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Cell pH and H^+ Secretion by S3 Segment of Mammalian Kidney: Role of H^+-ATPase and Cl^-

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Abstract. The role of H^+-ATPase in proximal tubule cell pH regulation was studied by microperfusion techniques and by confocal microscopy. In a first series of experiments, proximal S3 segments of rabbit kidney were perfused "in vitro" while their cell pH was measured by fluorescence microscopy after loading with BCECF. In Na^+- and Cl^--free medium, cell pH fell by a mean of 0.37 ± 0.051 pH units, but after a few minutes started to rise again slowly. This rise was of 0.17 ± 0.022 pH units per min, and was significantly reduced by bafilomycin and by the Cl^- channel blocker NPPB, but not by DIDS. In a second series of experiments, subcellular vesicles of proximal tubule cells of S3 segments of mouse kidney were studied by confocal microscopy after visualization by acridine orange or by Lucifer yellow. After superfusion with low Na^+ solution, which is expected to cause cell acidification, vesicles originally disposed in the basolateral and perinuclear cell areas, moved toward the apical area, as detected by changes in fluorescence density measured by the NIH Image program. The variation of apical to basolateral fluorescence ratios during superfusion with NaCl Ringer with time was 0.0018 ± 0.0021 min^{-1}, not significantly different from zero ($P > 0.42$). For superfusion with Na^+0 Ringer, this variation was 0.081 ± 0.015 min^{-1}, $P < 0.001$ against 0. These slopes were markedly reduced by the Cl^- channel blocker NPPB, and by vanadate at a concentration that has been shown to disrupt cytoskeleton function. These data show that the delayed alkalinization of proximal tubule cells in Na^+-free medium is probably due to a vacuolar H^+-ATPase, whose activity is stimulated in the presence of Cl^-, and dependent on apical insertion of subcellular vesicles. The movement of these vesicles is also dependent on Cl^- and on the integrity of the cytoskeleton.

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Key words: H^+-ATPase — Subcellular vesicles — Bafilomycin — Exocytosis — Cell pH regulation

Introduction

Among the mechanisms responsible for regulation of cell pH in proximal tubule, the most important is H^+-ion extrusion by Na^+/H^+ exchange [4]. A NHE-3 isoform of this exchanger is responsible for secretion of these ions into the tubule lumen [24, 32]. However, a number of additional H^+-ion and HCO_3^- transporters are present in the proximal tubule cell, contributing to regulate cell pH and H^+ secretion at the apical and basolateral membrane [26, 27]. It has been shown that in Na^+-free medium Cl^-/OH^- or Cl^-/HCO_3^- exchange may contribute in an important manner to cell pH regulation in rabbit proximal S2 segments [3, 27]. These exchangers are thought to be responsible for cell acidification observed when Cl^- is added to cells preincubated in NaCl-free medium.

The presence of a sodium independent mechanism of H^+ extrusion has been demonstrated in proximal tubule cells and attributed to a vacuolar H^+-ATPase [43]. This mechanism was postulated based on the inhibition of proximal bicarbonate reabsorption by bafilomycin, a specific inhibitor of this transporter [38]. It was also shown that this ATPase participates in the regulation of proximal tubule cell pH, where it is stimulated by the presence of CO_2/HCO_3^- in the medium [12, 19]. A number of studies have shown that this ATPase depends on a parallel chloride shunt, particularly in intracellular vesicles [23, 25], but also in isolated "in vitro" renal tubules [40] and in cultured renal cells [18]. Both transepithelial PD and bicarbonate reabsorption have been shown to depend on this shunt in cortical distal tubule, a segment where H^+-ATPase is an important means of luminal acidification [17, 41].

The role of vesicle trafficking and exocytosis in the

regulation of cellular and particularly epithelial H^+ transport has been recently documented [5, 13]. The V-type H^+-ATPase has been shown to be responsible for the acidification of intracellular organelles in several tissues; the incorporation, by exocytosis, of subapical vesicles into the apical membrane has been considered to be an important mechanism for the regulation of apical H^+ ion secretion in turtle bladder and in cortical collecting duct [21, 35].

In the present paper, we investigate several mechanisms of regulation of proximal tubule cell H^+ extrusion in isolated rabbit and mouse S3 segments, and the participation of chloride channels and subcellular vesicle movement in this process by confocal microscopy.

Materials and Methods

"In Vitro" Microperfusion of Proximal Tubule

Female white New Zealand rabbits were anesthetized by pentobarbital, their kidneys removed and about 1 mm thick slices were transferred to ice-cold normal Ringer solution, and proximal tubule S3 segments were isolated by microdissection. They were perfused "in vitro" using a modification of the methods originally described by Burg et al. [8, 30]. Briefly, segments were transferred to a thermostatically regulated acrylic perfusion chamber, and then cannulated with microperfusion pipettes on the stage of a Nikon Diaphot TMD inverted microscope. Solutions were changed by means of pneumatic valves; pressure was applied to perfusion solutions by means of gas cylinders, allowing for bath and luminal perfusion changes in a few seconds. The solutions used in the present experiments are given in Table 1. Their pH was adjusted to 7.4 at 37°C after bubbling the solutions with O_2 for 20 min in order to eliminate dissolved CO_2 (buffering was by Hepes buffer) and their osmolality to 300 ± 2 mOsm. The luminal solutions were filtered with 0.45 μm pore diameter Millipore filters before use.

Measurement of Cell pH

Tubules were loaded for 10 min with 10 μM BCECF-AM (Molecular Probes, Eugene, OR) in the bath. Images were acquired with an intensified CCD camera (Videoscope, Washington, DC) during the experiment, as described previously [19, 20]. The dye-loaded tubule cells were alternately excited at 440 and 490 nm, and their emission was monitored by the camera at 530 nm every 4 sec. The fluorescence excitation ratio, I_{490}/I_{440}, was displayed in pseudo-color on the computer monitor, and 5 to 8 cell areas per tubule (each containing approximately 2 to 3 cells) were defined for measurement. At the end of the perfusion experiments pH was standardized by the nigericin method, substituting the bath at 37°C with buffer solutions containing (in mM): 20 NaCl, 100 KCl, 10 K_2HPO_4 and 25 Hepes acid plus 10 μM nigericin (Molecular Probes, Eugene, OR) adjusted to pH 6.5, 7 and 7.5. In several instances, when standardization was not possible, a mean standard curve from previous experiments was used. Calculations and drawings were performed by the Excel program after import of the data obtained by the data acquisition program.

