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Characterization of Inside-Out Oriented H^+-ATPases in Cholate-Pretreated Renal Brush-Border Membrane Vesicles

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Summary. Exposure of porcine renal brush-border membrane vesicles to 1.2% cholate and subsequent detergent removal by dialysis reorients almost all N-ethylmaleimide (NEM)-sensitive ATPases from the vesicle inside to the outside. ATP addition to cholate-pretreated, but not to intact, vesicles causes H^+ uptake as visualized by the ΔpH indicator, acridine orange. The reoriented H^+-pump is electrogenic because permeant extravesicular anions or intravesicular K^+ plus valinomycin enhance H^+ transport. ATP stimulates H^+ uptake with an apparent K_m of 93 μM. Support of H^+ uptake and P_i liberation by ATP > GTP ≈ ITP > UTP indicates a preference for ATP and utilization of other nucleotides at lower efficiency. ADP is a potent, competitive inhibitor of ATP-driven H^+ uptake (K_i, 24 μM). Mg^{2+} and Mn^{2+} support ATP-driven H^+ uptake, but Ca^{2+}, Ba^{2+} and Zn^{2+} do not. 1 mM Zn^{2+} inhibits MgATP-driven H^+ transport completely. NEM-sensitive P_i liberation is stimulated by Mg^{2+} and Mg^{2+} and, unlike H^+ uptake, also by Ca^{2+} suggesting Ca^{2+}-dependent ATP hydrolysis unrelated to H^+ transport. The inside-out oriented H^+-pump is relatively insensitive toward oligomycin, azide, N,N′-dicyclohexylcarbodiimide (DCCD) and vanadate, but efficiently inhibited by NEM (apparent K_i, 0.77 μM), and 4-chloro-7-nitro-benzoxa-1,3-diazole (NBD-Cl; apparent K_i, 0.39 μM). Taken together, the H^+-ATPase of proximal tubular brush-border membranes exhibits characteristics very similar to those of "vacuolar type" (*V*-type) H^+-ATPases. Hence, *V*-type H^+-ATPases occur not only in intracellular organelles but also in specialized plasma membrane areas.

Key Words proximal tubule · brush border · H^+-ATPase · vacuolar

Introduction

Salt and water absorption in the proximal tubules of mammalian kidneys is intimately related to secretion of hydrogen ions (H^+) into the tubule lumen. To account for H^+ secretion, proximal tubule cells possess two types of H^+-transporting systems in their brush-border membrane: Na^+/H^+ exchangers and ATP-driven H^+-pumps (H^+-ATPases). The well-characterized Na^+/H^+ exchanger [2, 22] is responsible for the majority of H^+ transport across the brush-border membrane, whereas ATP-driven H^+-pumps are involved in a minor, Na^+-independent fraction of H^+ secretion.

The characteristics of the ATP-driven H^+-pumps have been studied with ATP-loaded brush-border membrane vesicles isolated from rat kidney [19, 20, 31]. H^+ efflux from these vesicles was inhibited by a carboxyl group reagent, N,N′-dicyclohexylcarbodiimide (DCCD). With respect to a sulfhydryl group reagent, N-ethylmaleimide (NEM), previous results are inconsistent since either no effect on H^+ efflux from ATP-loaded vesicles [19, 20] or an inhibition [31] was observed. The failure to demonstrate inhibition of H^+ efflux, however, was most likely due to the fact that NEM acts only from the inside of the brush-border membrane vesicles and has to be trapped intravesicularly before the onset of the experiment.

Searching for ATP hydrolysis possibly related to ATP-driven H^+ efflux we found DCCD- and NEM-blockable ATPase activities at the inside of brush-border vesicles [31]. Sensitivity to inhibitors, preference for ATP over other nucleotides, and activation by Mg^{2+} and Mn^{2+} suggested that these ATPases belong to the class of "vacuolar" H^+-ATPases (*V*-ATPases [11, 23, 28]). Although this conclusion is supported by immunohistological studies revealing the presence of putative H^+-ATPase subunits in the brush-border membrane [7] a functional proof requires detailed comparison of ATP-driven H^+ transport and ATPase activities in renal vesicles with characteristics of vacuolar H^+-ATPases from other sources. Such a study includes the determination of specificities and apparent affinities of the H^+-pump for nucleotides, divalent cations, and various inhibitors.

