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# $H^+$ Extrusion by an Apical Vacuolar-Type $H^+$-ATPase in Rat Renal Proximal Tubules

Z. Zimolo,* M.H. Montrose,** and H. Murer
Department of Physiology, University of Zürich, CH-8057 Zürich, Switzerland

**Summary.** The activity of $Na^+/H^+$-exchange and $H^+$-ATPase was measured in the absence of $CO_2/HCO_3$ by microfluorometry at the single cell level in rat proximal tubules (superficial $S_1/S_2$ segments) loaded with BCECF [2′7′-bis(carboxyethyl)5-6-carboxyfluorescein-acetoxymethylester]. Intracellular pH ($pH_i$) was lowered by a $NH_4Cl$-prepulse technique. In the absence of $Na^+$ in the superfusion solutions, $pH_i$ recovered from the acid load by a mechanism inhibited by 0.1 $\mu$M bafilomycin $A_1$, a specific inhibitor of a vacuolar-type $H^+$-ATPase. Readdition of $Na^+$ in the presence of bafilomycin $A_1$ produced an immediate recovery of $pH_i$ by a mechanism sensitive to the addition of 10 $\mu$M EIPA (ethylisopropylamiloride), a specific inhibitor of $Na^+/H^+$ exchange. The transport rate of the $H^+$-ATPase is about 40% of $Na^+/H^+$-exchange activity at a similar $pH_i$ (0.218 ± 0.028 *vs.* 0.507 ± 0.056 pH unit/min). Pre-exposure of the tubules to 30 mM fructose, 0.5 mM iodoacetate and 1 mM KCN (to deplete intracellular ATP) prevented a $pH_i$ recovery in $Na^+$-free media; readdition of $Na^+$ led to an immediate $pH_i$ recovery. Tubules pre-exposed to $Cl^-$-free media for 2 hr also reduced the rate of $Na^+$-independent $pH_i$ recovery. In free-flow electrophoretic separations of brush border membranes and basolateral membranes, a bafilomycin $A_1$-sensitive ATPase activity was found to be associated with the brush border membrane fraction; half maximal inhibition is at $6 \times 10^{-10}$ M bafilomycin $A_1$. It is concluded that in superficial rat proximal tubules a $H^+$-ATPase and $Na^+/H^+$ exchange are colocalized in the apical membrane; $H^+$ transport through the $H^+$-ATPase is affected by intracellular ATP and $Cl^-$ content. The transport capacity of apical $H^+$-ATPase is sufficient to explain $Na^+$-independent proximal tubular bicarbonate reabsorption.

**Key Words** bicarbonate reabsorption · $Na^+/H^+$-exchange · bafilomycin $A_1$ · ethylisopropylamiloride (EIPA)

## Introduction

Secretion of $H^+$ is the initial event leading to proximal tubular $HCO_3^-$-reabsorption (for review *see* [1]). $H^+$ secretion is to a large extent mediated by brush border membrane $Na^+/H^+$ exchange. However, in the absence of $Na^+/H^+$-exchange activity, either in the absence of luminal $Na^+$ and/or in the presence of inhibitors of $Na^+/H^+$ exchange, luminal acid secretion (bicarbonate reabsorption) continues, albeit at slower rates (for review *see* Ref. 1; e.g. *see* [7, 17]).

At a cellular level, this luminal acidification has been visualized as an alkalinization of intracellular pH ($pH_i$) which is only partially inhibited by $Na^+$ removal or addition of amiloride (for review *see* [1]; e.g. [14, 17]). At a subcellular level, studies on isolated brush border membrane have provided evidence for two proton extrusion systems: $Na^+/H^+$ exchange and $H^+$-ATPase (for review *see* [1, 6]; for $H^+$-ATPase *see* [13, 20, 23, 26]). In studies on brush border membrane N-ethylmaleimide-sensitive $H^+$-ATPase, it has been difficult to correlate the measured ATP-hydrolysis with functional $H^+$ extrusion, mainly because of the right-side-out orientation of this membrane preparation and inaccessibility of ATP to the intravesicular space. A further complication in the interpretation of these vesicle studies is a possible contamination of brush border membranes by other cellular organelles containing this enzyme-/transport-activity [12, 13, 18–20, 23, 26] (for review *see* [9]). However, recent studies with monoclonal antibodies raised against bovine kidney medulla $H^+$-ATPase have also shown immunolabeling of brush border membranes in rat proximal tubules; obviously, these experiments could not document proton-pump activity [5, 10]. Some authors have suggested that the $H^+$-ATPase is inactive while inserted in the brush border membrane [6].

The goal of this paper was to provide some functional evidence for ATP-dependent $H^+$-extrusion ($H^+$ – ATPase) in the plasma membrane of intact proximal tubular epithelial cells. By the analysis of alterations in cytosolic pH ($pH_i$) we could characterize some of the functional properties of this $H^+$-ATPase and could also obtain an estimate of its pump activity relative to $Na^+/H^+$-exchange activity. Finally, membrane fractionation experiments

* *Present address:* Department of Bone Biology and Osteoporosis Research, Merck, Sharp and Dohme Research Laboratories, WP26A/1010, West Point, PA 19486.