Preparation of Tubules for Confocal Microscopy

Straight proximal tubule S3 segments were microdissected in ice-cold Ringer solution from mouse kidney (Swiss) and transferred to a perfusion chamber (RC21B, Warner Instrument, Hamden, CT) with a disposable bottom made from a number 1 coverglass. This chamber was designed to maintain laminar flow, keeping movement of the tissue to a minimum, which is essential for the observations. The tubules were fixed to the disposable bottom slide by Cell-Tak (Becton Dickinson, Bedford, MA), allowing the tubules to be superfused without dislocation. 1 μl Cell-Tak solution was spread out in the central area of a 22 × 40 mm cover glass, and left to dry before use. The tubules were in a collapsed state, without luminal perfusion. They were superfused at room temperature (22 to 24°C) in order to be able to observe vesicle movement at a slower speed than that observed at 37°C. The solutions used for superfusion are given in Table 1. Perfusion was performed by gravity. Hepes buffered solutions were adjusted to pH 7.4 at room temperature.

Table 1. Solutions used for perfusion and superfusion experiments (mM)

Solution	1	2	3
NaCl	140	—	—
TMA Cl	—	140	—
TMA gluconate	—	—	140
K_2HPO_4	2.5	2.5	2.5
$CaCl_2$	1	1	—
Ca gluconate	—	—	3.5
$MgCl_2$	1	1	—
Mg gluconate	—	—	1
Glucose	5	5	5
Alanine	5	5	5
Hepes acid	5	5	5

TMA: tetramethyl ammonium.

Confocal Microscopy

The perfusion chamber was adapted to a Zeiss LSM confocal microscope. Subcellular vesicles were visualized by loading the tubules from the bath side with 10 μM acridine orange (AO) in sodium Ringer solution for a few minutes, or by lucifer yellow, given in drinking water (0.5 mM) for 24 to 48 hr before the experiments. The preparation was illuminated by means of an Argon laser at 488 nm, and the emission of fluorescence measured between 515 and 565 nm. A series of 15 images taken at variable intervals (10 sec to 1 min) was started while acid extrusion by the cells was stimulated by acidification of cell pH via superfusion with 0 Na^+ Ringer solutions, which blocks the function of the Na^+/H^+ exchanger. The images were stored on the hard disk of the system and then on CD for later analysis. They were analyzed by means of the NIH Image program on a Power MacIntosh computer. The movement of vesicles was followed by measuring fluorescence density in delimited areas on the basolateral and apical areas of the studied cells. The ratio of apical to basolateral fluorescence was followed with time; data were transferred to the Excel program for processing and drawing. A bleaching control was performed in several tubules following these ratios during superfusion with NaCl Ringer without stimulation of H^+ extrusion.

Most chemicals, including 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), acridine orange and lucifer yellow, were from Sigma, St. Louis, MO. 5-nitro-2-(3-phenylpropyl- amino) -benzoic acid (NPPB) was obtained from Dr. R. Greger, Univ. of Freiburg, Germany. Sodium vanadate (Na_3VO_4) was obtained from Dr. Clifford Slayman, Yale Univ. Statistical comparisons were performed by the Prism software (Graphpad, San Diego, CA) using Student's *t* test when two groups were compared or one group compared to zero, or by analysis of variance when more than two groups were compared, fol-

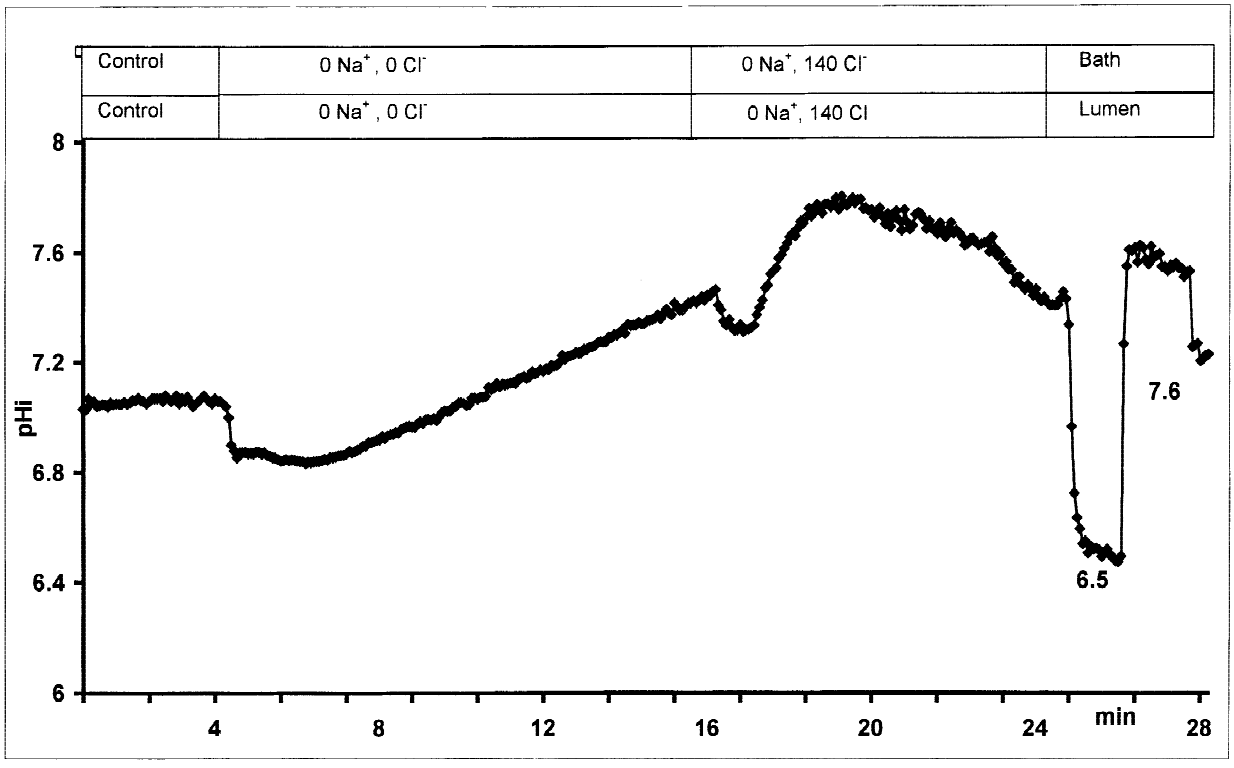

Fig. 1. Proximal tubule S3 segment cell pH (pH_i) during luminal and bath perfusion with $0Na^+/0Cl^-$ Ringer solution (sol. 3), followed by readdition of Cl^- to both solutions (sol. 2). A standardization curve is shown at the end of the experiment.

lowed by the Bonferroni multiple comparisons test. When standard deviations of groups were significantly different by Bartlett's test, data were compared by the Kruskal-Wallis nonparametric analysis of variance followed by Dunn's multiple comparisons test.