So far, detailed kinetic studies have not been possible because brush-border membrane vesicles are oriented right side out [9] and thus must be preloaded with divalent cations, ATP, and an ATP-

regenerating system to induce H^+ efflux. In this setting, the exact amount of intravesicular ATP is not known and, in addition, decreases rapidly with time due to hydrolysis. Similarly, the intravesicular concentrations of divalent cations and inhibitors are unknown. We have, therefore, developed a method to reorient the H^+-pump so that ATP added from the incubation medium causes uptake of hydrogen ions, i.e., an intravesicular acidification. With this experimental approach the extravesicular concentrations of all effectors of ATP-driven H^+ uptake can be easily controlled and maintained. The results, part of which has been published in preliminary form [8], support strongly the notion that the H^+-ATPase from renal brush-border membranes is a *V*-type ATPase.

Materials and Methods

Vesicle Preparation

Brush-border membrane vesicles were prepared from the cortex of pig kidneys by the Mg^{2+} precipitation method [4] and preloaded with 150 mM KCl, 5 mM HEPES, buffered with Tris to pH 7.0. Protein concentrations as determined according to Bradford [6] with bovine serum albumin as a standard was adjusted to 10 mg/ml, and vesicles were stored in liquid nitrogen. The purity of the membranes was occasionally checked by determination of the specific activity of the leucine aminopeptidase which was enriched 15.1 ± 1.9-fold over the starting homogenate in five preparations. In the same preparations, the specific activity of the $(Na^+ + K^+)$-ATPase, a marker for basolateral membranes, was 0.95 ± 1.0-fold of the starting homogenate proving the selective enrichment of brush-border membranes.

Detergent Treatment

At the day of the experiment, brush-border membrane vesicles were rapidly thawed at 37°C, cooled down again on ice and diluted with KCl buffer to a protein concentration of 3 mg/ml. Na cholate was added dropwise from a 10% (wt/vol) stock solution in water until the suspensions contained 1.2% (wt/vol) detergent. The probes were warmed up to 37°C for 1 min in order to complete solubilization. Then, the detergent was removed by overnight dialysis against a 100-fold volume of preloading buffer (150 mM KCl, 5 mM HEPES/Tris, pH 7.0) in Visking 8/32 dialysis tubes. In experiments not shown 1.2% cholate was found to be optimal for reorientation of H^+-pumps.

ATP-Driven H^+ Uptake

Intravesicular acidification due to ATP-driven H^+ uptake was monitored by the absorbance decrease of the ΔpH indicator, acridine orange, as detailed previously [26]. If not indicated otherwise, cholate-pretreated membranes (25–60 μg protein) were added to a cuvette containing 1 ml buffer with 150 mM KCl, 5 mM $MgCl_2$, 6 μM acridine orange, and 50 mM HEPES/Tris, pH 7.0. When the absorbance of the dye as measured by dual-wavelength photometry (492–540 nm) had stabilized, 20 μl of a 100 mM Tris-ATP solution (pH 7.0; final ATP concentration in the cuvette: 2 mM) was added and the absorbance decrease recorded. Inhibitors and ionophores were added to the vesicle suspensions 15 min or shortly before the onset of the experiment, respectively. Controls received the same amount of solvent (water for vanadate, azide, PCMBS, or ethanol for valinomycin, oligomycin, DCCD, NEM and NBD-Cl).

ATPase Assay

The ATPase activity was determined as in [31]. Cholate-pretreated vesicles (2 mg protein/ml) were either exposed to 0.1% Triton X-100 for 15 min at room temperature to gain access to intravesicular ATPases or received no detergent. Thereafter, they were diluted 10-fold with a buffer containing (final concentrations): 150 mM KCl, 5 mM $MgCl_2$, 50 mM HEPES/Tris, pH 7.0, and 0 or 100–200 μM NEM, as well as 0.5 mM vanadate, 2 mM azide, and 10 μg/ml oligomycin (to inhibit nonrelated ATPase activities). 15 min later the reaction was started by addition of Tris-ATP (final concentration 5 mM) and continued for 15 min at 37°C. After stopping the reaction with TCA, the liberated phosphate was determined colorimetrically [10].

