i\) recovery from the acid load was once again relatively slow. However, the late accelerated pH\(_i\) recovery occurred at an earlier point in time (cf. Figs. 1 and 5). When a second short NH\(_3\)/NH\(_4\)\(^+\) pulse was applied, the pH\(_i\) recovery from the acid load was very rapid. In five tubules, the initial rate of pH\(_i\) recovery (dpH\(_i\)/dt) was 0.04 ± 0.01 pH units/min, while pH\(_i\) recovery from a later acid load was more rapid, 1.73 ± 0.16 pH units/min (P < 0.001, equivalent to a \(f_h\) of 45.29 ± 3.89 pmol/mm ⋅ min, buffer capacity 41.65 ± 4.18 mmol ⋅ liter\(^{-1}\) ⋅ pH unit\(^{-1}\), pH\(_i\) changed from 7.46 to 7.06). In addition, the time required for pH\(_i\) to surpass prepulse values after an acid load was markedly shortened (control, n = 16, 934 ± 73 s; CO\(_2\)/HCO\(_3\), n = 5, 310 ± 32 s, P < 0.001). These results show that the removal of CO\(_2\)/HCO\(_3\) from the bath is not responsible for the initial, slow, Na\(^{+}\)-independent pH\(_i\) recovery from an acid load. However, the presence of CO\(_2\)/HCO\(_3\) shortens the time course of late Na\(^{+}\)-independent pH\(_i\) recovery and increases the magnitude of Na\(^{+}\)-independent pH\(_i\) recovery from a later acid load.

Because the presence of CO\(_2\)/HCO\(_3\) shortened the time course of late Na\(^{+}\)-independent pH\(_i\) recovery from an acid load, we next examined whether changes in Ca\(^{2+}\) could account for this phenomenon. The protocol for these studies was the same as in Fig. 3 B, except that all solutions contained CO\(_2\)/HCO\(_3\). The results were similar to those performed in the absence of CO\(_2\)/HCO\(_3\) (Fig. 6). The initial Ca\(^{2+}\) values in five tubules bathed in 135 mM Na\(^{+}\) were 84 ± 18 nM. After peritubular Na\(^{+}\) removal, Ca\(^{2+}\) transiently rose to a peak value of 546 ± 81 nM. Peritubular NH\(_3\)/NH\(_4\)\(^+\) addition caused Ca\(^{2+}\) to transiently decrease from 238 ± 35 to 149 ± 20 nM. Peritubular NH\(_3\)/NH\(_4\)\(^+\) withdrawal caused a transient increase in Ca\(^{2+}\) from 196 ± 28 to 267 ± 43 nM. Ca\(^{2+}\) then fell before slowly rising to 234 ± 28 nM over the next
Figure 5. (A) Na⁺-independent cell pH recovery after an acid load: presence of CO₂/HCO₃⁻. Tubules were initially bathed with a control solution containing 25 mM HCO₃⁻ (pH 7.4, solution 1, Table I) and luminally perfused with a Na⁺-free solution (pH 7.4, solution 7, Table I). The peritubular solution was then changed to a Na⁺-free solution containing 10 mM NH₄Cl and 25 mM HCO₃⁻ (pH 7.4, solution 8, Table I). After 4-5 min exposure to this solution, the peritubular solution was changed to an identical solution without NH₄Cl (pH 7.4, solution 7, Table I). After a variable period of 8-10 min, an acid load was again induced by a short NH₃/NH₄⁺ pulse (1 min).

Figure 6. Cell Ca²⁺ after an acid load: presence of CO₂/HCO₃⁻. The protocol followed was similar to that described in Fig. 3 except that all solutions contained CO₂/HCO₃⁻ (solutions 7-9).
5–10 min. Ca\(^{2+}\) values then slowly returned toward initial control Ca\(^{2+}\) values near the end of the experiment (130 ± 31 nM, 15 min after NH\(_3\)/NH\(_4^+\) removal). Of note, Ca\(^{2+}\) remained elevated after the acid load at a level similar to that in the studies performed in the absence of exogenous CO\(_2\)/HCO\(_3^-\). There was no significant difference between the Ca\(^{2+}\) values obtained in the presence or absence of CO\(_2\)/HCO\(_3^-\) at any time point. Thus, the more rapid, late, Na\(^+\)-independent pH\(_r\) recovery obtained in the presence of CO\(_2\)/HCO\(_3^-\) cannot be accounted for by measurable differences in Ca\(^{2+}\) changes. All subsequent studies were performed in the absence of CO\(_2\)/HCO\(_3^-\).

