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Relation of ATPases in Rat Renal Brush-Border Membranes to ATP-Driven H^+ Secretion

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Summary. In the presence of inhibitors for mitochondrial H^+-ATPase, ($Na^+ + K^+$)- and Ca^{2+}-ATPases, and alkaline phosphatase, sealed brush-border membrane vesicles hydrolyse externally added ATP demonstrating the existence of ATPases at the outside of the membrane ("ecto-ATPases"). These ATPases accept several nucleotides, are stimulated by Ca^{2+} and Mg^{2+}, and are inhibited by N,N′-dicyclohexylcarbodiimide (DCCD), but not by N-ethylmaleimide (NEM). They occur in both brush-border and basolateral membranes. Opening of brush-border membrane vesicles with Triton X-100 exposes ATPases located at the inside (cytosolic side) of the membrane. These detergent-exposed ATPases prefer ATP, are activated by Mg^{2+} and Mn^{2+}, but not by Ca^{2+}, and are inhibited by DCCD as well as by NEM. They are present in brush-border, but not in basolateral membranes. As measured by an intravesicularly trapped pH indicator, ATP-loaded brush-border membrane vesicles extrude protons by a DCCD- and NEM-sensitive pump. ATP-driven H^+ secretion is electrogenic and requires either exit of a permeant anion (Cl^-) or entry of a cation, e.g., Na^+ via electrogenic Na^+/D-glucose and Na^+/L-phenylalanine uptake. In the presence of Na^+, ATP-driven H^+ efflux is stimulated by blocking the Na^+/H^+ exchanger with amiloride. These data prove the coexistence of Na^+-coupled substrate transporters, Na^+/H^+ exchanger, and an ATP-driven H^+ pump in brush-border membrane vesicles. Similar location and inhibitor sensitivity reveal the identity of ATP-driven H^+ pumps with (a part of) the DCCD- and NEM- sensitive ATPases at the cytosolic side of the brush-border membrane.

Key Words H^+ secretion · H^+ ATPase · NEM · DCCD

Introduction

Secretion of protons into the tubule lumen is the primary step in absorption of Na^+, HCO_3^-, Cl^-, and water in kidney proximal tubules. Besides the well-documented Na^+/H^+ exchanger [4, 54], a second Na^+-independent transport system must exist in the luminal (brush-border) membrane of proximal tubule cells. Evidence for such a transport system stems from microperfusion [12, 15–17, 55] and electrophysiological experiments [24], and from intracellular pH determinations in vivo [3, 46, 56] and in isolated proximal tubules [8, 39, 51]. All these experiments revealed an H^+ secretion in the nominal absence of Na^+.

Na^+-independent proton secretion is most probably due to an H^+-ATPase in the brush-border membrane of proximal tubule cells. The binding of antibodies raised against an H^+-ATPase from bovine kidney medulla to the brush-border membrane of proximal tubules [27] supports this conclusion. However, despite numerous investigations it is not clear which ATPase activity in proximal tubules and in isolated brush-border membrane vesicles reflects the H^+-ATPase. The "Mg^{2+}-ATPase" measured in cortex homogenates [45], microdissected nephrons [32], and isolated brush-border membranes [5, 20, 31, 44] is stimulated by Ca^{2+} and other nucleotides besides ATP [13, 31, 44, 45]. This pattern is typical for ecto-ATPases found in other cells [14, 18, 28, 29, 40, 42] rather than for an ATPase involved in H^+ transport. The "bicarbonate-stimulated ATPase" in a rat kidney brush-border membrane preparation was related to ATP-driven H^+secretion [35, 36, 41, 43], but was later shown to be of mitochondrial origin [13, 31, 38]. Another candidate for H^+ secretion, the N,N′-dicyclohexylcarbodiimide (DCCD) and N-ethylmaleimide (NEM) sensitive ATPase was measured in microdissected and permeabilized proximal tubules [1], but a localization to the brush-border membrane was not demonstrated.

To relate ATPase in brush-border membranes to H^+ transport, nucleotide specificity, activation by divalent cations, and sensitivity to side group reagents must be compared with other well-described H^+-ATPases, e.g., from collecting ducts [2, 25, 26], proximal tubular endosomes [49], and intracellular organelles [47]. This indirect approach is necessary because a specific inhibitor for H^+-ATPases is not

known. ATPases involved in proximal tubular H^+ secretion should also be confined to the luminal membrane and have their catalytic site exposed to the cell interior. This pattern has not yet been demonstrated for any ATPase in proximal tubules. Finally, it must be shown that ATP-driven H^+ efflux from brush-border membrane vesicles has characteristics similar to ATPases thought to be involved in H^+ translocation. Previous investigations on ATP-driven H^+ secretion were performed on a crude brush-border membrane fraction [33, 37]. Thus, it remained open whether the ATP-driven H^+ secretion observed in these studies occurred indeed from brush-border membranes or rather from vesicles of intracellular organelles containing an H^+-ATPase.

The purpose of this study is the characterization of ATPases at the outside and inside of the brush-border membrane and their comparison with ATP-driven H^+ extrusion from purified brush-border membrane vesicles. Part of the results has been published in preliminary form [10, 11].

Materials and Methods

Preparation of Membrane Vesicles

Renal cortical brush-border membrane vesicles were isolated by the Mg^{2+}/EGTA precipitation method [7]. Basolateral membrane vesicles were purified by a Percoll density centrifugation technique [52]. The vesicles were loaded with a buffer containing 150 mM KCl and 10 mM HEPES titrated with Tris to pH 7.0 and stored in liquid nitrogen.

ATPase Measurements

Brush-border membrane vesicles were quickly thawed and diluted to a protein concentration of 2.5 mg/ml with preloading buffer containing no or 0.1% Triton X-100 (final concentration). After 15 min incubation at room temperature, the suspension was diluted fivefold and 50 μl (= 25 μg protein) were added to 450 μl of a prewarmed (37°C) reaction medium containing (in final concentrations) 5 mM ATP, 5 mM $MgSO_4$, 150 mM KCl, 50 mM HEPES/Tris, at pH 7.0, and 1 mM levamisole (to inhibit alkaline phosphatase), 2 mM ouabain (to inhibit (Na^+ + K^+)-ATPase), 5 μg/ml oligomycin (inhibitor of the mitochondrial H^+-ATPase), and 0.5 mM vanadate (inhibitor of (Na^+ + K^+)- and Ca^{2+}-ATPases). The applied inhibitor concentrations were high enough to completely block these ATPases as determined in separate experiments (*not shown*). The reaction was run at 37°C for 15 min and stopped by addition of 0.1 ml ice-cold trichloroacetic acid. The librated phosphate was determined colorimetrically [22]. Control experiments (*not shown*) revealed that P_i liberation was proportional to incubation time for up to 30 min and to protein amounts up to 100 μg. Protein was determined with Coomassie blue using bovine serum albumin as a standard [9]. All ATPase measurements were performed in quadruplicate on three to four membrane preparations and are presented as the mean ± SD.