Statistics

Two to four determinations were performed for each experimental condition. The results from three different membrane preparations were pooled and are shown as means ± SD. The statistical significance of differences was determined by Student's *t* test for unpaired data.

Materials

Nucleotides were purchased from Boehringer (Mannheim, FRG). Oligomycin was from Serva (Heidelberg, FRG). N,N'-dicyclohexylcarbodiimide (DCCD) was from Aldrich (Weinheim, FRG). Sodium cholate, N-ethylmaleimide (NEM), and 4-chloro-7-nitro-benzoxa-1,3-diazole (NBD-Cl) were obtained from Sigma (Munich, FRG). Vanadate was purchased from Merck (Darmstadt, FRG). The chemicals used in this study were of analytical grade.

Results

Reorientation of H^+-Pumps

The ΔpH indicator, acridine orange, was used to monitor ATP-driven intravesicular acidification in native and detergent-pretreated pig renal brush-border membrane vesicles. Addition of ATP to a suspension of intact vesicles hardly changes the absorbance of acridine orange. Likewise, addition of K^+ and H^+ ionophores, valinomycin and carbonylcyanide *p*-chloromethoxyphenylhydrazone (CCCP), to collapse a H^+-pump-generated ΔpH

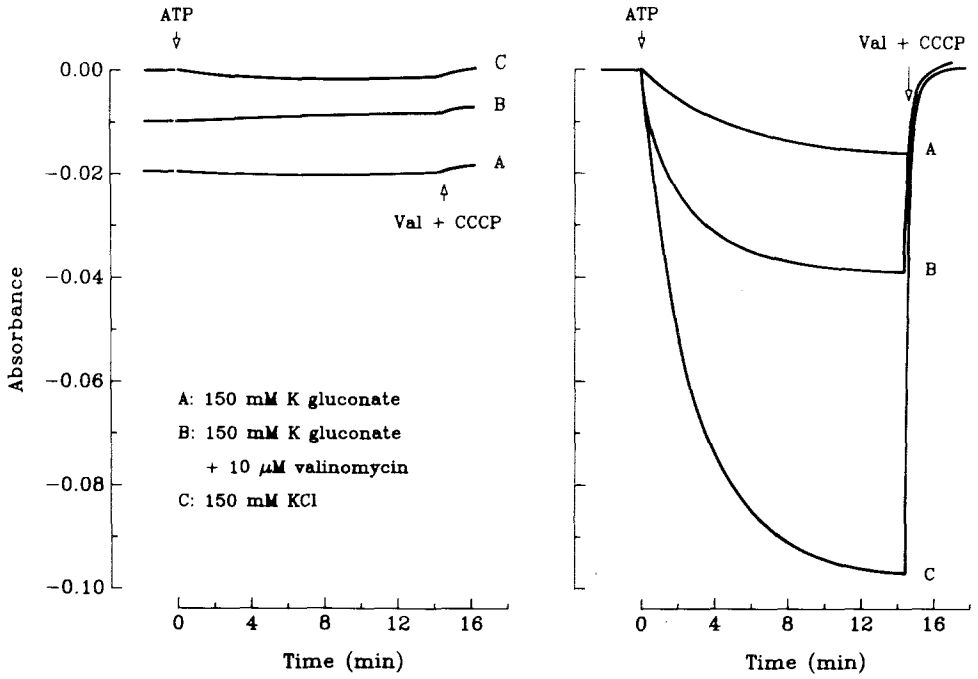

Fig. 1. ATP-driven H^+ uptake into porcine renal brush-border membrane vesicles before (left panel) and after transient exposure to cholate (right panel). Left panel: Pig renal brush-border membrane vesicles were preloaded with 150 mM K gluconate, 5 mM HEPES/Tris, pH 7.0, and added to buffers containing 150 mM K gluconate (A), 150 mM K gluconate plus 10 μM valinomycin (B), or 150 mM KCl (C). All buffers contained also 5 mM $MgSO_4$, 50 mM HEPES/Tris, pH 7.0, and 6 μM acridine orange. At the time indicated (ATP↓) 2 mM ATP was added to start H^+ uptake. Fifteen min later, 5 μM valinomycin (Val) and 2 μM carbonylcyanide *p*-chloromethoxyphenylhydrazone (CCCP) were added to dissipate the created ΔpH. Right panel: Porcine renal brush-border membrane vesicles were treated with 1.2% cholate and the detergent was then removed by overnight dialysis. Other conditions are as described for the left panel. For each curve, 60 μg protein (intact or cholate-pretreated) was used. Shown is a representative experiment