Results

Control cell pH during perfusion of rabbit kidney proximal S3 segments with sodium Ringer solution (solution 1, Table 1) was 7.11 ± 0.062 (n = 22 cells/9 tubules). Figure 1 shows the record of the time course of the pH of a group of cells during an experiment in which the tubule is initially perfused with Na^+ Ringers (solution 1), then, with 0 Na^+/0 Cl^- Ringers (solution 3) and finally with 0 Na^+ + Cl^- Ringer (solution 2). It is shown that after introduction of the 0 Na^+/0 Cl^- solution cell pH is reduced, but after a few minutes rises progressively, reaching a higher level than during the control period. This behavior has been shown before, and evidence was presented suggesting that it was due to the activation of an H^+-ATPase [12, 19]. After reintroduction of Cl^- in both lumen and bath, a biphasic response is shown, first with a fall in cell pH and then with a marked increase in this pH. Figure 2 gives cell pH in an experiment where first luminal Cl^- is reintroduced, with a consequent rise in cell pH, and then bath Cl^- is returned to normal, which causes a reduction of pH; this reduction may be due to basolateral Cl^-/OH^- or Cl^-/HCO_3^- exchange, an increase in basolateral Cl^- causing base extrusion and thus cell acidification. It is clear that the process occurring across the apical membrane is the opposite, there being thus no evidence for anion exchange at this site under the described experimental conditions. Figure 3 shows that the presence of 2×10^{-4} M DIDS in lumen does not eliminate the Na^+ independent pH recovery, and also not the alkalinizing effect of lumen Cl^-.

Figure 4 illustrates pH changes when the tubule is preincubated with 10^{-5} M NPPB, which has been described to block Cl^- channels [42], in the bath; it is shown that Na^+ independent pH recovery is reduced, and the effect of lumen Cl^- is reduced as compared to controls.

Figure 5 gives mean values of pH changes upon substitution of solutions performed in the experiments described above. These changes were measured 2 to 3 minutes after a substitution of perfusion solutions was performed. When normal Ringer solution (S 1) was substituted by 0 Na^+/0 Cl^- solution (S 3), cell pH decreased by 0.37 ± 0.051 (n = 32 cells/9 tubules) pH units. When S 3 solution was substituted by normal Ringer in the bath, cell pH also decreased (−0.57 ± 0.079 (17/6)). With normal Cl^- only in the lumen, pH increased by

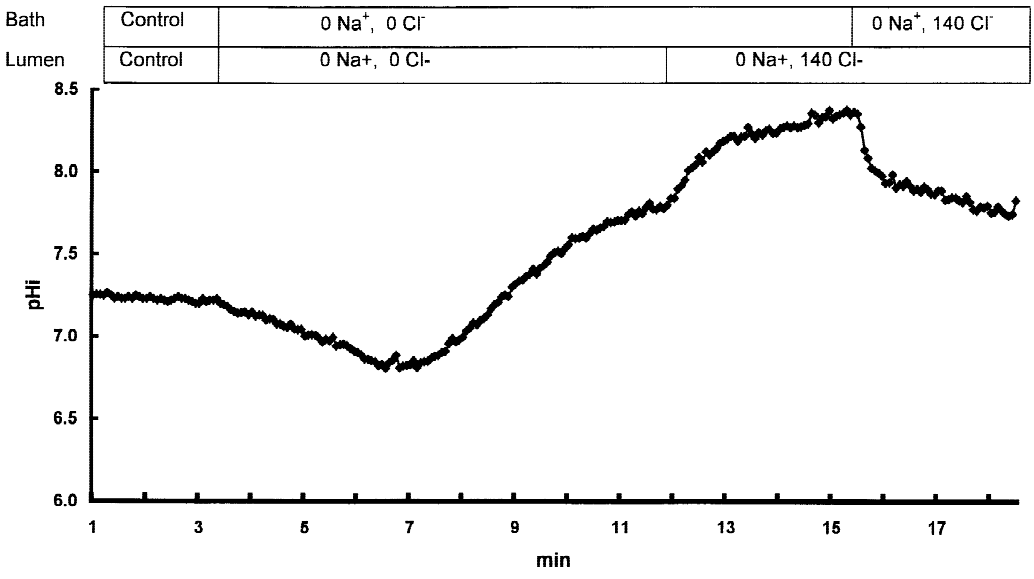

Fig. 2. Lumen and bath perfusion with low Na^+/Cl^- Ringer, followed by readdition of Cl^- in lumen and then in bath. *See* text for explanation.