**Effect of Peritubular Dimethyl-BAPTA**

To study the roles of increased Ca\(^{2+}\) levels in the response to acid loading, we next examined the effects of dimethyl-BAPTA, an intracellular Ca\(^{2+}\) chelating agent (Tsien, Pozzan, and Rink, 1982) on pH\(_r\) recovery from an acid load. As shown in Fig. 7 B, tubule preincubation with 100 \(\mu\)M peritubular dimethyl-BAPTA-AM for 20 min prevented all increases in Ca\(^{2+}\) during the protocol.

To examine pH\(_r\) recovery from an acid load in the absence of Ca\(^{2+}\) increases, tubules were preincubated for 20 min with dimethyl-BAPTA. The protocol followed was as in A.
similar to that in Fig. 2. Fig. 7A shows a representative tracing of the pH$_i$ response in these tubules. After acid loading, the rate of initial pH$_i$ recovery was markedly increased. In six tubules, the initial rate of pH$_i$ recovery was 0.27 ± 0.02 pH units/min, which was greater than that in control tubules, $P < 0.001$ (Fig. 8). Subsequent acid loads failed to demonstrate an increased rate of recovery, 0.21 ± 0.05 pH units/min ($P = \text{NS}$). In addition, pH$_i$ recovery was markedly delayed and pH$_i$ followed for at least 45 min after an acid load never recovered to values above the initial baseline pH$_i$. These studies suggested that changes in Ca$^{2+}$ are responsible for the initial inactivation and the delayed activation of the H$^+$ pump.

**Effect of Peritubular Na$^+$ Removal**

Because Na$^+$ removal caused both a spike and a sustained increase in Ca$^{2+}$, we next examined H$^+$ pump activity without peritubular Na$^+$ removal. In these studies,

![Figure 8: Initial rate of Na$^+$-independent cell pH recovery after an acid load: control, peritubular dimethyl-BAPTA, peritubular Na$^+$ plus amiloride, and absence of extracellular Ca$^{2+}$.]

Na$^+$-dependent pH$_i$ recovery was inhibited by 1 mM peritubular amiloride rather than Na$^+$ removal. We have previously demonstrated that 1 mM peritubular amiloride completely blocks Na$^+$-dependent pH$_i$ recovery in this segment (Hays and Alpern, 1990b). The protocol was similar to that of Fig. 3A except that amiloride was added instead of removing Na$^+$. Fig. 9 shows a representative tracing. Exposure to 1 mM peritubular amiloride resulted in a slow cell acidification. After peritubular NH$_3$/NH$_4^+$ addition and removal, the cells acidified further. In contrast to the studies where peritubular Na$^+$ was removed, the initial rate of pH$_i$ recovery from an acid load was more rapid. In addition, pH$_i$ recovered to a level greater than the initial baseline pH$_i$ values in a relatively short period of time. In seven tubules, initial dpH$_i$/dt was 0.18 ± 0.05 pH units/min, which was greater than in controls ($P < 0.005$), but not...
different from the initial rates obtained in the presence of dimethyl-BAPTA (Fig. 8). This study suggests that peritubular Na⁺ removal through changes in Ca⁺² inhibits the apical H⁺ pump. The fact that pHi recovered in these studies to values above baseline pH implies that late activation of the H⁺ pump was intact. Unfortunately, because amiloride and fura-2 fluoresce at similar wavelengths, we could not measure Ca⁺² with this protocol.

**Effect of Extracellular Ca⁺² Removal**

As noted above, peritubular Na⁺ removal caused a spike Ca⁺² increase and a sustained Ca⁺² increase. To examine the source for the Ca⁺² increase, and possibly to distinguish which phase of the Ca⁺² increase mediates the H⁺ pump inhibition, we repeated the studies in the absence of extracellular Ca⁺². The protocol was the same as that described in Fig. 3 A, with the exception that all solutions were Ca⁺² free and contained 2 mM EGTA.