ATP-Driven H^+ Efflux

To an ice-cold suspension of brush-border membrane vesicles (3–6 mg protein/ml) were added 5 mM ATP, a regenerating system consisting of 5 mM phosphoenolpyruvate and 0.1 mg phosphoenolpyruvate kinase, and a weakly permeant pH indicator, 6-carboxyfluorescein (250 μM) in 10 mM HEPES/Tris buffers, pH 7.0. The salts added during preloading varied and are therefore indicated in the figure legends. The mixture was quickly frozen in liquid nitrogen and then slowly thawed at 0°C to transiently open the vesicles [19]. Aliquots of the vesicle suspension (50 μl containing 0.3–0.6 mg protein) were then transferred into a cuvette containing 2 ml of buffer (room temperature; for composition *see* figure legends) and the fluorescence of 6-carboxyfluorescein was recorded continuously at 495 nm excitation and 525 nm emission wavelength. The fluorescence changes were calibrated with respect to intravesicular pH by suspending KCl-loaded vesicles in KCl buffers (150 mM KCl inside and outside the vesicles) of various pH in the presence of 5 μM of the K^+/H^+ exchanger, nigericin. The figures show redrawn representative traces from experiments on at least three membrane preparations.

Materials

Nucleotides (all trisodium salts) were purchased from Boehringer (Mannheim, FRG). Oligomycin, N-ethylmaleimide (NEM), diethylstilbestrol (DES), and *p*-chloromercuribenzoate (PCMB) were obtained from Serva (Heidelberg); N,N'-dicyclohexylcarbodiimide (DCCD) from Aldrich (Weinheim, FRG) or Calbiochem (Los Angeles, CA). Levamisole, *p*-chloromercuribenzenesulfonate (PCMBS), 4-chloro-7-nitro-benzoxa-1,3-diazole (NBD-Cl), and 6-carboxyfluorescein were purchased from Sigma (St. Louis, MO). The chemicals used were of analytical grade.

Results

Location of ATPases in the Brush-Border Membrane

All ATPase measurements have been performed in the presence of inhibitors for the mitochondrial H^+-ATPase, the (Na^+ + K^+)- and Ca^{2+}-ATPases from basolateral membranes and the alkaline phosphatase from brush-border membranes to abolish contributions by unrelated enzyme activities. Given these inhibitors, rat renal brush-border membrane vesicles hydrolyse ATP at a rate of 0.85 ± 0.041 μmol P_i/min and mg of protein (mean ± SEM from 25 membrane preparations). This ATP hydrolysis occurs with sealed brush-border vesicles (Fig. 1, upper panel, 0% Triton X-100). Since vesicles are impermeant to ATP and predominantly oriented right side out [6, 19, 21], ATP must have been degraded

by ATPases located at the outer (= extracellular) side of the brush-border membrane.

To find out whether additional ATPases are present at the cytoplasmic surface of the vesicles, we pretreated brush-border membranes for 15 min at room temperature with increasing concentrations of a detergent, Triton X-100. At about 0.04% Triton X-100, ATPase activity rises, indicating exposure of cryptic ATPases (Fig. 1, upper panel). Raising Triton X-100 concentrations further decreases P_i release, suggesting an inhibition of ATPases at higher detergent concentrations.

In the presence of an SH-group reagent, NEM, intact brush-border vesicles release P_i at a similar rate as without NEM (Fig. 1, top panel, open circles), revealing that the ATPases at the outside of the membrane are insensitive to NEM. Increasing Triton X-100 concentrations inhibit the activity of the NEM-insensitive ATPases. The rise in P_i release at intermediate detergent concentrations is completely prevented by NEM. Therefore, detergent-exposed ATPases at the inside of the membrane are blocked by NEM. Replotting of the NEM-sensitive ATPase activity as a function of detergent concentration (Fig. 1, bottom) shows that ATPases at the inner side of the brush-border membrane are best exposed at around 0.06% Triton X-100. Comparable results were obtained with 0.5% octylglucoside, 0.1% saponin, but not with cholate, which inhibited all ATPase activities (*data not shown*).

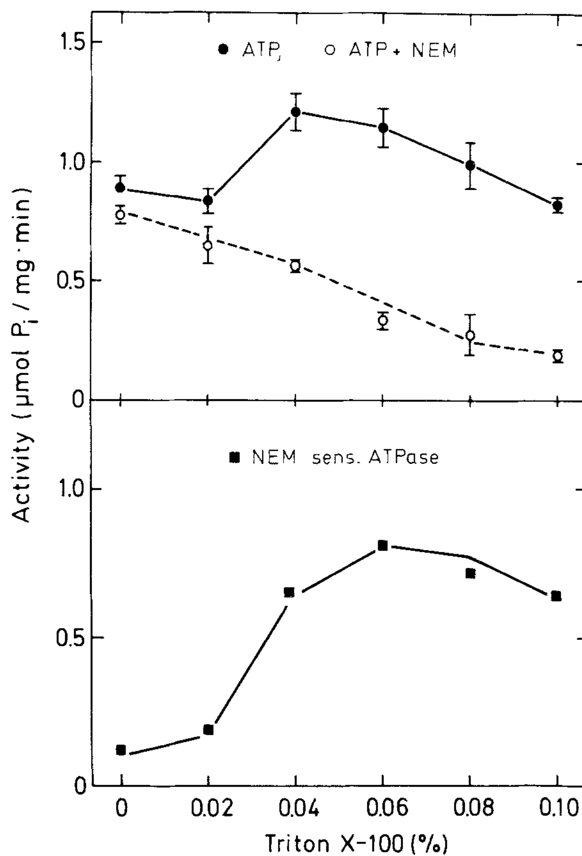

Fig. 1. Effect of Triton X-100 on ATPase activity in rat renal brush-border membranes. *Top:* The membranes were pretreated for 15 min at room temperature with the detergent concentrations indicated on the abscissa. Thereafter, 25 μg vesicle protein was added to assay buffers containing 5 mM ATP without (filled circles) or with 1 mM NEM (open circles) and incubated for 15 min at 37°C. The released P_i was determined colorimetrically and is shown at the ordinate. For further details of the buffer compositions, *see* Materials and Methods. *Bottom:* NEM-sensitive ATPase was calculated as the difference between P_i release in the presence of ATP and that in the presence of ATP + 1 mM NEM