** *Present address:* Department of Medicine, The Johns Hopkins University School of Medicine, Hunterian 515, 725 N. Wolfe Street, Baltimore, MD 21205.

permitted us to attribute $H^+$-ATPase activity to the brush border membrane fraction.

## Materials and Methods

### Preparation of Proximal Tubules and BCECF/AM Loading

A crude preparation of proximal tubules was obtained from 100-g male Wistar rats by cutting a 0.5–1.0 mm superficial slice and chopping it into small pieces (less than 1 mm) with a razor blade; tissue pieces were kept at 4°C in minimum essential medium (MEM; GIBCO), supplemented with 20 mM glucose and 1 mM adenosine but without phenol red. Prior to experiments on intracellular pH, tissue was incubated at 4°C for 30 min in this buffer containing 10 $\mu$M BCECF/AM [2'7'-bis(carboxyethyl)5-6-carboxyfluoresceinacetoxy methylester]. Tissue specimens were then immobilized by absorption on glass coverslips; these coverslips had been precoated with collagen by adding a solution of 2 mg/ml collagen in acetic acid and drying in air. Coverslips were then placed into a microscope chamber [15, 16] and superfused with the above medium containing 10 $\mu$M BCECF/AM. The chamber was subsequently centrifuged at 300 $\times$ $g$ for 1 min at 4°C. This procedure immobilized small tubule fragments onto the collagen-coated coverslip so that the chamber could be perfused without tubule movement. Prior to perfusion with different experimental solutions, loading with BCECF/AM was completed by further incubation for 30 min at room temperature without perfusion. $S_1$ and $S_2$ segments of proximal tubules were identified by attachment of glomerular structures and/or the physical appearance of the large cells in convoluted tubules. Cells from $S_1$ and $S_2$ segments were studied with no quantitative differences in results.

### Fluorescence Measurements

On the stage of an inverted microscope (Zeiss IM 35) proximal tubules were visualized so that the measuring area of a photomultiplier tube was focused on a single cell. The cells selected for study were either in single isolated tubules or were in tubules protruding from a group of several tubules. The microfluorometer was adjusted to monitor BCECF by excitation ratioing 390–440/475–490 nm and collecting emitted light between 515 and 565 nm as described previously [15, 16]; background correction was performed by subtracting the autofluorescence of comparable tubule cells without dye. Solutions used throughout the $pH_i$ measurement/perfusion experiments were nominally $CO_2/HCO_3^-$ free. The standard NaCl solution contained (in mM): 130 NaCl, 4 KCl, 1 $MgSO_4$, 1.7 $CaCl_2$, 1.0 $NaH_2PO_4$, 20 glucose, 20 HEPES adjusted with Tris to a pH of 7.4. Tetramethylammonium-chloride (TMACl) solution contained $TMA^+$ instead of $Na^+$, and $NH_4Cl$ solution contained 20 mM $NH_4^+$ and 110 mM TMA instead of $Na^+$. In experiments without chloride, all chloride salts ($Na^+$, $K^+$, $Ca^{2+}$, $TMA^+$, $NH_4^+$) were replaced by gluconate salts. Tetramethylammonium gluconate and ammonium gluconate were prepared by titration of corresponding hydroxides with D-gluconic acid lactones.

### Isolation of Plasma Membrane Vesicles by Free-Flow Electrophoresis

Crude plasma membrane isolation and free-flow electrophoresis was performed essentially as described by Heidrich et al. [11] with a slightly modified chamber buffer (in mM: 100 mannitol, 10 triethanolamine, 10 acetic acid adjusted with NaOH to a pH of 7.4) at 180 V/cm and 95 mA at 4°C using a Bender Hobein VAP22 apparatus. After separation, 90 fractions were collected and in each fraction $Na^+$-$K^+$-ATPase and leucine aminopeptidase (LAP) activity were assayed to measure the distribution of basolateral ($Na^+$-$K^+$-ATPase) and brush border membranes (LAP), respectively [2, 11].