Fig. 10 B shows a representative Ca⁺² tracing. After peritubular removal of Ca⁺², Ca⁺² decreased to a lower steady-state value. Under these conditions peritubular Na⁺ removal again resulted in a dramatic spike increase in Ca⁺², which rapidly fell back to
baseline. In contrast to the studies performed in the presence of extracellular Ca\(^{2+}\) (Fig. 3 B), there was no sustained increase in Ca\(^{2+}\). Ca\(^{2+}\) continued to slowly decrease and was not affected by peritubular NH\(_3\)/NH\(_4^+\) addition. Peritubular NH\(_3\)/NH\(_4^+\) withdrawal resulted in a small but rapid rise in Ca\(^{2+}\). Ca\(^{2+}\) then slowly fell during the remainder of the experiment.

In eight tubules, initial Ca\(^{2+}\) was 113 ± 26 nM and fell to 81 ± 13 nM (P < 0.01) upon removal of peritubular Ca\(^{2+}\). After peritubular Na\(^+\) removal Ca\(^{2+}\) rose to 628 ± 184 nM (P < 0.001) before falling back to 74 ± 13 nM within 90 s. Prior to NH\(_3\)/NH\(_4^+\) withdrawal Ca\(^{2+}\) had slowly fallen to 60 ± 10 nM, before rising to 103 ± 17 nM upon NH\(_3\)/NH\(_4^+\) removal (P < 0.001). These studies demonstrate that peritubular Na\(^+\) removal results in a release of Ca\(^{2+}\) from intracellular stores (spike Ca\(^{2+}\) increase). The sustained increase in Ca\(^{2+}\) requires extracellular Ca\(^{2+}\) and may be due

\[\text{Peritubular [Na\(^+\)], mM} \quad 135 \quad 0\]
\[\text{Peritubular [NH\(_3\)/NH\(_4^+\)], mM} \quad 0 \quad 10 \quad 0\]
\[\text{Peritubular [Ca\(^{2+}\)], mM} \quad 1.6 \quad 0 \quad \text{EGTA}\]

**Figure 10.** (A) Na\(^+\)-independent cell pH recovery after an acid load: absence of extracellular Ca\(^{2+}\). The protocol followed was identical to that described in Fig. 4 except that all solutions contained no added Ca\(^{2+}\) and 2 mM EGTA. (B) Cell Ca\(^{2+}\) during an acid load: absence of extracellular Ca\(^{2+}\). The protocol followed was as in A.
to Na\(^+/\)Ca\(^{2+}\) exchange. In addition, peritubular NH\(_3\)/NH\(_4^+\) removal also results in release of Ca\(^{2+}\) from intracellular Ca\(^{2+}\) stores. The small magnitude of this spike in these studies may be due to depletion of cell Ca\(^{2+}\) stores by the previous Ca\(^{2+}\) spike, and the continued absence of extracellular Ca\(^{2+}\).

Fig. 10A shows a representative tracing of the time course for pH\(_i\) with this protocol. Removal of extracellular Ca\(^{2+}\) had no effect on pH\(_i\). Peritubular Na\(^+\) removal again resulted in a slow cell acidification. After peritubular NH\(_3\)/NH\(_4^+\) removal, pH\(_i\) recovered at a rapid initial rate similar to that found in the dimethyl-BApTA studies. However, in contrast to the dimethyl-BApTA studies, pH\(_i\) recovered to a level greater than the initial control pH\(_i\) values.

In six tubules, the initial rate of pH\(_i\) recovery was 0.20 ± 0.05 pH units/min, which was greater than that in control studies (0.06 ± 0.02 pH units/min, P < 0.05; see Fig. 8), but not different from that in the dimethyl-BApTA studies (0.27 ± 0.02 pH units/min, P = NS). Thus, the slow rate of initial Na\(^+\)-independent pH\(_i\) recovery correlates best with the sustained increase in Ca\(^{2+}\) which occurs upon peritubular Na\(^+\) removal. One of the two Ca\(^{2+}\) spikes, which were present with zero extracellular Ca\(^{2+}\) but absent with dimethyl-BApTA, probably signal the late H\(^+\) pump activation.