Characteristics of ATPases at the Outside and Inside of the Brush-Border Membrane

As a good approximation, ATPase activity in the absence of detergent reflects the enzymes at the outside of the membrane. P_i release in the presence of 0.1% Triton X-100 is an estimate of ATPases at the inside of the brush-border membrane, the external enzymes being nearly completely inhibited by the detergent (*cf.* Fig. 1). Table 1 summarizes the properties of ATPases at the outside and inside of the brush-border membrane. The ATPases are not influenced by monovalent cations with exception of lithium, which causes 15–18% inhibition. Among the anions, 25 mM thiocyanate and nitrate show small inhibition of the outer ATPases, but more than 40% inhibition of the detergent-exposed ATPases at the inside of the membrane. Activation by divalent cations and nucleotide specificity exhibit also remarkable differences. ATPases at the outside of the brush-border membrane are nearly fully activated by 5 mM Ca^{2+}, whereas the ATPases at the inside show in the presence of Ca^{2+} only 41% of their full activity. Ba^{2+} and Zn^{2+} hardly stimulate the ATPases. The detergent-exposed ATPases clearly prefer ATP over ITP and GTP, whereas the enzymes at the outside of the membrane hydrolyse all added nucleotides. ITP and GTP are hydrolysed even more effectively than ATP.

Finally, as also shown in Table 1, the ATPases differ with respect to their sensitivity towards putative inhibitors. Following 15 min preincubation, the carboxyl group reagent N,N′-dicyclohexylcarbodiimide (DCCD; 1 mM) is the only effective inhibitor of ATPases at the outside of the brush-border membrane. The SH-group reagents PCMB and PCMBS, and an inhibitor of various H^+-ATPases, DES, show maximally 20% inhibition. Another SH-reagent, NEM, even stimulates the external ATPase after preincubation. The ATPases at the

Table 1. Characteristics of ATPases at the outside and inside of rat renal brush-border membrane vesicles

	ATPase at the outside (% of control)	ATPase at the inside (% of control)
Monovalent cations		
Potassium	100.0 ± 6.7 (12)	100.0 ± 4.2 (13)
Rubidium	101.5 ± 9.6 (12)	96.4 ± 20.4 (12)
Sodium	102.4 ± 20.6 (13)	97.7 ± 12.9 (12)
Lithium	82.0 ± 9.7 (13)[a]	84.5 ± 14.0 (12)[a]
Tetramethylammonium	107.7 ± 14.7 (12)	104.7 ± 14.6 (13)
Anions		
Chloride	100.0 ± 9.3 (14)	100.0 ± 9.8 (14)
Gluconate	95.8 ± 5.6 (13)	89.3 ± 9.0 (14)[b]
Sulfate	102.8 ± 5.6 (13)	101.0 ± 7.3 (14)
Thiocyanate	90.5 ± 8.4 (14)[b]	60.2 ± 11.1 (11)[a]
Nitrate	90.1 ± 6.7 (13)[b]	51.9 ± 14.1 (14)[a]
Divalent cations		
Magnesium	100.0 ± 8.0 (12)	100.0 ± 6.8 (12)
Manganese	67.5 ± 13.8 (15)[a]	111.0 ± 15.0 (15)
Calcium	88.9 ± 12.0 (14)[b]	41.4 ± 9.8 (15)[a]
Barium	18.3 ± 11.8 (14)[a]	17.3 ± 7.9 (15)[a]
Zinc	19.6 ± 11.9 (15)[a]	14.3 ± 10.6 (15)[a]
No addition	4.9 ± 5.9 (15)[a]	9.2 ± 5.4 (15)[a]
Nucleotides		
ATP	100.0 ± 6.6 (14)	100.0 ± 8.2 (13)
ITP	143.2 ± 14.5 (13)[a]	54.2 ± 8.3 (12)[a]
GTP	112.6 ± 14.6 (13)[b]	33.5 ± 15.6 (13)[a]
UTP	123.4 ± 20.2 (13)[a]	16.8 ± 16.7 (13)[a]
CTP	102.9 ± 14.5 (13)	9.1 ± 7.5 (13)[a]
Inhibitors		
No addition	100.0 ± 4.6 (12)	100.0 ± 6.4 (12)
DCCD	35.8 ± 15.5 (12)[a]	12.5 ± 5.7 (11)[a]
DES	81.6 ± 16.8 (12)[b]	69.8 ± 10.8 (12)[a]
NBD-Cl	98.7 ± 5.0 (12)	41.6 ± 4.6 (12)[a]
NEM	119.3 ± 20.0 (12)[b]	12.5 ± 5.7 (11)[a]
PCMB	88.0 ± 8.0 (12)[a]	29.3 ± 9.2 (12)[a]
PCMBS	81.4 ± 7.2 (12)[a]	27.0 ± 9.6 (12)[a]

ATPases at the outside were determined with intact brush-border membrane vesicles; ATPases at the inside with Triton X-100-pretreated membranes (0.1% Triton X-100; 2.5 mg/ml protein; 15 min preincubation at room temperature). The control ATPase assay was performed with 150 mM KCl, 5 mM ATP, 5 mM $MgCl_2$, 20 mM HEPES/Tris, at pH 7.4, and P_i release set to 100%. For "monovalent cations," 150 mM K^+ was replaced by the indicated cations. For "divalent cations," 5 mM Mg^{2+} was replaced by an equivalent amount of indicated cations. For "nucleotides" 5 mM ATP was replaced by 5 mM of other nucleotides. For "inhibitors," vesicles were preincubated 25 min at room temperature with either no inhibitor or 1 mM DCCD, 1 mM NEM, 100 μM DES, 10 μM NBD-Cl; 50 μM PCMB; or 500 μM PCMBS. The influence of anions was measured in the presence of 125 mM gluconate and 25 mM of indicated anions (K^+ salts). The table shows mean ± SD from the number of determinations indicated in the brackets (three membrane preparations). [a] $P < 0.01$; [b] $P < 0.05$.

cytoplasmic membrane side are severely inhibited by all tested agents including the NH_2-group reagent NBD-Cl.