### Measurements of Bafilomycin $A_1$ ($BA_1$)-Sensitive ATPase

$BA_1$-sensitive ATPase was measured by a $P_i$-liberation assay similar to that described by Dean et al. [8]. Briefly, vesicles (25 $\mu$g protein) were incubated for 15 min at 37°C in 200 $\mu$l of the following incubation medium (in mM): 250 sucrose, 100 KCl, 5 $MgSO_4$, 1 ouabain, 1 levamisole, 5 disodium ATP, 10 HEPES adjusted with Tris to a pH of 7.0; oligomycin was added at a concentration of 5 $\mu$g/ml and incubation was performed in the presence or absence of bafilomycin $A_1$ (usually 0.1 $\mu$M). After this incubation, 1 ml was added of a solution containing 2.9% ascorbic acid (wt/vol), 0.48% ammonium molybdate (wt/vol), 2.86% sodium dodecyl sulfate (wt/vol) in 0.48 N HCl, and samples were left on ice for 10 min. This was followed by an incubation at 37°C for 15 min, after addition of 1.5 ml of a solution containing 2% Na-arsenite (wt/vol), 2% Na citrate (wt/vol), 2% (wt/vol) acetic acid in $H_2O$. Absorbance at 705 nm was used to measure $P_i$ release. The bafilomycin $A_1$-sensitive ATPase activity was expressed as the difference in ATPase activity with and without inhibitor. Prior to ATPase measurements, vesicles were permeabilized with 0.5% (wt/vol) octyl-beta-D-glucopyranoside (octylglucoside treatment) at 30°C for 15 min.

### Chemicals

Unless indicated, all chemicals were obtained from Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany), or Sigma (St. Louis, MO) and were of the finest grade available. Ethylisopropyl amiloride (EIPA) was the gift from Dr. Thomas Friedrich (Frankfurt/M, Germany), and bafilomycin $A_1$ was the gift from Prof. Dr. Karlheinz Altendorf (Osnabrück, Germany). $H_2$DIDS and BCECF/AM were from Molecular Probes (Eugene, OR).

## Results

The activity of plasma membrane $H^+$-extrusion mechanisms of isolated rat proximal tubular epithelial cells were measured by single cell microfluorometry of intracellular pH ($pH_i$), using cells loaded with BCECF [1, 14, 15, 16]. Changes in $pH_i$ were always recorded in bicarbonate-free media and were monitored exclusively from cells located at the end of proximal tubular fragments, in order to ensure that both luminal and contraluminal sides were exposed to perfusion solutions. For the present experiments it was crucial to minimize the time of isolation and to eliminate the exposure of tissues to enzymes frequently used in preparing isolated tubular segments, such as collagenase and hyaluronidase. If these enzymes were used to prepare a tubular sus-

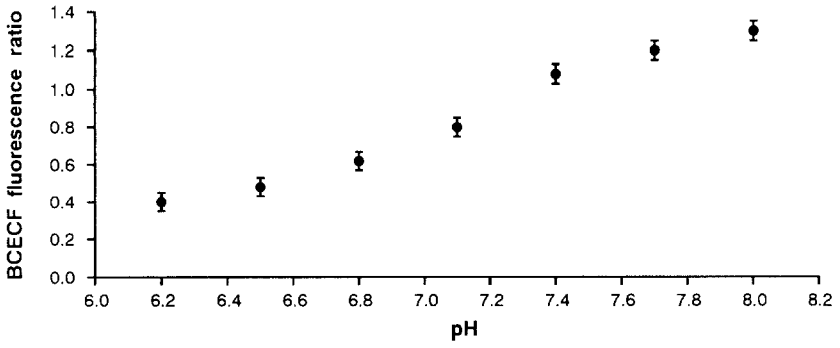


**Fig. 1.** Calibration of the BCECF fluorescence signal from the proximal tubules. Tubules were perfused with high $K^+$ (110 mM) solution with addition of 5 $\mu$M nigericin. Different media pHs were tested and the fluorescence ratio readings, from alternative excitation of BCECF at 390–440 nm and 475–490 nm, were plotted against extracellular pH (mean ± SE; $n$ = 3).

pension, $Na^+$-independent $H^+$-extrusion activity was either diminished or completely absent (*data not shown*).

To validate the use of BCECF in this preparation, the potassium/nigericin calibration method was used as originally described by Thomas et al. [25] to correlate the fluorescence ratios to $pH_i$ [15, 16]. The curve that was determined (shown in Fig. 1) was similar to the calibration curves obtained previously in the same microfluorometer, using established renal cell lines grown on permeant supports [15, 16]. The mean steady-state $pH_i$ of proximal tubular cells perfused with NaCl solution was 7.45 ± 0.04 ($n$ = 25).

In most of the following experiments, $H^+$-extrusion mechanisms were studied after cellular acidification created by the classical $NH_4^+$-prepulse technique [3, 15, 16]. After perfusion with the standard NaCl solution (*see* Materials and Methods) the tissue was perfused with a $Na^+$-free TMA medium containing 20 mM $NH_4Cl$ (replacing isoosmotically TMA-Cl). The presence of $NH_4Cl$ caused a cellular acidification (Fig. 2*a*) in contrast to most other previously examined cell types (e.g. in squid giant axons, OK cells and LLC-$PK_1$ cells) where addition of $NH_4Cl$ produces transient alkalinization (e.g. [3, 15, 16]). Since the acidification was not inhibited by 10 $\mu$M EIPA (*data not shown*), it is unlikely to be due to reversal of $Na^+/H^+$ exchange. The data suggest the presence of a significant $NH_4^+$-influx pathway, although it was not possible to identify the mechanism of $NH_4^+$-ion uptake. The rapid acidification was not prevented by 1 mM furosemide (inhibitor of $Na^+/K^+/2Cl^-$ symport), 10 mM tetraethylammonium, 10 mM $BaCl_2$ or 2 mM chloroquine (blockers of various $K^+$ channels) (*data not shown*). After $pH_i$ reached an apparent steady state, $NH_4Cl$ was removed and replaced by the TMA-Cl solution (Fig. 2*b*). The resulting further acidification was followed by a strong alkalinization and an overshoot in $pH_i$ over the resting $pH_i$ value (Fig. 2*c*). This recovery from an acid load was observed in the complete absence of $Na^+$,