results in a very small increase in absorbance (Fig. 1, left). This result suggests that almost all intact vesicles are oriented right side out with ATP-splitting sites of H^+-pumps at the inside. In these vesicles, ATP cannot cause uptake of protons.

Vesicles that have been transiently exposed to cholate show a different picture. The absorbance of acridine orange clearly decreases when ATP is added to cholate-pretreated vesicles (Fig. 1, right). Valinomycin + CCCP revert the signal proving uptake of H^+ into an intravesicular space and creation of a transmembrane ΔpH. Thus, cholate pretreatment must have reoriented H^+-pumps from the inside of intact vesicles to the outside of detergent-exposed vesicles. Also shown in Fig. 1 is a stimulation of H^+ uptake by valinomycin (in the presence of K gluconate) and by chloride replacing gluconate in the extravesicular medium. These data reveal the electrogenicity of the reoriented ATP-driven H^+-pump which requires K^+ exit (via valinomycin) or entry of permeant anions, e.g., Cl^-, for charge compensation.

In order to estimate which fraction of H^+-ATPases was reoriented we measured the NEM-sensitive ATPase activities in intact and cholate-pretreated vesicles. Triton X-100 was included in some of the measurements to gain access to ATPases at the vesicle inside [31]. Intact vesicles (not pretreated with cholate) hydrolyse ATP in a largely NEM-independent fashion. This ATP hydrolysis is most probably due to NEM-insensitive ATPases located at the outside of the vesicle's membrane (Table 1, first line). With Triton X-100 total ATPase increases from 0.237 to 0.403 μmol P_i/mg · min suggesting exposure of additional enzymes located at the inside of the vesicles. These Triton-exposed ATPases are strongly inhibited by NEM (Table 1, second line). The ATP hydrolysis remaining in the presence of NEM is smaller with Triton (0.072 μmol P_i/mg · min) than without (0.212 μmol P_i/mg · min) revealing a strong inhibitory effect of Triton on NEM-insensitive ATPases. Thus, as found earlier for the rat kidney [31], Triton has two effects: it inhibits NEM-insensitive ATPases at the outside and exposes NEM-sensitive ATPases at the inside (cytoplasmic side) of pig renal brush-border membrane vesicles.

After a transient exposure to cholate the vesi-

Table 1. NEM-sensitive ATPase in intact and cholate-pretreated porcine renal brush-border membrane vesicles

Triton X-100	ATPase (μmol P_i/mg · min)		
	−NEM	+NEM	ΔNEM
A: Intact vesicles			
−	0.237 ± 0.050	0.212 ± 0.036	0.025 ± 0.036
+	0.403 ± 0.040	0.072 ± 0.023	0.331 ± 0.023[a]
B: Cholate-pretreated vesicles			
−	0.331 ± 0.045	0.037 ± 0.007	0.294 ± 0.008
+	0.349 ± 0.042	0.012 ± 0.014	0.337 ± 0.014[a,b]

ATPase activity as determined by the liberation of phosphate was measured in intact vesicles (A) and in vesicles from the same preparations that had been transiently solubilized with cholate (B). Intact and cholate-pretreated vesicles were exposed to 0 or 200 μM N-ethylmaleimide (NEM) and 0 or 0.1% Triton X-100 for 15 min at room temperature. All measurements were performed in the presence of azide, oligomycin and vanadate to block nonrelated ATPase activities. Shown are means ± SD from 12 measurements (three preparations).

[a] $P < 0.001$ (+ Triton *versus* − Triton).