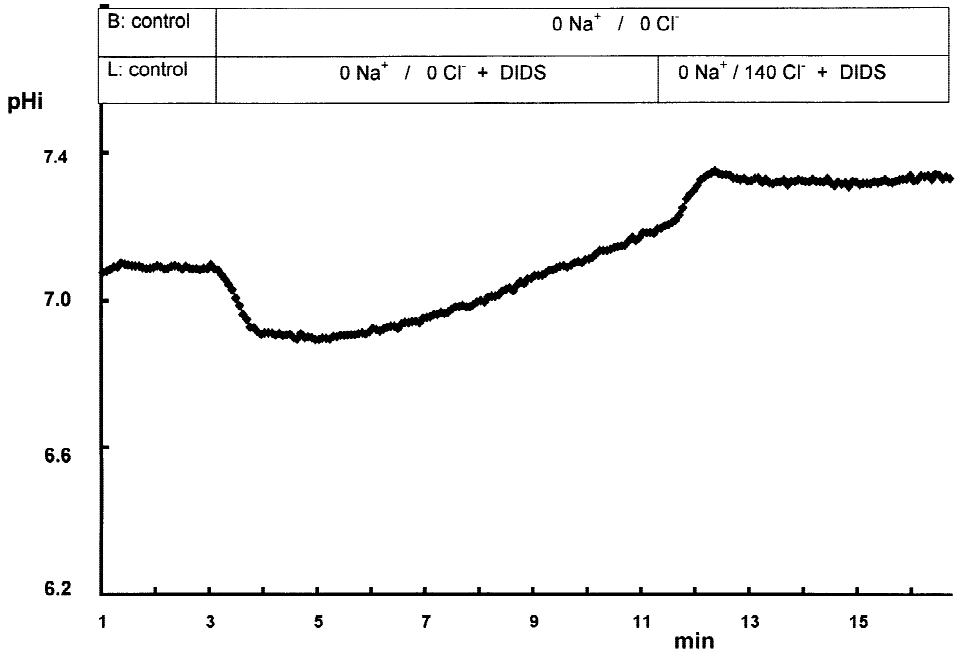

Fig. 3. S3 segment perfusion with 2×10^{-4} M DIDS in lumen and bath. First, low Na^+/Cl^- Ringer plus DIDS was perfused in both bath and lumen. Then, DIDS with Cl^- was added to lumen perfusion.

0.74 ± 0.091 (32/8), which was reduced (without statistical significance, $P > 0.05$) to 0.59 ± 0.15 (6/2) with 0.2 mM DIDS in the lumen, an inhibitor of anion exchange; it was significantly ($P < 0.01$) reduced to −0.008 ± 0.022 (9/2) with 1 μM bafilomycin in the lumen, a specific blocker of the vacuolar H^+-ATPase, and to 0.15 ± 0.040 (25/5) ($P < 0.01$) after preincubation with 10 μM NPPB. Figure 6 gives the rate of change of cell pH (ΔpH/min) during perfusion with several solutions. After perfusion with 0 Na^+/0 Cl^-, cell pH fell to a more acid value, and then started to rise slowly; the rate of rise was measured during a period of 4 min after the rate of pH increase stabilized; this rate was 0.17 ± 0.022 (35/9) pH units per min. It was reduced, but not significantly ($P > 0.05$) with 0.2 mM DIDS in the lumen to 0.095 ± 0.020 (8/2), significantly ($P < 0.01$) reduced with 1 μM luminal bafilo-

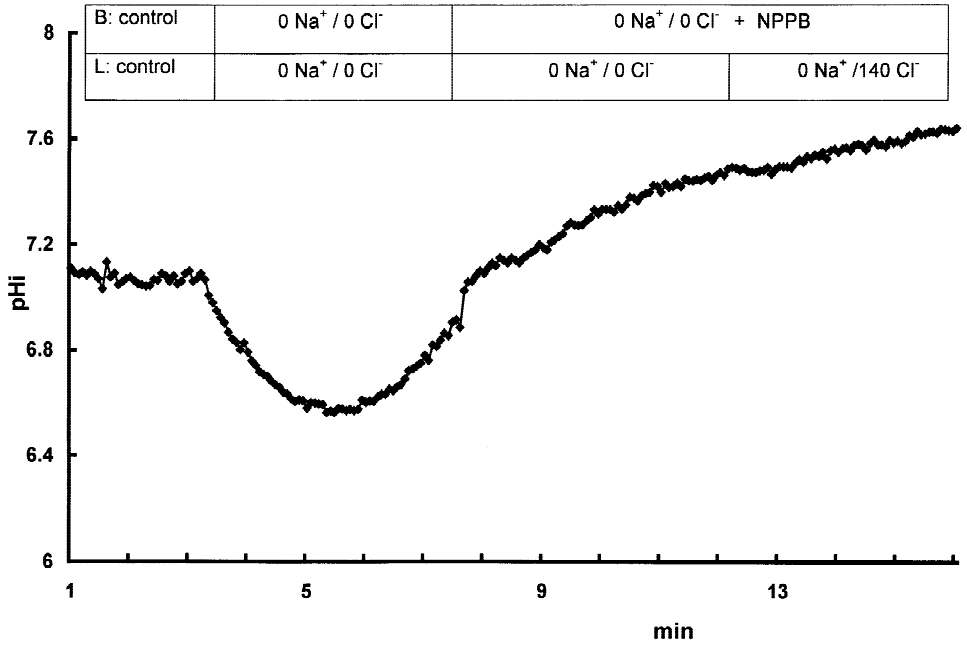

Fig. 4. S3 segment perfusion after incubation (min 8 to 12) with 10 μM NPPB in the bath.

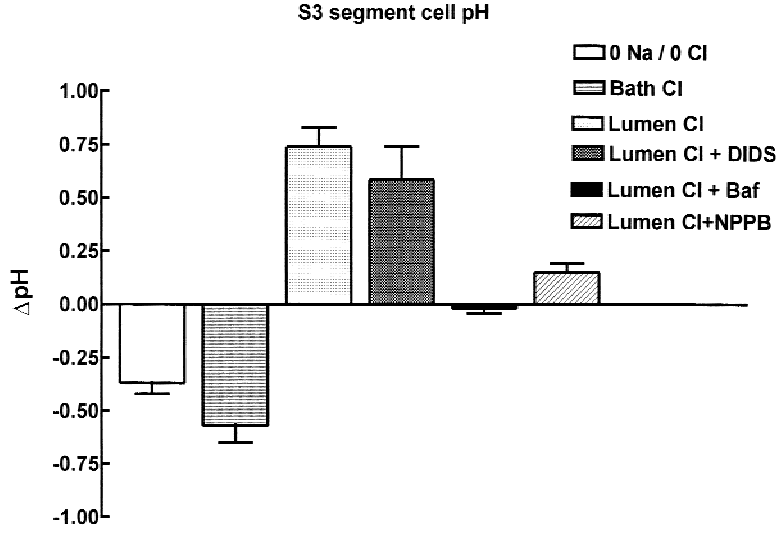

Fig. 5. Summary of maximal pH changes between control vs 0 Na^+/0 Cl^-, change of 0 to normal Cl^- in bath, and of 0 to normal Cl^- in lumen, in the absence and presence of 0.2 mM DIDS in lumen, of 1 μM bafilomycin in lumen or of preincubation with 10 μM NPPB in bath. Means ± SE.

mycin, to 0.078 ± 0.013 (13/2) pH units/min, and to −0.011 ± 0.008 (8/2) ($P < 0.001$) after preincubation for 5 min with 1–10 μM sodium vanadate. At this concentration, vanadate disrupts the function of the cytoskeleton and thereby impairs vesicle movement, without direct effect on H^+-ATPase [28]. After preincubation with 10 μM NPPB, a blocker of Cl^- channels, which are essential for the function of H^+-ATPase, this rate fell to 0.032 ± 0.009 (26/5).