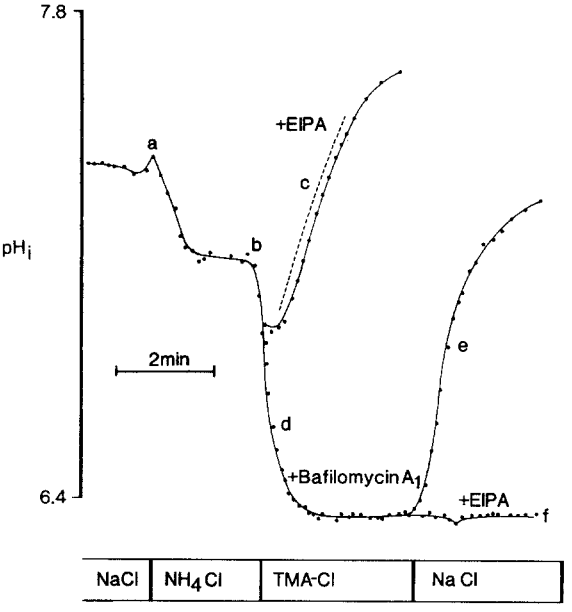


**Fig. 2.** Presence of bafilomycin $A_1$ ($BA_1$)-sensitive, $Na^+$-independent $pH_i$ recovery in rat proximal tubule cells. After perfusion with a NaCl solution, a $NH_4Cl$ solution (*see* Materials and Methods) provoked an acidification (*a*). After removing $NH_4Cl$ solution further rapid acidification occurred (*b*), followed by a $pH_i$ recovery in a $Na^+$-free medium (TMACl) (*c*). This recovery (0.218 ± 0.028 $pH_i$ unit/min) (*c*) was not inhibitable by 10 $\mu$M EIPA (*c*, dotted line) or 100 $\mu$M $H_2$DIDS (*not shown*). In the presence of 0.1 $\mu$M $BA_1$ (*d*), acidification due to $NH_4Cl$ removal was stronger and $pH_i$ did not recover. Tubules treated with $BA_1$, after perfusion with NaCl medium, exhibited $Na^+/H^+$-exchange activity (0.507 ± 0.056 $pH_i$ unit/min (*e*), blockable by 10 $\mu$M EIPA (*f*).

was not dependent on extracellular chloride (*not shown*) and was not sensitive to 10 $\mu$M EIPA (Fig. 2*c*; dotted line) or 100 $\mu$M $H_2$DIDS (blocker of several anion exchange mechanisms; *data not shown*).

Bafilomycin $A_1$ ($BA_1$) has recently been described as a potent inhibitor of vacuolar (*V*) ATPases [4]. Addition of 0.1 $\mu$M $BA_1$ completely blocked the $Na^+$-independent recovery in $pH_i$ and caused larger acidification after $NH_4Cl$ removal (Fig. 2*d*). The in-

hibition of $Na^+$-independent recovery by 0.1 $\mu$M $BA_1$ strongly suggests that $Na^+$-independent $pH_i$ recovery is related to a (*V*) ATPase. In the continuous presence of $BA_1$, subsequent addition of $Na^+$ provoked an immediate return of $pH_i$ to resting values (Fig. 2*e*). This $Na^+$-dependent recovery in $pH_i$ was prevented by 10 $\mu$M EIPA, documenting that it represents $Na^+/H^+$ exchange (Fig. 2*f*). Therefore, these experiments show that two separate mechanisms mediate proton extrusion from the cytosol of rat proximal tubular epithelial cells: a vacuolar type $H^+$-ATPase and $Na^+/H^+$ exchange. If the $pH_i$ recovery rates are calculated over the same ranges of $pH_i$ (6.7 to 7.0), thus with similar intracellular buffering capacities, it is possible to compare rates of $Na^+/H^+$ exchange (0.507 ± 0.056 pH unit/min; $n$ = 7) to those of $H^+$-ATPase (0.218 ± 0.028 pH unit/min; $n$ = 9). Thus, over a $pH_i$ range close to physiological values, $H^+$ extrusion by $H^+$-ATPase is about 40% that by $Na^+/H^+$ exchange.