### Effect of Cell Alkalinization

In the next series of studies we investigated whether the transient NH\(_3\)/NH\(_4^+\)-induced alkalinization contributes to the initial inactivation of the H\(^+\) pump. We therefore examined the pH\(_i\) recovery from an acid load induced by sudden addition of 40 mM peritubular Na propionate. This protocol eliminates the transient alkalinization that occurs with the NH\(_3\)/NH\(_4^+\) prepulse method.

The protocol was the same as in Fig. 9, but cells were acid loaded by Na propionate addition to the bath. Fig. 11 shows a representative tracing of the pH\(_i\) response with this protocol. After peritubular amiloride addition, the cells slowly acidified. 40 mM peritubular Na propionate addition resulted in a further rapid cell acidification. pH\(_i\) recovery from this acid load occurred at a rapid rate. In six tubules, initial pH\(_i\) recovery from an acid load was 0.19 ± 0.05 pH units/min, which was not different from the initial rate of pH\(_i\) recovery seen with NH\(_3\)/NH\(_4^+\) prepulse, performed under similar conditions (the presence of peritubular amiloride and Na\(^+\)). These studies demonstrate that the alkalinization that occurs with NH\(_3\)/NH\(_4^+\) prepulse does not contribute to the initial inhibition of the H\(^+\) pump. The late increase in pH\(_i\) above baseline levels was present with this protocol.

### Effect of Cytoskeletal Inhibitors

The last series of studies examined the effects of the cytoskeletal disrupting agents colchicine and cytochalasin B on pH\(_i\) recovery from an acid load. The protocols used were identical to that in Fig. 2 except that tubules were preincubated for at least 30 min in the presence of either 0.5 mM peritubular colchicine or 20 μM peritubular cytochalasin B. In addition, either colchicine or cytochalasin B was present in all bathing solutions throughout the experiment.

A typical tracing of the colchicine experiments is shown in Fig. 12. In these studies, the initial rate of pH\(_i\) recovery from an acid load was very slow. In six tubules, the initial rate of pH\(_i\) recovery was 0.005 ± 0.004 pH units/min, a rate significantly
Peritubular (propionate), mM

| pH | 0 | 40 |
|----|---|----|

Peritubular Amiloride, mM

| pH | 0 | + |

Peritubular [Na+], mM

| pH | 135 |

**Figure 11.** Na⁺-independent pH, recovery after an acid load: induced by Na propionate in the presence of peritubular Na⁺ and amiloride. Before the experimental tracing shown above, tubules were initially bathed with a control solution (pH 7.4, solution 1, Table I) and luminally perfused with a Na⁺-free, CO₂/HCO₃⁻-free solution (pH 7.4, solution 2, Table I). 10 min before experimentation the peritubular solution was changed to a CO₂/HCO₃⁻-free solution substituting 40 mM gluconate for Cl⁻ (pH 7.4, solution 11, Table I) and continued for the first 3–4 min of the experimental tracing above. The peritubular solution was then changed to an identical solution containing 1 mM amiloride for 5 min. Peritubular amiloride was continued throughout the remainder of the experiment. The acid load was then induced by changing the peritubular solution to an identical solution substituting 40 mM Na propionate for 40 mM Na gluconate (solution 12).

**Figure 12.** Na⁺-independent cell pH recovery after an acid load: presence of colchicine. The protocol followed was similar to that described in Fig. 2, except that tubules were preincubated and continuously exposed to colchicine.
slower than control tubules (control, 0.06 ± 0.02 pH units/min; P < 0.02). In addition, pH recovery from a later acid load was also significantly inhibited (control, 0.63 ± 0.09 pH units/min; colchicine, 0.33 ± 0.07 pH units/min, P < 0.02). However, the time required for pH to return to control (prepulse) levels after an acid load was not significantly different from that obtained in control tubules (control [n = 16], 934 ± 73 s; colchicine [n = 6], 986 ± 124 s, P = NS). Thus, although peritubular colchicine resulted in a mild inhibition of H⁺ pump activity in response to both an initial and later acid load, it did not inhibit the late increase in pH above prepulse levels.