The results shown in Table 1 represent lumped characteristics of ATPases located at the outside and of those at the inside of the brush-border membrane. The data do not allow discrimination between a single type of ATPase or several ATPases at the outside of the membrane. Likewise, the characteristics of detergent-exposed ATPases may reflect one or more ATPases at the inside of the brush-border membrane. The results do, however, clearly indicate the presence of distinct ATPases at both aspects of the brush-border membrane.

Sidedness of ATPases

Next, we determined which of the ATPases are located only in the brush-border membrane (as expected for H^+-ATPases) and which occur also in

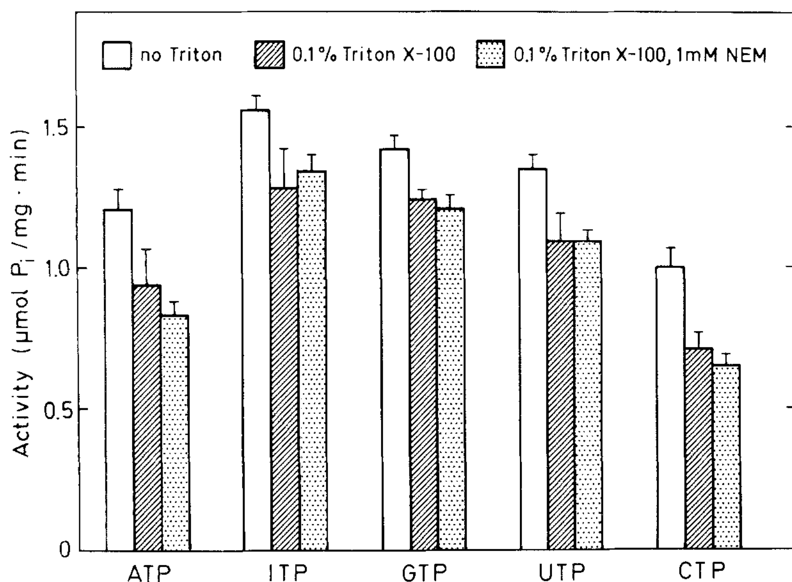

Fig. 2. ATPase activities in rat renal basolateral membrane vesicles. The membranes were pretreated without (open bars) or with (hatched and stippled bars) 0.1% Triton X-100 and added to buffers containing 5 mM of the indicated nucleotides, and 0 (open and hatched bars) or 1 mM NEM (stippled bars). ATPase reaction was stopped after 15-min incubation at 37°C

basolateral membranes. Various nucleotides were added to rat renal basolateral membranes and the release of P_i was determined. Again, the experiment was performed in the presence of several inhibitors to abolish contributions from other ATPases in basolateral membranes, (Na^+ + K^+)- and Ca^{2+}-ATPases, and from contaminations by alkaline phosphatase and mitochondrial H^+-ATPase. The results are shown in Fig. 2. Basolateral membrane vesicles hydrolyse all nucleotides at comparable rates. Triton X-100 (0.1%) causes a small inhibition of ATPase activity. Since NEM is ineffective in the presence of Triton X-100, the detergent does not expose a cryptic NEM-sensitive ATPase activity. The NEM-sensitive ATPases are, therefore, restricted to the luminal membrane where they occur at the inside of the membrane. As opposed, NEM-insensitive ATPases hydrolyzing all nucleotides occur in luminal and contraluminal membranes.

ATP-Driven H^+ Secretion

To test for a possible relation between NEM-sensitive ATPases at the inside of the brush-border membrane and ATP-driven H^+ transport we loaded vesicles with ATP, an ATP-regenerating system and the weakly permeant pH indicator, 6-carboxyfluorescein, to measure H^+ efflux. For loading we used a previously published freeze/thaw method [19], which allows incorporation of impermeant substances into brush-border membrane vesicles after their purification.

First, we investigated the applicability of trapped 6-carboxyfluorescein as an indicator for intravesicular pH. Brush-border membrane vesicles were loaded with the dye in the absence or presence of KCl. K^+-free vesicles were then suspended in KCl buffers, K^+-containing vesicles in K^+-free buffers to create transmembrane K^+ gradients. In the presence of the K^+/H^+ exchanger, nigericin, an inward K^+ gradient causes H^+ efflux; an outward K^+ gradient, H^+ influx. The corresponding intravesicular pH changes should alter the fluorescence of trapped carboxyfluorescein, but not the fluorescence of dye bound to the outside of the vesicle membrane or dissolved in the incubation medium. Figure 3 shows the fluorescence changes expected for a trapped dye, i.e., a fluorescence increase with intravesicular alkalinization and a decrease with acidification.

Similar, but smaller fluorescence changes were recorded when the protonophor CCCP was used instead of nigericin (Fig. 3, right). This finding indicates that brush-border membranes are permeable to K^+ even without nigericin. K^+ movement across the membrane requires H^+ movement in the opposite direction for charge compensation. All ionophore-driven changes in intravesicular pH could be abolished by the presence of 50 mM NH_4Cl, which acts as a permeant buffer and thereby dissipates transmembrane pH differences (*data not shown*).