In the following, experiments using the technique of confocal microscopy to study the role of vesicle traffiking in the cellular acid extrusion process will be discussed. Figure 7 gives several sequential images of an S3 proximal segment which was loaded with acridine orange and then acidified by superfusion with solution 2 (0 Na^+). It is noted that at the start of the experiment, the largest concentration of vesicles is around the nucleus and at the basolateral side of the cells. As acidification of the cell proceeds, the density of vesicles at the apical pole of the cells increases, suggesting their transfer toward this pole. This process was quantified by determining the fluorescence density in apical and basolateral areas of different cells from the tubule images. Figure 8 shows the time course of Fap/Fbl (apical over basolateral

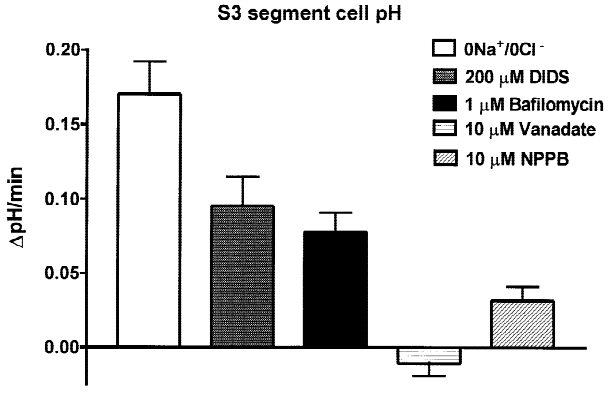

Fig. 6. Rate of cell pH alkalinization per min. during bath and lumen perfusion with $0Na^+/0Cl^-$, with 0.2 mM DIDS in lumen, 1 μM bafilomycin in lumen or after preincubation with 10 μM vanadate or NPPB in bath. Means ± SE.

fluorescence ratios) of 3 cells of the experiment shown in Figure 7. It is noted that these ratios increase markedly, especially after the first 10 min of stimulation. This does not occur when these tubules are superfused with NaCl Ringer solution (S 1), as shown in Fig. 9, where the Fap/Fbl ratios show only minor change with time. Mean slopes (changes of Fap/Fbl with time) of lines such as shown on Figs. 8 and 9, were calculated over a period between 2 min after the start of superfusion, and 15 min of superfusion. For NaCl Ringer superfusion, mean slopes were 0.0018 ± 0.0021 min^{-1} (n = 6 cells/2 tubules), not significantly different from zero ($P > 0.42$). For superfusion with 0 Na^+ Ringer, solution 3, they were 0.081 ± 0.015 min^{-1} (10/5), $P < 0.001$ against 0.

At the start of a minority of experiments there was only a group of vesicles present at the luminal border, which lost part of their fluorescence during the remainder of the experiment. Figure 10 shows such a behavior in a cell loaded with acridine orange and acidified by Na^+ 0 Ringer superfusion. The graphical representation of this situation is given in Fig. 11, in which the reduction of apical fluorescence is clearly apparent.

A group of tubules was superfused with Ringer solutions containing 10 μM vanadate, an agent known to impair vesicle movement. When cell acidification was induced by superfusion with 0 Na^+ and hepes-buffered solutions (sol. 3), the rate of change of Fap/Fbl ratios was 0.0035 ± 0.0013 (7/2) min^{-1}. When 10 μM NPPB, a blocker of Cl^- channels, was added to the superfusion solutions, rates of change of Fap/Fbl ratios during 0 Na^+ Ringer acidification were 0.0077 ± 0.0065 (6/3) min^{-1}. Fig. 12 compares these rates to those found during NaCl and 0 Na^+ Ringer superfusion, and it is shown that both vanadate and NPPB reduce them significantly as compared to 0 Na^+ control by nonparametric Anova ($P < 0.01$).

Discussion

Proximal tubule segments have significant H^+-ATPase activity, which has been observed during "in vivo" microperfusion studies in rat and rabbit kidney [12, 19, 38]. In these studies, it was observed that a sodium independent transport mechanism was present that was inhibited by n-ethyl maleimide or by bafilomycin, which are known to impair the function of vacuolar H^+-ATPase. Several studies have also detected this transporter in isolated perfused tubules or cultured proximal tubule cells, including those from S1, S2 and S3 segments [2, 7, 22]. In addition, a number of studies has demonstrated the participation of subcellular vesicle exocytosis in the stimulation of H^+ ion transport mediated by H^+-ATPase. Stimulation of acid secretion thought to be due to apical membrane insertion of these pumps by a membrane fusion process has been advocated in turtle bladder [9] and in cortical collecting duct [36, 39].

In the present work, we have confirmed the presence of a sodium independent transport mechanism in proximal S3 segments of rabbit kidney leading to a delayed cellular alkalinization after incubation in a sodium-free medium. Kurtz et al [27] have described a sodium independent mechanism which they showed to be chloride dependent and obtained evidence for cellular alkalinization by Cl^-/OH^- exchange in rabbit proximal superficial S2 segment. In our experiments, the addition of chloride to the bath fluid led to cell acidification, as expected from an anion exchange mechanism. When Na^+ and Cl^- were removed from the bath and lumen, a progressive slow cell alkalinization occurred, which was not eliminated by DIDS (Fig. 3). This agent caused a delay in the rate of the described alkalinization, but without reaching statistical significance (Fig. 5). This observation suggests that the alkalinization, although it might present a participation of Cl^-/OH^- exchange, was not dependent on anion exchange in a major way. In addition, when added to lumen after preincubation in 0 Na^+-0 Cl^- solution, Cl^- ions induced an accelleration of cell alkalinization, which is in the opposite direction as expected by the above described anion exchange mechanism, which would respond with cell acidification upon increase in external Cl^-. The addition of Cl^- might be related to the interaction of a Cl^- current with H^+-ATPase activity. There is evidence that H^+-ATPases of the vacuolar type are electrogenic, and that they need a parallel Cl^- current to shunt the generated potential [1, 37]. An additional, non electrical coupling of H^+-ATPase and Cl^- has also been proposed, based on studies in renal medullary microsomes, in which the stimulatory role of Cl^- was observed even in the presence of K^+/valinomycin shunting vesicle PD; this coupling was denoted *electrogenic symport* [25]. Evidence for H^+-ATPase and Cl^- interaction has also been found during "in vivo" microperfusion studies in cortical distal tubule of the rat, in which a