To show that the $Na^+$-independent $pH_i$ recovery rate was dependent on cellular ATP content, we included protocols known to lower ATP content in most cells. The combined application of 1 mM KCN (inhibitor of oxidative phosphorylation), 0.5 mM iodoacetate (IAA; inhibitor of glycolysis) and 30 mM fructose (hexokinase substrate prevented $Na^+$-independent $pH_i$ recovery (Fig. 3*d*), but not $Na^+$-dependent recovery ($Na^+/H^+$ exchange) (Fig. 3*e*). *V*-ATPases [9], as well as kidney and macrophage endosomal ATPase are $Cl^-$ dependent [12, 19, 21, 23, 24]. In tubules exposed for 2 hr to $Cl^-$-free media (replacement by gluconate), $H^+$-pump activity was reduced (Fig. 3*c*). Control experiments were performed using tubules from the same preparation maintained in the standard NaCl solution. This control group maintained normal $H^+$-extrusion activity for 2 hr (*data not shown*). This suggests that removal of $Cl^-$, and not tissue degradation, is responsible for the decrease in $H^+$ extrusion in the experimental group. Thus, $Na^+$-independent $pH_i$ recovery seems to be related to a $Cl^-$-dependent, ATP-dependent mechanism.

Measuring $Na^+$-independent $H^+$ extrusion resulted in alkalinization which was above normal steady state observed in NaCl medium (*see* Fig. 2). This alkalinization (pH = 7.93 ± 0.08; $n$ = 15) could not be returned to the normal steady-state value unless NaCl medium was re-added; the rate of this recovery (alkali extrusion) was 0.251 ± 0.022 pH unit/min ($n$ = 6). This phenomenon was not affected by replacements of $Cl^-$ with gluconate (*data not shown*) and also was not inhibited by 10 $\mu$M EIPA (*data not shown*). However, as indicated in Fig. 4, this return to resting $pH_i$ after readdition of $Na^+$ was sensitive to 100 $\mu$M $H_2$-DIDS: readdition of NaCl

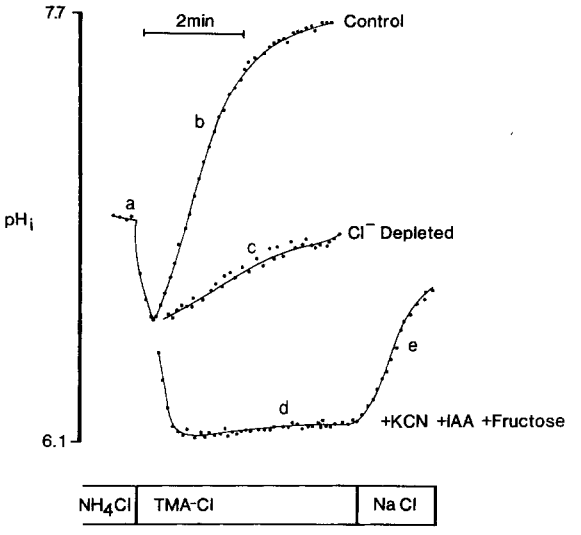


**Fig. 3.** $Cl^-$ and ATP dependence of the $Na^+$-independent $pH_i$ recovery rate. $NH_4Cl$ was withdrawn (*a*) to produce an acid load (*see also* Fig. 2) and a normal $Na^+$-independent $pH_i$ recovery rate was observed (*b*). Tubules pre-exposed to $Na^+$-gluconate medium for 2 hr/37°C, in order to decrease the intracellular $Cl^-$ content, gave much smaller $pH_i$ recovery rate analyzed in the $Cl^-$-free medium (*c*). In cells exposed to 30 mM fructose, 0.5 mM iodoacetate (IAA) and 1 mM KCN for 30 min at 4°C (to decrease the intracellular ATP content), $Na^+$-independent $pH_i$-recovery rate was completely inhibited (*d*) (always in the presence of $Cl^-$-containing media). The $Na^+/H^+$-exchange activity remained in the presence of KCN, IAA and fructose (*e*).

medium in the presence of 100 $\mu$M $H_2$DIDS did not lead to a fall in $pH_i$; removal of $H_2$-DIDS in the continuous presence of NaCl initiated an immediate $pH_i$-recovery mechanism which leveled off at the resting $pH_i$, observed in normal NaCl-perfusion conditions.