[b] Not significantly different (intact vesicles *versus* cholate-pretreated vesicles).

cles exhibit NEM-sensitive ATPase activity which is measurable even without Triton (Table 1, third line). With Triton, the amount of NEM-sensitive ATPase activity increases only by 14% suggesting that cholate treatment reorients most of NEM-sensitive ATPases to the outside of the vesicles (Table 1, fourth line). The activities of NEM-sensitive ATPases measured in the presence of Triton X-100 are equal in intact and cholate-pretreated membranes, suggesting a complete recovery of these enzymes. In contrast, the NEM-insensitive ATPases have been largely inactivated as shown by the small ATP hydrolysis remaining in the presence of NEM.

The activities of marker enzymes were completely recovered similar to NEM-sensitive ATPases. The specific activities of leucine aminopeptidase (LAP), alkaline phosphatase and γ-glutamyltranspeptidase were 91.3 ± 17.9%, 98.8 ± 14.5%, and 91.8 ± 25.8%, respectively, of the activities measured in the intact vesicles before cholate treatment (means ± SD from 6–8 determinations performed on three preparations).

Anion Dependence of the Reoriented H^+-Pump

The electrogenic H^+-pump can be stimulated by extravesicular chloride (*cf.* Fig. 1). The effect of other extravesicular anions on ATP-driven H^+ uptake into cholate-pretreated vesicles is shown in Table 2. H^+ uptake is lowest with gluconate, sulfite and sulfate, intermediate with chloride, bromide, iodide and nitrate, and highest with thiocyanate. Stimulation of H^+ uptake seems to increase with increasing permeability of the anions and, hence, with their ability to allow for charge compensation for pumped hydrogen ions. Whether the anions have also direct stimulatory or inhibitory effects on the pump was not tested further.

Table 2. Effect of external anions on ATP-driven H^+ uptake into cholate-pretreated vesicles

Anion (25 mM)	H^+ uptake (% of control)	*n*
Sulfate	33.45 ± 9.73	9
Gluconate	41.02 ± 7.74	9
Sulfite	58.56 ± 13.12	9
Chloride (control)	100.0 ± 7.80	10
Iodide	100.11 ± 19.40	9
Bromide	111.47 ± 26.88	9
Nitrate	124.13 ± 22.56	13
Thiocyanate	293.65 ± 53.40	9

Cholate-pretreated vesicles (30 μg protein/assay) were added to buffers containing 25 mM K salts of the indicated anions, 125 mM K gluconate, 5 mM $MgSO_4$, 5 mM HEPES/Tris, pH 7.0, and 6 μM acridine orange. The absorbance decrease of acridine orange upon addition of 2 mM ATP was recorded and expressed in % of the absorbance changes observed with 25 mM chloride. The data are collected from three preparations using *n* determinations.

Nucleotide Specificity

Figure 2 shows that the absorbance changes of acridine orange and, thus, H^+ uptake are a hyperbolic function of ATP concentration. The inset (a Lineweaver-Burk diagram) reveals a single, saturable mechanism with an apparent K_m of 93 μM ATP.

The nucleotide specificity of H^+ uptake is depicted in Table 3. The highest uptake occurs with 5 mM ATP. With 5 mM each of GTP and ITP, roughly one-third, and with UTP a tenth, of the activity is observed, suggesting that these nucleotides are also accepted by the H^+-ATPase. CTP does not support H^+ uptake. Also shown in Table 3 are combinations of nucleotides. H^+ uptake with 0.5 mM ATP plus 5 mM of any other nucleotide is smaller than with 0.5 mM ATP alone. The degree of inhibition of H^+ uptake decreases in the order GTP > ITP > UTP ≈ CTP. The inhibitions indicate that besides ATP also GTP, ITP, UTP and CTP interact with the pump resulting in a retardation of H^+ uptake.