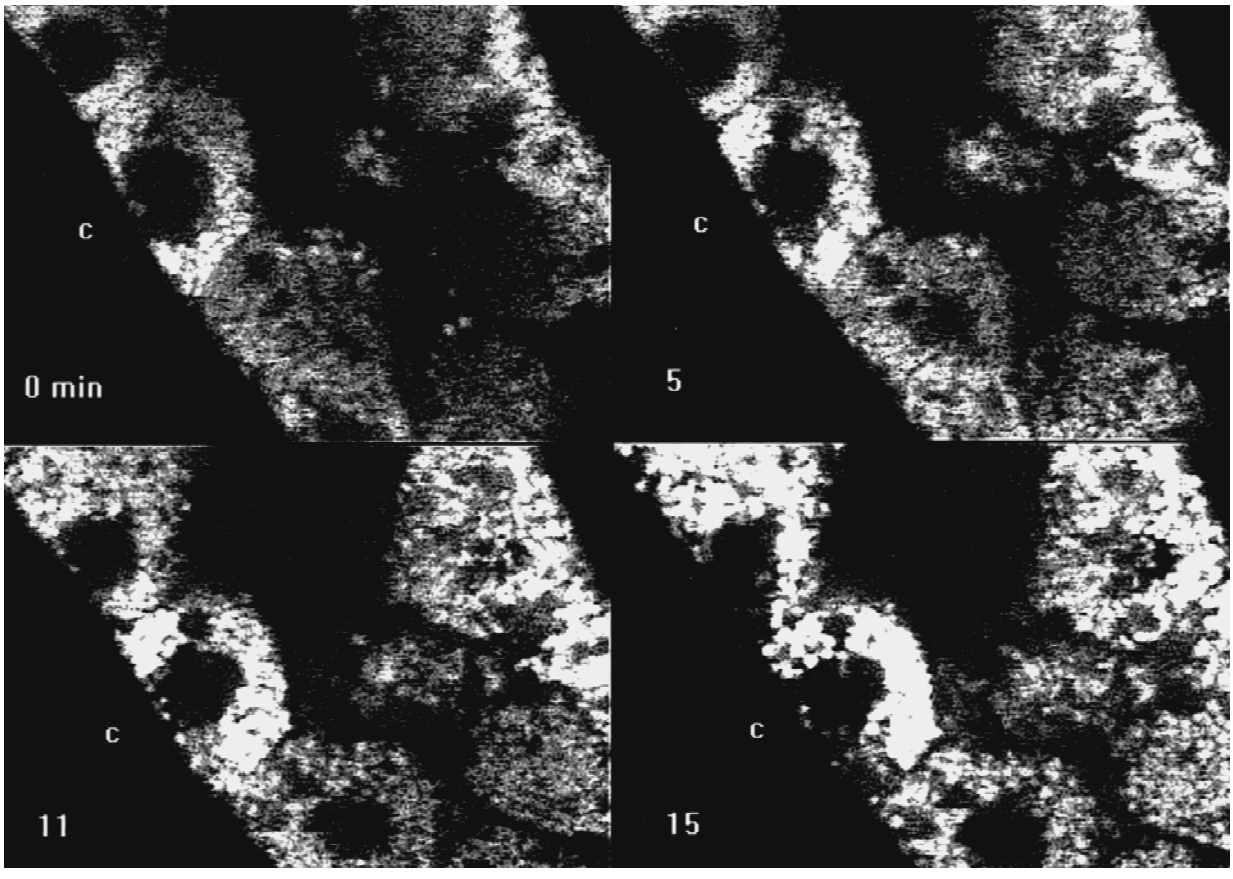

Fig. 7. Confocal microscopy images of mouse S3 segment cells preincubated with acridine orange and superfused with 0 Na^+ Ringer (sol. 2). Sequence of images is 2, 6, 11 and 15 min after start of superfusion. Note movement of vesicles of cell c.

chloride channel blocker, NPPB, was found to increase the negativity of transepithelial lumen PD and to inhibit luminal acidification [16]. In addition, in isolated rat proximal tubules both low Cl^- medium and NPPB impaired H^+-ATPase-dependent cell pH recovery [40]. In the present studies, we found that NPPB impaired both the recovery of cell pH in the absence of Na^+ and cell alkalinization due to luminal perfusion with Cl^-, suggesting that both these processes may be related to H^+ ATPase activity. The action of NPPB during luminal and basolateral perfusion with 0 Cl^- solutions is more difficult to explain. However, intracellular Cl^- may not be reduced to zero during the perfusions, and could possibly be responsible for a shunting current even under these conditions [11].

It has been shown that vanadate, besides being an inhibitor of p-ATPases such as the Na^+/K^+-ATPase, impairs subcellular vesicle movement and exocytosis probably by affecting cytoskeleton proteins [15, 28], although it may, albeit at more elevated concentrations than used in the present studies, also impair the activity of H^+-ATPase by an ADP-dependent mechanism [14]. In our experiments on isolated perfused rabbit proximal S3 segments, the rate of recovery of cell pH in Na^+-free medium was almost abolished in the presence of 10 μM vanadate; this finding suggests that the activation of H^+-ATPase observed in our experiments may be dependent on subcellular vesicle insertion into the apical membrane.

The possibility of subcellular vesicle insertion into apical membrane was followed up by an investigation using confocal microscopy to visualize these organelles and follow their movement. These studies were performed on collapsed proximal S3 segments of the mouse kidney, which allows for incubation of this tissue with different solutions acting from the basolateral side. Two vesicle markers were used: acridine orange, a diffusive fluorescent substance that is concentrated in acid organelles, with fluorescence proportional to H^+ ion concentration, that can be added to the medium and is taken up rapidly by the tissues. And lucifer yellow, a fluorescent substance of larger molecular weight (457 D), which is given by mouth and taken up by endocytosis. Since the time course of Fap/Fbl change obtained by the use of these markers was not different, these results were pooled.