From the experiments described above, conclusions on the cellular location (basolateral *vs.* brush border) of the $H^+$-extrusion systems cannot be given. To assess the plasma membrane location of the $BA_1$-inhibited $H^+$-pump activity (*see* Fig. 2) we measured $BA_1$-sensitive ATPase activity in separated brush border (BBM) and basolateral membranes (BLM). We used a free-flow electrophoretic procedure for membrane separation; applying this method of isolation, contamination of BBM and BLM by endocytic vesicles, lysosomes, mitochondria and endoplasmic reticulum is minimal [11]. Figure 5*A* shows the separation of BBM (leucine-aminopeptidase activity, LAP) and BLM ($Na^+$-$K^+$-ATPase activity). In crude plasma membranes (PM), prior to application to the free-flow electrophoresis procedure, LAP was enriched by a factor of 4.7 ± 1.0 ($n$ = 3) over activity in the homogenate and $Na^+$-$K^+$-ATPase enrichment was 7.0 ± 1.0 fold ($n$ =

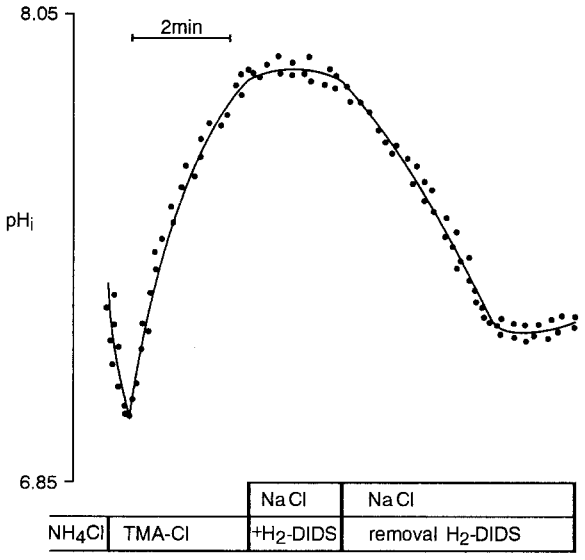


**Fig. 4.** $Na^+$-dependent, $H_2$DIDS-inhibitable alkali extrusion. In $CO_2/HCO_3^-$-free media, $Na^+$-independent $H^+$ extrusion produces intracellular alkalinization (mean $pH_i$ = 7.93 ± 0.08) which cannot be reversed in the absence of $Na^+$ (*not shown*). By addition of $Na^+$ to the extracellular fluid the $pH_i$ returns to resting $pH_i$ values observed in normal $Na^+$-perfusion conditions. This $Na^+$-dependent alkali extrusion was reversibly inhibited by 100 μM $H_2$DIDS; as indicated in the figure, the addition of $Na^+$ in the presence of $H_2$DIDS does not lead to a recovery of $pH_i$, removal of $H_2$DIDS is followed by alkali extrusion. The rate of this recovery was 0.251 ± 0.022 pH unit/min.

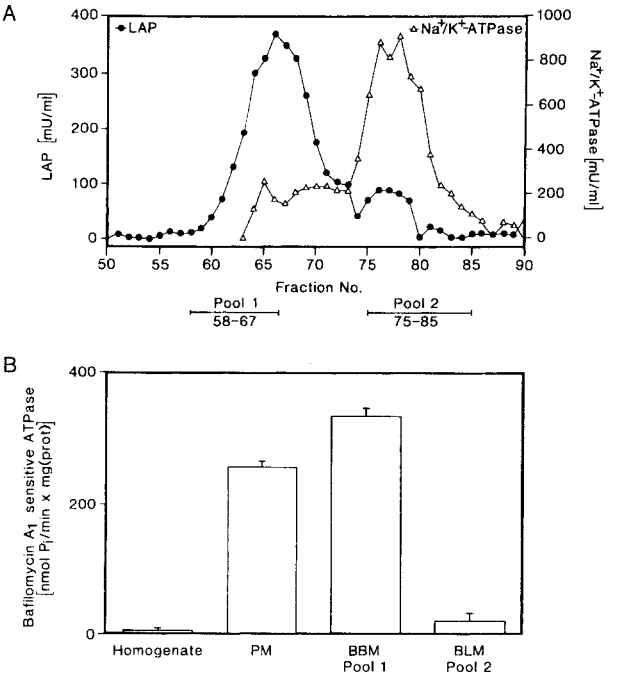


**Fig. 5.** Localization of the vacuolar type (*V*) $H^+$-ATPase in brush border membranes. Crude plasma membrane vesicles were isolated and separated by free-flow electrophoresis (*A*) After separation, 90 fractions were collected and in each fraction $Na^+/K^+$-ATPase and leucine aminopeptidase (LAP) activity were assayed as the measure for the distribution of the basolateral ($Na^+$-$K^+$-ATPase) and the brush border membranes (LAP), respectively. (*B*) Fractions from free-flow electrophoresis were pooled as indicated in *A* and enzyme activities ($H^+$-ATPase) measured in homogenates of kidney cortex slices and plasma membranes (PM) applied to free-flow electrophoresis and in basolateral (BLM) and brush-border membranes (BBM).