To support a possible interaction of other nucleotides with the H^+-pump we measured NEM-sensitive liberation of inorganic phosphate from ATP,

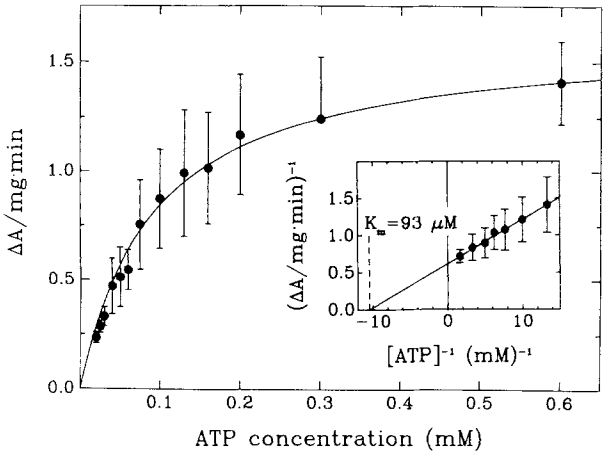

Fig. 2. Dependence of H^+ uptake into cholate-pretreated vesicles on ATP concentration. Brush-border membrane vesicles were exposed to cholate and dialyzed against 150 mM KCl, 5 mM HEPES/Tris, pH 7. Thirty μg vesicle protein was added to 1 ml buffer containing 150 mM KCl, 5 mM $MgCl_2$, 50 mM HEPES/Tris, pH 7, and 6 μM acridine orange. H^+ uptake was started by addition of ATP in concentrations indicated on the abscissa. The ordinate shows the initial rate of H^+ uptake as determined from the absorbance decrease of acridine orange (ΔA/mg · min). The *inset* is a Lineweaver-Burk diagram of the same data. Each point is the mean ± SD from six determinations (three preparations)

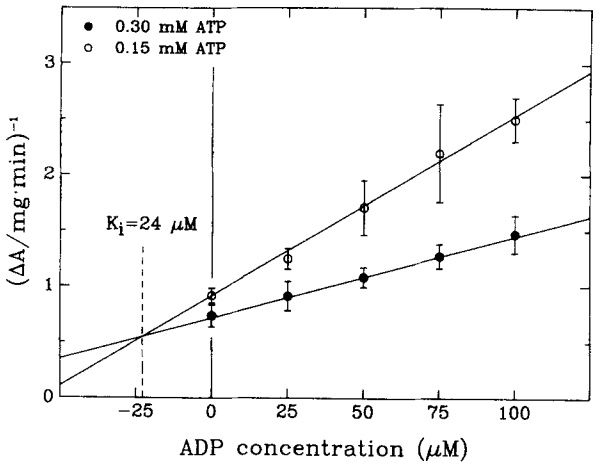

Fig. 3. Inhibition of ATP-driven H^+ uptake by ADP. To cholate-pretreated vesicles suspended in KCl buffer with acridine orange (*see* legend to Fig. 2) were added 0.15 (open circles) or 0.3 mM ATP (filled circles) and ADP in concentrations indicated on the abscissa. The reciprocals of the resulting absorbance decrease of acridine orange are depicted on the ordinate (Dixon plot). The data are pooled from three preparations (means of six determinations ± SD)

GTP, UTP, ITP, and CTP with cholate-pretreated vesicles. The highest rate of NEM-sensitive P_i liberation is observed with ATP as substrate (Table 4). With GTP, ITP, and UTP approximately half of the rate, and with CTP a tenth, is seen as compared to

Table 3. Nucleotide specificity of the reoriented H^+-pump

Addition	H^+ uptake		*n*
	(ΔA/mg · min)	(% of control)	
A: Activation by 5 mM nucleoside triphosphates			
ATP	1.06 ± 0.10	100 ± 9.43	6
GTP	0.37 ± 0.02[a]	34.9 ± 1.89[a]	6
ITP	0.31 ± 0.04[a]	29.2 ± 3.77[a]	6
UTP	0.12 ± 0.02[a]	11.3 ± 1.89[a]	5
CTP	0	0	3
B: Combination of 0.5 mM ATP and 5 mM nucleoside triphosphates			
ATP	1.20 ± 0.20	100 ± 16.7	9
ATP + GTP	0.52 ± 0.08[a]	43.3 ± 6.67[a]	9
ATP + ITP	0.71 ± 0.12[a]	59.2 ± 10.0[a]	9
ATP + UTP	0.85 ± 0.14[a]	70.8 ± 11.7[a]	9
ATP + CTP	0.89 ± 0.08[a]	74.2 ± 6.67[a]	9