A typical tracing of the cytochalasin B experiments is shown in Fig. 13. Once again, the initial rate of pH recovery from an acid load was slow. In five tubules, the initial rate of recovery was 0.08 ± 0.02 pH units/min, a value not significantly different from controls (0.06 ± 0.02 pH units/min). However, the pH recovery from a later acid load was significantly inhibited (control, 0.63 ± 0.09 pH units/min; cytochalasin B, 0.33 ± 0.10 pH units/min, P < 0.05). In addition, the time required for pH to return to control prepulse values was significantly prolonged (control [n = 16], 934 ± 73 s, cytochalasin B [n = 5], 2498 ± 393 s, P < 0.0001). This inhibition of the late pH recovery with cytochalasin B suggests that cytoskeletal microfilaments participate in the late pH recovery.

**FIGURE 13.** Na⁺-independent cell pH recovery after an acid load: presence of cytochalasin B. The protocol followed was similar to that described in Fig. 2, except that tubules were preincubated and continuously exposed to cytochalasin B.

| Peritubular [Na⁺], mM | 0 | 0 |
|------------------------|---|---|
| Peritubular [NH₃/NH₄⁺], mM | 0 | 0 |

**DISCUSSION**

**Na⁺-independent pH, Recovery from an Acid Load**

Previously, we found that Na⁺-independent pH recovery from an acid load is characterized by an initial brief, slow alkalinization followed by a variable period of pH stabilization lasting 3–12 min (Hays and Alpern, 1990b). The cell then begins to alkalinize at gradually increasing rates, raising pH above the initial pre-acid load values. This pattern of Na⁺-independent pH recovery is similar to that observed in the S₃ segment of the rabbit proximal tubule by Nakhoul, Lopes, Chaillet, and Boron.
(1988) after prolonged incubation in Na⁺-free solutions. Thus, in both of these segments initial pHᵢ recovery is slow, followed by an acceleration in pHᵢ recovery over time, eventually leading to pHᵢ values greater than the pre-acid load pHᵢ. In addition, the initial rate of Jᵢ measured in our studies (0.47 ± 0.13 pmol/mm·min) could not account for the measured rate of net HCO₃⁻ absorption found in the OMCD (8–10 pmol/mm·min [Hays et al., 1986]). In the present studies the rate of pHᵢ recovery from acid loads repeated serially in the same tubule gradually increased to a rate 14-fold greater than the initial pHᵢ recovery. These studies suggest an initial inactivation followed by a subsequent activation of the apical membrane H⁺ pump.

**Initial Inactivation of the H⁺ Pump**

The initial inactivation of the apical membrane H⁺ pump is due to a sustained increase in Ca²⁺ which occurs in response to removal of peritubular Na⁺. This conclusion is based on the following observations: (a) The initial slow rate of pHᵢ recovery occurs whether Na⁺ has been replaced with choline or N-methyl-D-glucosamine and in both cases a sustained increase in Ca²⁺ occurs. (b) When the basolateral membrane Na⁺/H⁺ antiporter was inhibited by amiloride rather than peritubular Na⁺ removal, the initial rate of the H⁺ pump was rapid. (c) In the presence of Na⁺ removal, when all Ca²⁺ increases were prevented by dimethyl-BAPTA, the initial rate of the H⁺ pump was rapid. (d) In the presence of Na⁺ removal, when the sustained increase in Ca²⁺ was prevented by removal of extracellular Ca²⁺, the initial rate of the H⁺ pump was rapid. Thus, based on these experiments, inactivation of the H⁺ pump correlates with the sustained increase in Ca²⁺ rather than with the spike increase in Ca²⁺.

There are many examples of regulation of transporter activity by sustained changes in Ca²⁺. Inhibition of apical membrane transporters by sustained increases in Ca²⁺ has been demonstrated for the apical membrane Na⁺ channel of the toad bladder (Chase and Al-Awqati, 1983). A similar inhibition of apical membrane Na⁺ transport, probably involving an Na⁺/H⁺ exchanger, has been postulated to occur with chronic increases in proximal tubule Ca²⁺ (Friedman, Figueiredo, Maack, and Windhager, 1981). In addition, sustained increases in Ca²⁺ have been shown to increase Cl⁻/HCO₃⁻ exchange activity in UMR-106 cells (Green, Yamaguchi, Kleeman, and Muallem, 1990). Finally, sustained increases in Ca²⁺ activate K⁺ channels (Blatz and Magleby, 1987).