Since the previous experiment proved the suitability of trapped 6-carboxyfluorescein, we next loaded vesicles with ATP, ATP-regenerating system and dye to detect ATP-driven H^+ efflux. Figure 4 shows that ATP-loaded vesicles alkalinize their interior, i.e., they eject protons. Vesicles loaded with pH indicator, but not with ATP, show a small apparent alkalinization, which is due to a slow dye leakage. As estimated from the dye fluorescence in the supernatant of sedimented vesicles, dye leakage

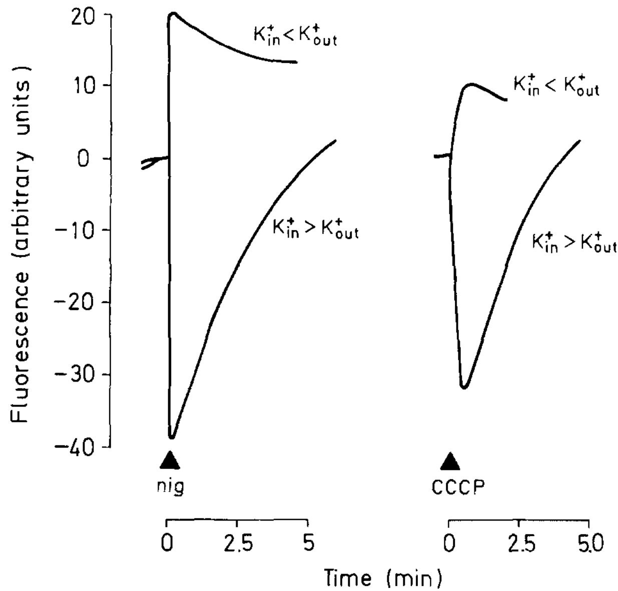

Fig. 3. K^+/H^+ exchange visualized by intravesicularly trapped 6-carboxyfluorescein. Brush-border membrane vesicles were loaded by freeze/thawing with 250 μM 6-carboxyfluorescein in 10 mM HEPES/Tris buffers, pH 7.0, containing 150 mM tetramethylammonium chloride ($K^+_{in} < K^+_{out}$) or 150 mM KCl ($K^+_{in} > K^+_{out}$). At zero time, the vesicles were suspended in buffers (HEPES/Tris, at pH 7.0) containing 150 mM KCl ($K^+_{in} < K^+_{out}$) or 150 mM tetramethylammonium chloride ($K^+_{in} > K^+_{out}$) and either 5 μM of the K^+/H^+ exchanger nigericin (left) or the protonophor CCCP (right). The fluoresence changes of trapped 6-carboxyfluorescein were recorded continuously. Shown are redrawn traces

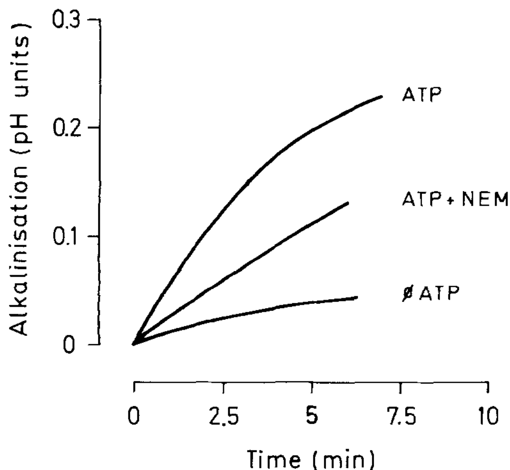

Fig. 4. ATP-driven intravesicular alkalinization and inhibition by NEM. Brush-border membrane vesicles were preloaded without (*ø ATP*) or with ATP (*ATP*), an ATP-regenerating system, 6-carboxyfluorescein as a pH indicator, and 0 or 1 mM NEM as indicated. The preloading buffers contained in addition 150 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES/Tris, pH 7.0. H^+ efflux was followed fluorimetrically in a buffer containing 150 mM KCl, 20 mM HEPES/Tris, pH 7.0

Table 2. Effect of putative inhibitors on ATP-driven H^+ efflux

Inhibitor	Conc (mM)	Initial rate of alkalinization (%)	
		Control	Experimental
DCCD	0.25	100.0 ± 15.5 (8)	17.2 ± 10.7 (6)[a]
NEM	1.0	100.0 ± 19.1 (11)	62.8 ± 26.0 (9)[b]
PCMB	0.1	100.0 ± 10.2 (6)	10.3 ± 14.6 (5)[a]
Vanadate	1.0	100.0 ± 13.4 (5)	109.8 ± 12.2 (3)
Levamisole	1.0	100.0 ± 11.8 (8)	110.2 ± 12.7 (6)
Oligomycin	0.01 g/ml	100.0 ± 11.9 (8)	103.6 ± 15.9 (8)

ATP-driven H^+ efflux was monitored as fluorescence increase of trapped 6-carboxyfluorescein in the presence of 150 mM KCl without (control) or with the indicated compounds. The initial rates of fluorescence increase were determined by drawing a tangent to the earliest portions of the recorded fluorescence *vs.* time curves. The fluorescence decrease under control conditions was set to 100%. The table shows mean ± SD from the number of determinations indicated in the brackets (two to four preparations). [a] $P < 0.001$; [b] $P < 0.01$. (experimental *vs.* respective controls).

does not exceed 10% per hr and is independent of the presence of intravesicular ATP (*data not shown*). The ATP-dependent alkalinization is inhibited by nigericin, CCCP, NH_4Cl (*data not shown*), and by 1 mM intravesicular NEM (Fig. 4). Extravesicular NEM was ineffective. These data demonstrate the presence of an NEM-sensitive ATP-driven H^+ pump.

To further characterize this H^+ pump, we tested putative inhibitors. As indicated in Table 2, NEM inhibits the initial rate of intravesicular alkalinization (H^+ efflux) by about 40%. DCCD, at 0.25 mM, and 0.1 mM PCMB cause greater than 80% inhibition. The inhibitors of ($Na^+ + K^+$)-ATPase and Ca^{2+}-ATPase, vanadate, of alkaline phosphatase, levamisole, and of the mitochondrial H^+-ATPase, oligomycin, did not decrease ATP-driven H^+ efflux. The inhibitory pattern of ATP-driven H^+ efflux fits completely that of the detergent-exposed ATPases at the inside of the brush-border membrane (*cf.* Table 1). The greater effect of DCCD and PCMB on H^+ pumping than on ATPases may be due to additional effects, e.g., an action as protonophors [48], or to the presence of other DCCD- and PCMB-insensitive ATPases not related to H^+ extrusion.