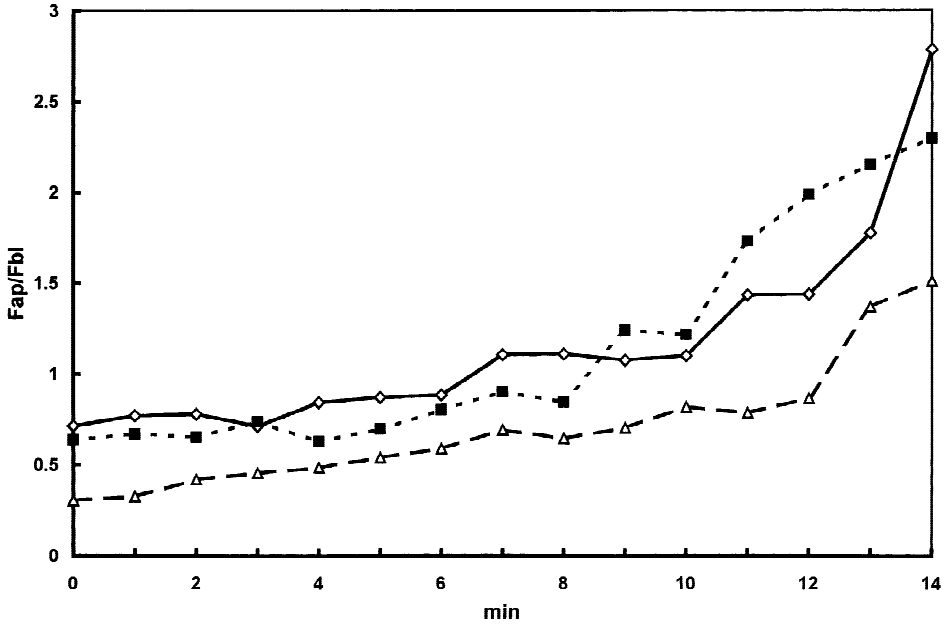

Fig. 8. Time course of apical to basolateral fluorescence density ratio (Fap/Fbl) in three cells of mouse S3 segment loaded with acridine orange and superfused with 0 Na^+ Ringer.

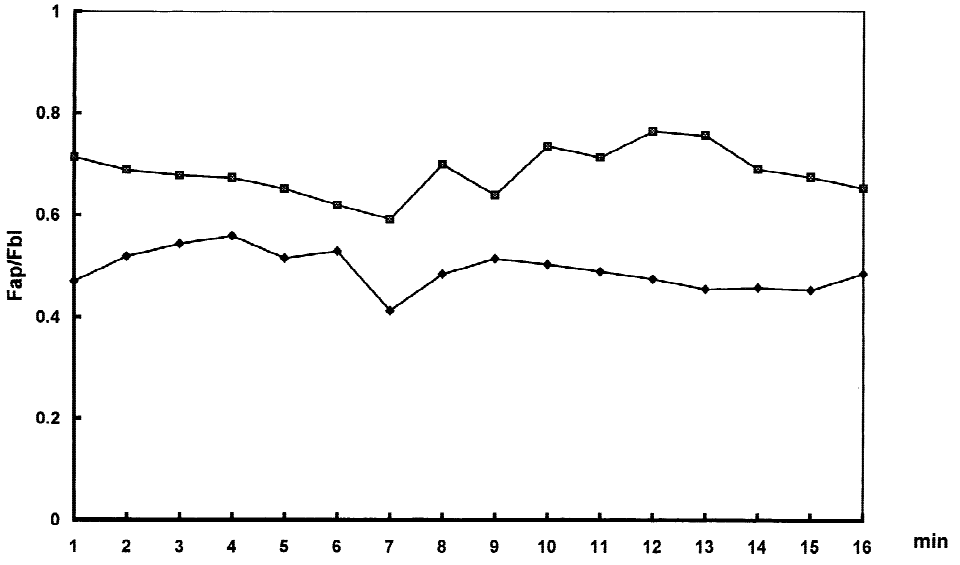

Fig. 9. Time course of Fap/Fbl in S3 cells superfused with NaCl Ringer (sol. 1).

Evaluation of the movement of vesicles was made by analysis of tubule images, measuring fluorescence density in an apical as compared to a basolateral area and following the ratio of these densities with time. In a number of experiments, these ratios increased with time, which is exemplified in Figs. 7 and 8. The time course of these ratios is thought to represent the transfer of vesicles from basolateral to apical sectors of the cell, but could also be due to the disappearance of perinuclear vesicles and appearance of new apical ones. In other experiments, only apical vesicles were present, and these reduced their fluorescence with time of perfusion. The reduction in apical fluorescence may be due to fusion of apical vesicles with the apical membrane, insertion of their membrane, and emptying of their contents into the lumen, as had been suggested for the studies in turtle bladder and collecting duct [9, 39]. It may also be due, however, to emptying of vesicles into the lumen, without