3). In pool 1, the BBM fraction, LAP activity was enriched 12.5 ± 3.2 fold ($n$ = 3), whereas $Na^+$-$K^+$-ATPase activity was enriched 2.7 ± 0.1 fold of that in the homogenate. In pool 2, the BLM fraction, $Na^+$-$K^+$-ATPase activity was enriched 21.0 ± 5.1 ($n$ = 3) and LAP activity 3.2 ± 0.4 ($n$ = 3) fold. In Fig. 5, not all of the $BA_1$-sensitive ATPase activity from the crude plasma membrane fraction (PM) is copurified with LAP. This is likely to be due to either the presence of $BA_1$-sensitive ATPase in intracellular organelles which are eliminated during free-flow electrophoresis, or to degradation of the ATPase during electrophoresis. The vesicles obtained by the free-flow electrophoresis were permeabilized with 0.5% (wt/vol) octylglucoside in order to expose both sides of the membrane to the ATPase assay procedure. Figure 5*B* shows that there is a significant $BA_1$-sensitive ATPase activity, measured by a phosphate liberation assay, in crude plasma membranes (PM). Figure 5*B* also demonstrates that $BA_1$-sensitive ATPase activity is concentrated in the BBM fractions and almost absent in the BLM fractions. Together with experiments given in Fig. 2, the observation presented in Fig. 5 is strong evidence for the $BA_1$-sensitive $H^+$ extrusion being associated with the $BA_1$-sensitive $H^+$-ATPase activity located in the brush border membrane.

Since, at higher concentrations, $BA_1$ is known to inhibit other ATPases in addition to the *V*-type [4], we tested the sensitivity of the oligomycin-, ouabain- and levamisole-resistant ATPase activity to increasing concentrations of $BA_1$ (Fig. 6). 50% inhibition was observed at a $BA_1$ concentration of around 0.6 nM, a value far below that reported for inhibition of $F_1F_0$-ATPases (50% inhibition at μM concentration of $BA_1$; [4]). In comparison to N-ethylmaleimide ($IC_{50}$ in μM range) $BA_1$ is at least $10^4$ times more potent as an inhibitor of vacuolar $H^+$-ATPases.

## Discussion

In the present experiments it was important to minimize any lengthy manipulation of the tissue and to exclude enzymatic treatment, which might damage

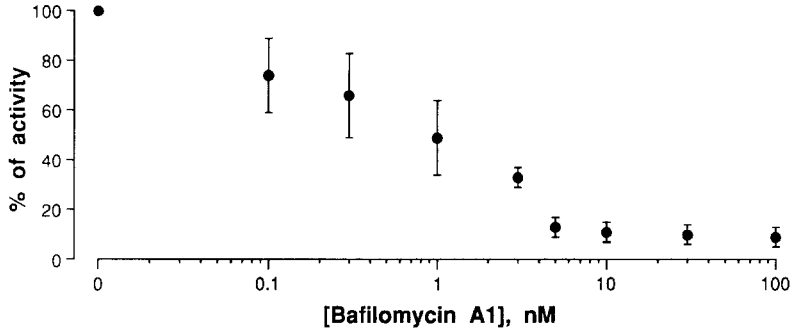


**Fig. 6.** Bafilomycin $A_1$ inhibition curve for oligomycin-, levamisole-, ouabain-resistant ATPase in brush border membranes (BBM). BBM obtained by the free-flow electrophoresis separation (*see* Fig. 5, *pool 1*) were treated with 0.5% (wt/vol) octylglucoside and exposed to various concentrations of bafilomycin $A_1$. ATPase assay was performed as described in Materials and Methods. Total oligomycin-, laevamisole-, ouabain-resistant ATPase activity in these experiments was 219 ± 50 nmol $P_i$/min · mg protein ($n$ = 3). Bafilomycin $A_1$-insensitive ATPase (also oligomycin-, laevamisole-, ouabain-resistant) was 22.3 ± 11.7 nmol $P_i$/min · mg protein ($n$ = 3). The values represent percent inhibition of the former activity in the presence of different concentrations of bafilomycin $A_1$. $I_{50}$ for bafilomycin $A_1$ was 0.61 ± 0.15 nM ($n$ = 3).

some plasma membrane proteins. Only under such conditions do proximal tubules have the ability to extrude protons after acid load in the absence of $Na^+$. The fact that the observed $Na^+$-independent $H^+$-extrusion activity (Fig. 2) and $H^+$-ATPase in purified brush border membranes (Fig. 5) are inhibited by low concentration of bafilomycin $A_1$ strongly suggests the presence of a functional vacuolar $H^+$-ATPase pump in the BBM. Indeed, studies with monoclonal antibodies have demonstrated the presence of the vacuolar proton pump in the proximal tubular brush border membrane [5]. Furthermore, $Na^+$-independent $H^+$ extrusion (bicarbonate reabsorption) has been shown earlier (for review *see* [1]; e.g. [7, 14, 17, 22, 28], although the mechanisms involved in this phenomenon have been difficult to define.