Figure 5, top, shows the influence of anions present inside and outside the ATP-loaded vesicles on H^+ extrusion. Weakly permeant anions like gluconate or sulfate inhibit intravesicular alkalinization as compared to chloride. This finding suggests that H^+ extrusion is electrogenic and requires anion exit for charge compensation. This interpretation is supported by data shown in Fig. 5, bottom. In the

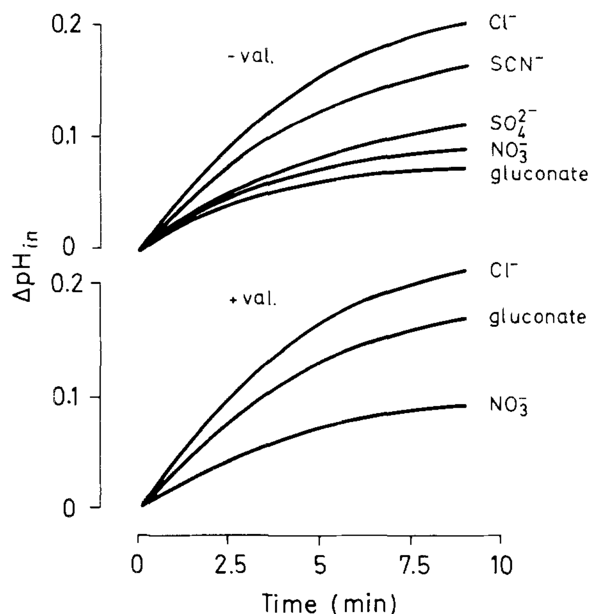

Fig. 5. Effect of anions on ATP-driven intravesicular alkalinization. *Top:* Membrane vesicles were loaded with ATP, ATP-regenerating system, 6-carboxyfluorescein, 150 mM of the indicated anions as K^+ salts, 5 mM $MgCl_2$, 20 mM HEPES/Tris, pH 7.0. H^+ efflux was measured in buffers of identical salt composition (no anion gradients present). *Bottom:* The buffers contained in addition 10 μM valinomycin

presence of a K^+ ionophor, valinomycin, inhibition of intravesicular alkalinization by gluconate is much smaller because K^+ entry can compensate for electrogenic H^+ efflux. Control experiments (*not shown*) revealed that valinomycin stimulates H^+ efflux only in the presence of K^+ ruling out nonspecific effects of the ionophor on the H^+ pump. The inhibition by thiocyanate and nitrate cannot be overcome by valinomycin indicating a direct inhibition of the H^+ pump, which is in agreement with the effect of these anions on detergent-exposed ATPases (*cf.* Table 1).

Since data shown in Fig. 5 suggested that the H^+ pump is electrogenic, we looked for the effect of D-glucose and L-phenylalanine on H^+ extrusion. Both substrates are cotransported with Na^+ across the brush-border membrane and thereby carry positive charges into the vesicles. If H^+ pump and Na^+-coupled substrate transporters are localized in the same membrane, D-glucose and L-phenylalanine should provide the charge compensating counterion (Na^+) during electrogenic H^+ efflux. Table 3 shows that the initial rate of intravesicular alkalinization is accelerated by D-glucose and by L-phenylalanine by 49–57%. Both substrates are ineffective in the absence of Na^+ ruling out nonspecific effects of ATP-driven H^+ efflux. These data reveal the coexistence

Table 3. The effect of D-glucose and L-phenylalanine on ATP-driven H^+ extrusion

Salt	Substrates	Initial rate of alkalinization (%)	
		Control	Experimental
KCl	D-glucose	100.0 ± 11.8 (5)	94.2 ± 10.0 (6)
NaCl	D-glucose	100.0 ± 18.1 (20)	149.2 ± 30.9 (18)[a]
KCl	L-phenylalanine	100.4 ± 8.7 (3)	98.8 ± 9.1 (3)
NaCl	L-phenylalanine	100.0 ± 19.3 (12)	156.7 ± 22.8 (7)[a]

Intravesicular alkalinization was studied in vesicles loaded with ATP and either 150 mM KCl or NaCl and suspended in buffers containing 150 mM KCl or NaCl, respectively (no cation gradients present), and 0 (control) or 25 mM D-glucose or 10 mM of L-phenylalanine. The initial rates of alkalinization were determined by the fluorescence increase of trapped 6-carboxyfluorescein and controls set to 100%. Shown are mean ± SD of the number of determinations given in brackets. [a] $P < 0.001$ (experimental *vs.* respective control).

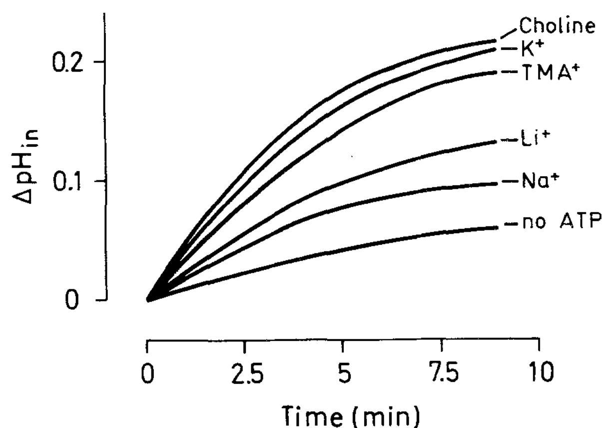

Fig. 6. The influence of cations on ATP-dependent intravesicular acidification. Vesicles were loaded with 150 mM of the indicated cations as Cl^- salts, 5 mM $MgCl_2$, 20 mM HEPES/Tris, pH 7.0, and ATP, ATP-regenerating system and 6-carboxyfluorescein. The vesicles were suspended in buffers containing the same cations as the preloading buffer (no cation gradient present)

of ATP-driven H^+ pumps and Na^+-coupled transport systems in the same vesicles.

The cation dependence of ATP-driven H^+ extrusion is displayed in Fig. 6. In the presence of chloride, which allows for charge compensation during electrogenic H^+ extrusion, highest rates of intravesicular alkalinization were observed with choline, K^+ and tetramethylammonium. Li^+ and Na^+ inhibit ATP-driven H^+ efflux although Li^+ had only a small and Na^+ no inhibitory effect on brush-border membrane ATPases. In three membrane preparations, initial intravesicular alkalinization was 61.3 ± 15.9% with Li^+ and 35.3 ± 9.0% with Na^+ of the rate observed with K^+ (means ± SD).