A: To cholate-pretreated brush-border membranes (25 μg protein/assay) suspended in buffers containing 150 mM KCl, 5 mM $MgCl_2$, 5 mM HEPES/Tris, pH 7.0, and 6 μM acridine orange, 5 mM of the indicated nucleotides were added. B: Vesicles treated as above received 0.5 mM ATP together with 5 mM of the indicated nucleotides. The initial absorption decrease of acridine orange (H^+ uptake) following nucleotide addition was determined and expressed as ΔA/mg protein · min or in % of the respective controls. Shown are means ± SD from *n* determinations (three preparations).
[a] $P > 0.001$ *versus* ATP.

ATP. This finding suggests that the NEM-sensitive H^+-ATPase accepts and hydrolyses a variety of nucleotides although at different efficiencies.

During the experiments on nucleotide specificity of the H^+-pump we found a complete inhibition of ATP-driven H^+ uptake by 5 mM ADP. To determine the kinetics of this inhibition H^+ uptake was studied with two ATP concentrations and various ADP concentrations. The data as plotted in a Dixon diagram (Fig. 3) reveal two straight lines that intersect above the abscissa suggesting a competitive inhibition of ATP-driven H^+ uptake by ADP. The K_i as determined from the intersection point is 24 μM, indicating a high affinity of the H^+-pump for ADP.

Specificity for Divalent Cations

Full activity of ATP-driven H^+ uptake requires the presence of Mg^{2+} as can be seen from Table 5,*A*. Also at 5 mM concentration, Mn^{2+} supports 82% of the activity seen with Mg^{2+}. The cations Ca^{2+}, Ba^{2+} and Zn^{2+} do not stimulate H^+ uptake. In Table 5,*B* the combined affects of 0.5 mM Mg^{2+} and 5 mM of the other cations is shown. ATP-driven H^+ uptake is not significantly changed when 5 mM of Mn^{2+}, Ca^{2+} or Ba^{2+} are offered together with 0.5 mM

Table 4. Nucleotide-specificity of the NEM-sensitive ATPase in cholate-pretreated vesicles

Nucleotide	ATPase (μmol P_i/mg · min)			
	−NEM	+NEM	ΔNEM	%
ATP	0.304 ± 0.042	0.057 ± 0.009	0.252 ± 0.046	100 ± 18.0
GTP	0.198 ± 0.025	0.059 ± 0.006	0.139 ± 0.025	54.8 ± 10.0[a]
ITP	0.197 ± 0.020	0.068 ± 0.007	0.129 ± 0.020	51.0 ± 7.83[a]
UTP	0.189 ± 0.031	0.067 ± 0.006	0.122 ± 0.031	48.4 ± 12.3[a]
CTP	0.091 ± 0.020	0.059 ± 0.006	0.033 ± 0.020	13.1 ± 7.98[a]

Phosphate liberation was determined with cholate-pretreated vesicles in the presence of 5 mM Mg^{2+} and 5 mM of the indicated nucleotides. 15 min before the start of the reaction, the vesicles were exposed to 0 or 100 μm N-ethylmaleimide (NEM). Phosphate release is expressed in μmol/min · mg protein (first three columns). The last column shows the NEM-sensitive activity in % of the control (ATP). Each datum is the mean ± SD of 10–12 determinations (pooled from three preparations).
[a] $P < 0.001$ with respect to ATP.

Table 5. Specificity of the reoriented H^+-pump for divalent cations

Addition	H^+ uptake		n
	(ΔA/mg · min)	(% of control)	
A: Activation by 5 mM divalent cations			
Mg^{2+}	1.0 ± 0.11	100 ± 6.23	6
Mn^{2+}	0.82 ± 0.07[a]	82.0 ± 3.43[a]	6
Ca^{2+}	0	0	6
Ba^{2+}	0	0	6
Zn^{2+}	0	0	6
B: Combination of 0.5 mM Mg^{2+} and 5 mM divalent cations			
Mg^{2+}	0.91 ± 0.13	100 ± 4.86	10
Mg^{2+} + Mn^{2+}	0.91 ± 0.15 NS	100 ± 7.13 NS	8
Mg^{2+} + Ca^{2+}	0.82 ± 0.14 NS	88.6 ± 6.08 NS	9
Mg^{2+} + Ba^{2+}	0.82 ± 0.10 NS	88.6 ± 5.02 NS	8
Mg^{2+} + Zn^{2+}	0	0	6