**Late Activation of the H⁺ Pump**

After the acid load, pHᵢ eventually achieved levels higher than the pre-acid load steady-state value. Such a change in steady-state pHᵢ may be attributable to an increase in the activity of an H⁺ extruding mechanism, or a decrease in the activity of a base extruding mechanism. Since these studies were performed with the Na⁺/H⁺ antiporter inhibited, the only H⁺ extruding mechanism that is active is the H⁺ pump. The data shown in Fig. 2, where H⁺ pump activity was examined sequentially, demonstrated a steady increase in H⁺ pump activity following the acid load.

The only protocol in which pHᵢ did not return to levels above the prepulse steady-state pHᵢ was the one where dimethyl-BAPTA was added. In addition, this is the only protocol where a late NH₃/NH₄⁺ pulse led to the same rate of H⁺ pump activity as the original pulse. These data strongly suggest that the late activation of
the H⁺ pump is mediated by an increase in Ca²⁺. While it is possible that the failure to observe late activation of the H⁺ pump in these studies was due to the short duration of cell acidification, a similar short duration of cell acidification was seen in the studies with amiloride and in the studies with zero extracellular Ca²⁺, but both of these protocols were associated with steady-state pH, values greater than prepulse.

By comparing the different protocols, it is also possible to determine which Ca²⁺ change was responsible for the late activation. Since late activation was achieved in the studies where amiloride rather than Na⁺ removal was used, Na⁺ removal-induced changes in Ca²⁺ are not required for the effect. This result is further confirmed by the studies with removal of extracellular Ca²⁺ where the sustained increase in Ca²⁺ in response to Na⁺ removal was prevented and once again a high late pH was obtained. In the studies where weak acid addition (Na propionate) was used, pH recovered to levels above prepulse levels, suggesting that the NH₃/NH₂⁺-induced alkalinization with its secondary Ca²⁺ decrease is not required. This conclusion agrees with the results of Nakhoul et al. (1988), who also found a late increase in pH with cell acidification induced by Na⁺ removal, where once again there was no cell alkalinization.

Thus, the late activation of the H⁺ pump and increase in steady-state pH correlates best with cell acidification. The fact that this stimulation is inhibited by dimethyl-BAPTA suggests that the increase in Ca²⁺ induced by cell acidification is key. These results are reminiscent of the results of Gluck, Cannon, and Al-Awqati (1982), Cannon, van Adelsberg, Kelly, and Al-Awqati (1985), and van Adelsberg and Al-Awqati (1986) in the turtle urinary bladder, and of Schwartz and Al-Awqati (1985) in the proximal and collecting tubules of the kidney, where sudden decreases in pH induced by CO₂ or butyric acid addition led to exocytotic insertion of apical membrane, presumably containing H⁺ pumps. In the studies of Cannon et al. (1985) it was demonstrated that the acid load was associated with an increase in Ca²⁺ and that blocking the Ca²⁺ increase with MAPTAM blocked the exocytosis.

In addition, we found that the microfilament-disrupting agent, cytochalasin B, caused a marked delay in the late activation of the H⁺ pump. The microtubule-disrupting agent, colchicine, inhibited H⁺ pump activity at all time points, but had no effect on the late increase in pH above prepulse levels. These studies suggest that microfilaments more than microtubules participate in the late activation of the H⁺ pump. Stetson and Steinmetz (1983) also found that cytochalasin B, and to a lesser extent colchicine, markedly inhibited the CO₂-induced increase in turtle bladder H⁺ secretion, a process believed to be mediated by the fusion of H⁺ pump containing subapical vesicles with the luminal (apical) membrane (Gluck et al., 1982; Stetson and Steinmetz, 1983; Cannon et al., 1985; van Adelsberg and Al-Awqati, 1986). Thus, late activation of the H⁺ pump occurs in response to cell acidification and a secondary spike increase in Ca²⁺, and is dependent upon microfilament function. These results, along with those of Al-Awqati and co-workers (Gluck et al., 1982; Cannon et al., 1985; Schwartz and Al-Awqati, 1985; van Adelsberg and Al-Awqati, 1986), suggests a role for exocytotic insertion of H⁺ pumps.