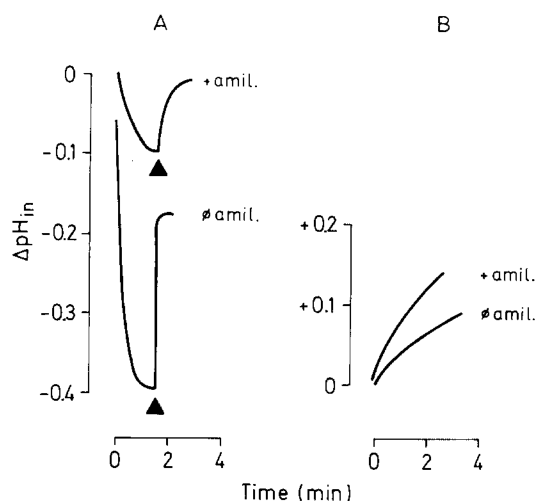

Fig. 7. Irreversible inhibition of Na^+/H^+ exchange (*A*) and stimulation of ATP-driven intravesicular alkalinization (*B*) after photoaffinity labeling with amiloride. Brush-border membrane vesicles prepared in 150 mM KCl, 10 mM HEPES/Tris, pH 7.0, were incubated with 0 or 5 mM amiloride and irradiated at 350 nm for 10 min in a photoreactor [23]. Unbound amiloride was removed by washing the vesicles in buffers containing 100 mM NaCl and 50 mM KCl. Then, the vesicles were preloaded with ATP, ATP-regenerating system, and dye in 100 mM NaCl/50 mM KCl. For further details, *see* legend to Fig. 4. Immediately after freeze/thawing, the vesicles were used to demonstrate ATP-driven H^+ extrusion (*B*). Approximately 3 hr later, when trapped ATP was hydrolyzed, further aliquots of the vesicles were suspended in Na^+-free buffers (100 mM tetramethylammonium chloride, 50 mM KCl) and Na^+ efflux-driven intravesicular acidification (= Na^+/H^+ exchange) was studied (*A*). Arrow heads: addition of NaCl to the cuvette

The inhibitory effects of Na^+ and Li^+ suggest the coexistence of H^+ pump and $Na^+(Li^+)/H^+$ exchanger in the same vesicles: protons pumped out by the ATPase cycle back into the vesicles via the Na^+/H^+ exchanger. This effect should vanish with an inhibitor of the antiporter, amiloride. However, amiloride present in solution quenches the fluorescence of 6-carboxyfluorescein and therefore its action cannot be investigated directly. Fortunately, it is possible to use amiloride similarly to its 6-bromo-derivative [23] as a photolabel to irreversibly inactivate Na^+/H^+ exchange. To this end, brush-border vesicles were irradiated with UV light for 10 min in the absence or presence of amiloride. After washing off unbound amiloride, vesicles were loaded with Na^+, ATP, ATP-regenerating system, and 6-carboxyfluorescein. Figure 7*A*, shows that ATP-depleted, Na^+-loaded vesicles irradiated without amiloride acidify their interior when suspended in a Na^+-free medium (Na^+/H^+ exchange). This acidification is strongly reduced in vesicles irradiated with amiloride, indicating irreversible inhibition of the Na^+/H^+ exchanger.

In the absence of an outward Na^+ gradient ($Na^+_{in} = Na^+_{out}$), ATP-loaded vesicles alkalinize their interior (Fig. 7*B*). Alkalinization in vesicles irradiated with UV light proves that the ATP-driven H^+-pump is still operative and not impaired by light. Following irradiation with amiloride, vesicles exhibit an increased alkalinization. In three preparations, vesicles irradiated with amiloride alkalinized their interior with 153.8 ± 26.5% of the rate observed with vesicles irradiated without amiloride (mean ± SD; $n = 14$; $P < 0.001$). Obviously, the irreversible inhibition of Na^+/H^+ exchange in these vesicles prevents back-cycling of protons. This finding indicates the coexistence of ATP-driven H^+ pump and Na^+/H^+ exchanger in the same vesicles.

Discussion

ATPases at the Outside of the Brush-Border Membrane

Brush-border membrane vesicles are impermeant to ATP and largely oriented right side out [6, 19, 21]. Therefore, hydrolysis of ATP added externally to intact vesicles indicates the presence of one or more ATPases at the outside of the brush-border membrane. These enzymes are stimulated by Ca^{2+} and Mg^{2+} and hydrolyse several nucleotides. They are hardly influenced by monovalent cations and anions and are insensitive to oligomycin, ouabain, vanadate, levamisole, and NEM. The only potent inhibitor found so far is the carboxyl group reagent, DCCD.

The properties of the ATPase(s) at the outside of the brush-border membrane resemble closely those described for ecto-ATPases in a variety of cells. All these ecto-enzymes are stimulated by Mg^{2+} and Ca^{2+}, hydrolyse several nucleotides, and are insensitive to inhibitors of mitochondrial H^+-ATPase and cation translocating ATPases of plasma membranes [14, 18, 28, 29, 40, 42]. Most of the ecto-ATPases are weakly inhibited or completely resistant to NEM [28, 29, 42], some are considerably inhibited, although only at NEM concentrations 10-fold higher than those applied in our study [18]. DCCD proved as a weak [28] or potent inhibitor [29, 42] of ecto-ATPases. Similar properties lead us to conclude that ATPase(s) at the outside of the brush-border membrane belong to a family of ecto-enzymes found in many cells. The role of these enzymes is not yet known.

An ATPase activity with characteristics similar to ecto-ATPases was also found in renal basolateral membranes. These enzymes are most probably also

located at the outside of the basolateral membrane, although we cannot prove this assumption, since our basolateral membrane vesicles show a random orientation. Triton X-100, which at higher concentration inhibits the ecto-ATPases in brush-border membranes, had a much smaller effect on ATPase activity in basolateral membranes. The reason for this difference is not known.

An activation by Ca^{2+} [5, 20, 31, 32, 44, 45] and by other nucleotides besides ATP [13, 31, 44, 45] has also been observed in earlier experiments on ATPases in cortex homogenates, isolated tubules, and membrane vesicles, suggesting that many of the previous investigations including partial purification [44] have been performed on ecto-ATPases. Our data showing the presence of Mg^{2+}-ATPases in both brush-border and basolateral membranes are in agreement with some earlier investigations [30, 53] and disagree with others, which have demonstrated a Ca^{2+} or Mg^{2+}-activated ATPase exclusively in the luminal [20] or contraluminal [34] membrane of proximal tubule cells. There is at present no explanation for the discrepancy between our work and that of van Erum et al. [20]. The discrepancy to the findings of Kinne-Saffran and Kinne [34], however, is only apparent. These authors used a detergent which, similar to Triton X-100, may have inactivated ecto-ATPases in luminal, but not in contraluminal membranes (*cf.* Figs. 1 and 2 in this study). Activation of the enzyme by Ca^{2+} and nucleotide specificity clearly indicate that this enzyme is in all likelihood not involved in Ca^{2+} transport as previously suggested.