In the absence of $Na^+$, $H^+$ extrusion overshoots the steady-state $pH_i$, and addition of $Na^+$ returns $pH_i$ to the steady-state $pH_i$ (observed under $Na^+$-perfusion conditions), via $H_2$DIDS-sensitive, EIPA-insensitive, $Cl^-$-independent mechanism (Fig. 4 and *data not shown*). Although the precise nature of this latter transport mechanism was not investigated in the present study, the possibility exists that it represents $Na[OH^-]HCO_3^-$ cotransport. This hypothesis presumes that sufficient cellular Na and base equivalents are available for net efflux. Such a mechanism could be operative in nominally bicarbonate-free solutions due to cellular $CO_2$ production and carbonic-anhydrase activity or due to efflux of hydroxyl ions. This DIDS-sensitive transport mechanism seems to have the potency to sense $pH_i$ and to reduce its transport rate near resting $pH_i$. An alternate interpretation is that at resting $pH_i$ the rate of this alkali extrusion mechanism could match the rate of $H^+$-extrusion mechanism. The detailed study of this phenomenon was not in the scope of the present paper but could be a basis for further investigations.

The data presented do not allow one to draw any conclusions concerning the activity of the $Na^+$-independent $H^+$-extrusion system at resting $pH_i$ in the presence of full $Na^+$. By the technique used (measurement of $pH_i$), inhibition of the pump by bafilomycin $A_1$ in the presence of $Na^+$ cannot result in intracellular acidification as $Na^+/H^+$-exchange activity compensates and keeps $pH_i$ at resting values. On the other hand, any $pH_i$ alteration (acidification) or also a constant $pH_i$ after amiloride inhibition of $Na^+/H^+$ exchange might be the result of (only partial) inhibition of $Na^+/H^+$ exchange which can (in part) be compensated by the $H^+$ pump.

It is known that in acidifying epithelia $H^+$-ATPase might be recruited from an intracellular compartment to the apical surface, e.g. due to acidification of intracellular space [14, 22, 27]. Two lines of evidence argue against the involvement of such a mechanism in the present study: (i) the presence of colchicine (100 μM after 30 min of preincubation) had no influence in the present study on the rate of $Na^+$-independent bafilomycin-sensitive $H^+$ extrusion (0.161 ± 0.051 pH unit/min in the absence of colchicine and 0.211 ± 0.054 in the presence of colchicine; $n$ = 3; pH range for measuring of Na-independent $pH_i$ recovery was between 6.7 and 7.0). This makes an exocytic insertion rather unlikely, in contrast to a previous study on rabbit $S_3$-proximal tubule seg-

ments [14]. (ii) Bafilomycin $A_1$-sensitive ATPase was also present in brush border membrane vesicles isolated from rat renal superficial cortex from the same animals not pre-exposed to an acidotic condition.

Finally, we believe that the activity measured in isolated brush border vesicles and the activity measured in $H^+$ extrusion by microfluorometry is related to an identical molecular structure. The following arguments are in favor of such an interpretation: (i) $H^+$-ATPase as well as $H^+$ extrusion are inhibited by low bafilomycin $A_1$ concentrations; (ii) $H^+$ extrusion is reduced by lowering intracellular [$Cl^-$], and vacuolar ATPase enzyme activity in intracellular organelles is known to require $Cl^-$ for optimum activity [19]; (iii) other investigators have documented a brush border location of vacuolar type $H^+$-ATPase by immunohistochemical methods [5]; this enzyme activity is known to be sensitive to bafilomycin $A_1$ [4, 9].

Considering the physiological role of the proximal tubular brush border $Na^+$-independent $H^+$ pump, it is very likely that this mechanism plays a significant role in acid extrusion. It may also excrete protons at significant rates when intratubular pH is low, since $Na^+/H^+$ exchange is known to be inhibited by acidic pH in the extracellular medium. Thus, this $H^+$ extrusion system might become more prominent with progressing/increased intratubular acidification. Furthermore, the $Na^+$-independent $H^+$-extrusion mechanism might also operate at more alkaline intracellular $pH_i$ values than $Na^+/H^+$ exchange (*see* presented data). Thus, the relative contribution of $Na^+$-independent $H^+$ extrusion might also become more important with progressing intracellular alkalosis.

In conclusion, this study presents functional as well as biochemical data on brush border membrane location of a vacuolar type $H^+$-ATPase in renal proximal tubules. These observations extend previous biochemical information on NEM-sensitive ATPase activity in isolated brush border membranes [13, 18–20, 23, 26], as well as on $Na^+$-independent $H^+$ extrusion [7, 14, 17, 22, 28] by attributing it to a bafilomycin $A_1$-sensitive, $Cl^-$-dependent vacuolar type (*V*) ATPase. Furthermore, the present data suggest that the rate of this $H^+$-extrusion activity in brush border membrane is sufficient to secrete the fractional amount of protons necessary for the $Na^+$-independent mode of $HCO_3$-reabsorption in the proximal tubules (ca. 30–40%).

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