**Effect of Na⁺ and pH on Ca²⁺**

The spike increase in Ca²⁺ that occurs upon removal of peritubular Na⁺ appears to be secondary to release of Ca²⁺ from intracellular stores. These results are found whether Na⁺ is replaced with choline or with N-methyl-D-glucosamine. Smith et al.
(1989) found that removal of extracellular Na⁺ from human skin fibroblasts, smooth muscle cells, and canine endothelial cells triggers inositol polyphosphate production, resulting in a large increase in Ca²⁺ release from intracellular stores. The sustained increase in Ca²⁺ seen in our studies may be due to inhibition of Ca²⁺ efflux on the Na⁺/Ca²⁺ exchanger or activation of a plasma membrane Ca²⁺ channel. Recent evidence suggests the presence of basolateral membrane Na⁺/Ca²⁺ exchange in a number of mammalian renal tubular segments (Gmaj, Murer, and Kinne, 1979; Friedman et al., 1981; Snowdowne and Borle, 1985; Talor and Arruda, 1985; Bourdeau and Lau, 1990).

In turtle urinary bladder (von Adelsberg and Al-Awqati, 1986) and in our experiments, Ca²⁺ increased with cell acidification and decreased with cell alkalinization. This is in contrast to vascular smooth muscle (Siskind, McCoy, Chobanian, and Schwartz, 1989), embryonic ventricular muscle (Kim and Smith, 1987), and lymphocytes (Grinstein and Goetz, 1985), where cytoplasmic alkalinization has been shown to increase Ca²⁺. In squid axons (Mullins and Requena, 1979) an alkaline pH, induced by periaxonal 10 mM NH₄Cl lowers both the resting level of Ca²⁺ and the increase in Ca²⁺ seen upon axonal stimulation. This effect has been proposed to be secondary to the enhanced buffering of Ca²⁺ seen with alkaline pH (Mullins and Requena, 1986). The fact that Ca²⁺ increased during an acid load in the OMCD, even in the absence of extracellular Ca²⁺ suggests that Ca²⁺ is released from an intracellular source.

**Physiologic Implications**

An important question in all cells, and especially in epithelia that transport H⁺ ions, is, what is the relative role of the H⁺ pump and the Na⁺/H⁺ antiporter? Numerous studies have been performed in which this question has been addressed by examining the effect of complete Na⁺ removal on the physiologic process, either pH, defense or transepithelial H⁺ secretion. Implicit in these studies is the assumption that Na⁺ removal has no effect on the H⁺ pump. The present studies demonstrate that in the OMCD, Na⁺ removal causes an initial inactivation of the H⁺ pump. In addition, our results and those of Al-Awqati and co-workers (Gluck et al., 1982; Cannon et al., 1985; Schwartz and Al-Awqati, 1985; van Adelsberg and Al-Awqati, 1986) and Nakhoul et al. (1988) suggest that inhibition of the Na⁺/H⁺ antiporter by drugs such as amiloride or Na⁺ removal, maneuvers that acidify the cell, may lead to eventual increases in the activity of the H⁺ pump. Removal of CO₂/HCO₃⁻ from all the solutions, to decrease any contribution of Cl⁻/HCO₃⁻ exchange to the results, may also affect the H⁺ pump. Although studies performed in the presence of CO₂/HCO₃⁻ did not prevent the initial inactivation of the H⁺ pump induced by Na⁺ removal, the presence of CO₂/HCO₃⁻ enhanced the late Na⁺-independent pH recovery process. In similar studies, Chen and Boron (1990) found that Na⁺-independent pH recovery from an acid load was stimulated by the presence of basolateral CO₂/HCO₃⁻ in the S₅ segment of the proximal tubule. Once again, a maneuver designed to isolate out H⁺ pump activity may, in and of itself, lead to inhibition of the H⁺ pump. Thus, these types of studies must be interpreted with great caution.

The present studies also demonstrate that sustained increases in Ca²⁺ can inhibit the apical membrane H⁺ pump. This may serve as a physiologic regulator of H⁺
pump activity. Future studies are required to examine the role of sustained Ca²⁺ changes in physiologic regulation. Finally, our studies demonstrate that rapid cell acidification associated with a spike increase in Ca²⁺ leads to a delayed activation of the H⁺ pump, which may be mediated by exocytotic insertion of H⁺ pumps.

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