ATPases at the Inside of the Brush-Border Membrane

Pretreatment of rat renal brush-border membrane vesicles with 0.1% Triton X-100 exposed additional ATPases. Their catalytic site faces the intracellular fluid, a pattern that is expected for ATPases involved in the export of intracellular protons. We believe that these ATPases have not been measured so far since previous investigators have not used detergents [35–38, 41, 43]. The key characteristics of the endo-ATPases are insensitivity to oligomycin, ouabain, vanadate, levamisole and monovalent cations. Thiocyanate and nitrate inhibited as well as DCCD and a series of NH_2- (NBD-Cl) and SH-group reagents (NEM, PCMB, PCMBS). Although the detergent-exposed ATPase activity may reflect more than one type of enzyme, the overall pattern matches those reported for ATP-driven H^+ transport and H^+-ATPases in endosomes [48–50], clathrin-coated vesicles, lysosomes, endoplasmic reticulum and Golgi apparatus [47] as well as for ATP-driven H^+ secretion in collecting ducts [25, 26] and amphibian urinary bladders [2]. These similarities suggest strongly that (part of) the ATPases at the inside of the brush-border membrane belong to the vacuolar type H^+-ATPases and are involved in H^+ transport across the membrane.

ATP-Driven H^+ Transport

Brush-border membrane vesicles loaded with ATP extrude protons and thereby alkalinize their interior. These results confirm and extend previous studies, which revealed an acidification of the extravesicular medium in the presence of ATP-loaded vesicles [33, 37]. Our approach to load a pH indicator together with ATP into the vesicles has several advantages over previous measurements with pH electrodes. Due to the relatively small intravesicular volume, H^+ translocation across the vesicles membrane changes intracellular pH much more than extravesicular pH leading to a greater sensitivity of the trapped dye method used here. Moreover, pH changes are detected only in those vesicles that have transiently opened during freeze/thawing. Vesicles that remained closed neither took up ATP nor the pH indicator. Finally, the recently described freeze/thaw method [19] made it possible to load ATP into purified brush-border membrane vesicles. Loading with ATP during tissue homogenization as used earlier does not allow for a complete, time-consuming purification of brush-border membrane vesicles since the trapped ATP is rapidly hydrolysed even in the cold [33].

ATP-driven proton extrusion was inhibited by thiocyanate, nitrate, NEM, DCCD, and PCMB. Oligomycin, vanadate and levamisole were ineffective in complete agreement with the effect of these compounds on the detergent-exposed ATPase at the inside of the brush-border membrane. NEM was only effective when loaded together with ATP into the vesicles before the onset of the H^+ efflux measurement. The insensitivity towards NEM of H^+ efflux from ATP-loaded vesicles found earlier [37] may have been caused by insufficient entry of NEM into the vesicles. Therefore, it seems unnecessary to postulate a new type of NEM-insensitive H^+ pump in the brush-border membrane. Based on similar inhibitor sensitivities, we rather postulate that (part of) the NEM-sensitive ATPases at the inside of the brush-border membrane function as ATP-driven H^+ pumps.

In agreement with previous studies [33], the stimulation of H^+ secretion by chloride or by K^+/valinomycin indicated that the H^+-ATPase is electrogenic. The H^+-ATPase shares this property with all vacuolar type ATPases and with the H^+-

ATPases from collecting ducts [25, 26] and amphibian bladders [2]. Such an electrogenic pump would also explain the bicarbonate-dependent, lumen-positive transepithelial potential difference, which was detected earlier in electrophysiological measurements on the intact kidney [24].

It could be argued that ATP-driven H^+ extrusion monitored with the trapped pH indicator did not occur in brush-border membrane vesicles but rather in vesicles of intracellular origin that contaminated our preparation. To address this problem, we tested for a colocalization of ATP-driven H^+ pump and transport systems known to be situated in the brush-border membrane. Two lines of evidence reveal the location of the H^+ pump in the brush-border membrane. First, ATP-driven H^+ extrusion was stimulated by D-glucose and L-phenylalanine in the presence of sodium. During cotransport with Na^+ across the brush-border membrane, D-glucose and L-phenylalanine provide the charge-compensating cation for electrogenic ATP-driven H^+ secretion. The stimulation is only possible if H^+-ATPase and Na^+/D-glucose or Na^+/L-phenylalanine cotransporters coexist in the same vesicle. Second, the inhibition of ATP-dependent H^+ extrusion by Na^+ and Li^+ indicated the coexistence of H^+ pump and Na^+/H^+ exchanger in the same vesicles: protons pumped out by the H^+-ATPase can cycle back into the vesicles in the presence of intravesicular Na^+ or Li^+, both being substrates of the Na^+/H^+ exchanger. The irreversible inhibition of Na^+/H^+ exchanger by photoaffinity labeling with amiloride stimulated ATP-dependent intravesicular alkalinization proving that it was the Na^+/H^+ exchanger that allowed for the back-cycling of protons.

The colocalization of the ATP-driven H^+ pump, Na^+/H^+ exchanger and Na^+-coupled substrate transporters in the same vesicles, which has so far not been demonstrated, indicates that these systems are situated in vivo in close neighborhood on the microvillus. This localization suggests that ATP-driven H^+ pumps are genuine to the brush-border membrane and do not, or only partially, originate from endosomes, which have fused with the luminal membrane. This conclusion is supported by the fact that brush-border membrane vesicles exhibit a threefold higher specific NEM-sensitive ATPase activity as compared to purified endosomes (0.74 ± 0.051 *versus* 0.25 ± 0.016 μmol P_i/min · mg protein, respectively; mean ± SD from 19 brush-border and seven endosome preparations). During acidosis, the brush-border H^+-ATPases may be supplemented by endosomal H^+-ATPases to increase proximal tubular H^+ secretion as suggested earlier [2].

In summary, our data reveal the presence of different ATPases at the outside and the inside of rat renal brush-border membranes. The NEM-sensitive ATPases located at the cytosolic side are restricted to the luminal cell side and (part of them) are responsible for NEM-sensitive H^+ secretion, which account for approximately one-third of the overall proton secretion in the mammalian proximal tubule [15–17, 46].

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