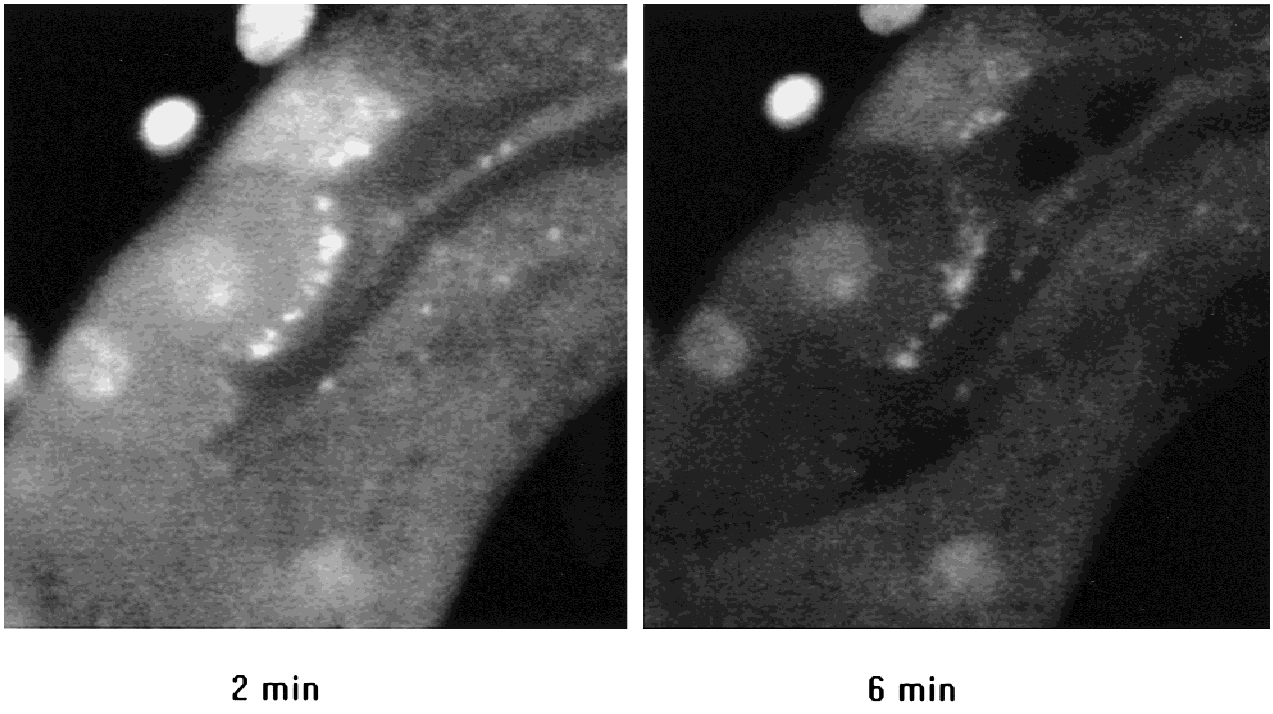

Fig. 10. Confocal microscopy images of S3 segment with cell containing a row of apical vesicles during superfusion with Na^+0 Ringer.

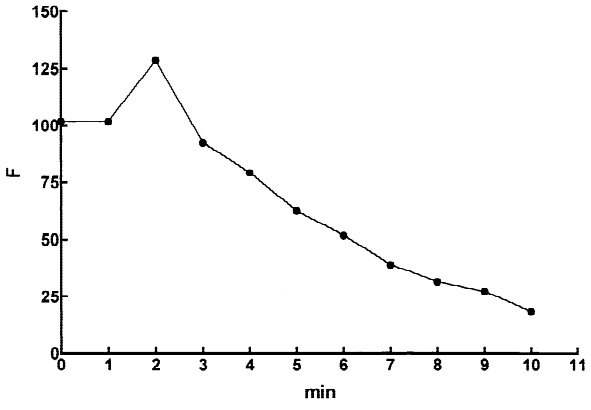

Fig. 11. Time course of apical vesicle fluorescence (image series of Fig. 10) of proximal tubule S3 segment cell.

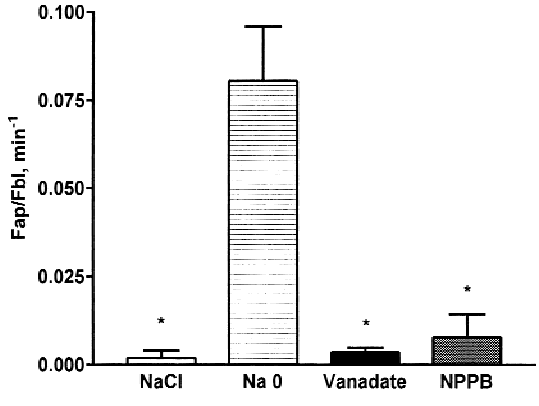

Fig. 12. Rates of change of Fap/Fbl of S3 segment cells superfused with Na Cl and Na^+0 Ringer in the presence and absence of 10 μM vanadate or NPPB. Means ± SE. *, $P < 0.01$.

membrane fusion. We have obtained images supporting also the latter conclusion (*see* Figs. 10 and 11), that is, some of the apical vesicles remain evident until the end of the experiment, losing their fluorescence progressively. Such a behavior has been found in apical vesicles of pancreatic acinar cells, by means of atomic force microscopy, showing that these vesicles dock to specific sites of apical depressions, without losing their identity while emptying their contents into the gland lumen [34]. Our observations suggest that two distinct behaviors of subcellular vesicles may be observed during cell acidification. In most experiments with Na^+ 0 Ringer superfusion as used in the present experiments, there is a transfer of vesicle fluorescence from basolateral or perinuclear sites to apical sites. The two types of behavior observed in these experiments may be a sequence of events, activation of acid secretion by tubule cells having a first phase of transfer of vesicles toward the apical membrane, and a second one involving vesicle fusion and/or emptying at the apical membrane.

Superfusion with 1 to 10 μM vanadate or 10 μM NPPB Ringer solution markedly impaired the progression of vesicles described above. For the case of vana-

date, this observation indicates that normal function of cell cytoskeleton is essential for the handling of acidifying vesicles during activation of cellular H^+ extrusion, since vanadate at this concentration has been shown to affect cytoskeleton protein assembly [15, 28]. The action of vanadate on the function of the cytoskeleton has been recently confirmed [10], and its function as an inhibitor of protein tyrosine phosphatases has been proposed as its mechanism of action, which leads to the disassembly of actin filaments in glomerular podocyte cells using 40 μM vanadate [33]. The present results support our previous observation that colchicine impairs the stimulation of H^+-ATPase by angiotensin II, indicating that the cytoskeleton may be involved in activation of this H^+-ion transporter [40]. On the other hand, the role of Cl^- and NPPB in H^+-ATPase activation has been demonstrated both in subcellular organelles [6, 25, 29] and in renal tubule cell acidification [16, 40]. In the present work, we demonstrate that also vesicle trafficking is significantly affected by chloride channel blocking. It is not known what might be the mechanism of this action of NPPB. However, it has been shown that bafilomycin A1, a specific inhibitor of vacuolar H^+-ATPase, also impairs vesicle exocytosis in neutrophils [31]. Studies from our laboratory have shown that incubation of renal tubules and MDCK cells in Cl^--free medium leads to impairment of the H^+ extrusion activity of H^+-ATPase in a way similar to that observed upon using NPPB [18, 40]. Thus, it appears that the maintenance of activity of the vacuolar H^+-ATPase may be important for exocytotic vesicle movement.

Since the main transporter responsible for acid secretion in renal proximal tubules is the Na^+/H^+ exchanger, it is possible that it may also be activated by the mechanism of vesicle trafficking. The participation of vesicle insertion of Na^+/H^+ exchangers during superfusion with 0 Na^+ Ringer solution is unlikely, since their activity is abolished under these conditions. Although it is known that in most cells subcellular vesicle acidification is mediated by H^+-ATPases, the existence of additional vesicles involved in the regulation of Na^+/H^+ exchange activation in proximal tubule epithelial cells, even in the absence of external Na^+ cannot be ruled out. Additional studies will be necessary to investigate this question.

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