A: To cholate-pretreated brush-border membranes suspended in buffers containing 150 mM KCl, 5 mM ATP, 5 mM HEPES/Tris, pH 7.0, and 6 μM acridine orange, 5 mM of the indicated divalent cations were added as chloride salts. B: Vesicles treated as above received 0.5 mM $MgCl_2$ together with 5 mM of the indicated divalent cations. The initial absorption decrease of acridine orange (H^+ uptake) following nucleotide addition was determined and expressed as ΔA/mg protein · min or in % of the respective controls. Shown are means ± SD from n determinations (three preparations).
NS: not statistically significant.
[a] $P < 0.01$ (control : Mg^{2+}).

Mg^{2+}. The inability of Ca^{2+} and Ba^{2+} to support H^+ uptake in the absence of Mg^{2+}, and to inhibit it in the presence of Mg^{2+}, suggests that these divalent cations do not interfere with ATP-driven H^+ translocation. In contrast, Zn^{2+} completely inhibits ATP-driven H^+ uptake in the presence of Mg^{2+}.

When the requirement for divalent cations was tested also for NEM-sensitive P_i liberation, i.e., H^+-ATPase activity, the results shown in Table 6 were obtained. Mn^{2+} stimulates P_i liberation better than Mg^{2+}. Stimulation by Ca^{2+} of NEM-sensitive ATPase activity amounts to 46% of that seen with Mg^{2+}, whereas Ba^{2+} produced less than a tenth of activity. It is obvious that the degrees of stimulation of H^+-uptake and of NEM-sensitive ATPase activity differ for the various divalent cations. It is possible that Mn^{2+} and Ca^{2+} support a partial reaction of the enzyme, i.e., ATP hydrolysis without H^+ transport. Alternatively, other NEM-sensitive ATPases are present which are stimulated by Mn^{2+} and Ca^{2+} and are not involved in H^+ transport.

Five mM of Zn^{2+} abolished ATP-driven H^+ uptake into cholate-pretreated vesicles (*cf.* Table 5). The kinetics of Zn^{2+} inhibition are shown in Fig. 4. Zn^{2+} inhibits ATP-driven H^+ uptake completely at 1 mM concentration. The Dixon representation of the data (*inset*) does not reveal a straight line indicating a complex action of Zn^{2+} on the H^+-pump.

Sensitivity to Inhibitors

To further characterize the H^+-pump in cholate-pretreated vesicles we tested the effect of putative inhibitors. Table 7 summarizes the results of these experiments. Azide and oligomycin at concentrations greater than those required for complete inhibition of the mitochondrial ATP synthase (F_0F_1 ATPase) decrease ATP-driven H^+ uptake by roughly 25%. N,N′-dicyclohexylcarbodiimide (DCCD) which inhibits F_0F_1 ATPases at 1–2 μM [11], also decreases ATP-driven H^+ uptake although only at relatively high concentrations (200 μM). Vanadate which at 0.1 mM completely inhibits phosphorylating ATPases as, e.g., the (Na^+ + K^+)-

Table 6. Dependence on divalent cations of the ATPase in cholate-pretreated vesicles

Cation	ATPase (μmol P_i/mg · min)			
	−NEM	+NEM	ΔNEM	%
Mg^{2+}	0.295 ± 0.022	0.041 ± 0.010	0.254 ± 0.025	100 ± 9.82
Mn^{2+}	0.406 ± 0.082	0.018 ± 0.009	0.387 ± 0.038	152.3 ± 15.0[a]
Ca^{2+}	0.158 ± 0.022	0.041 ± 0.008	0.117 ± 0.022	45.8 ± 8.77[a]
Ba^{2+}	0.038 ± 0.014	0.020 ± 0.012	0.017 ± 0.012	6.6 ± 4.78[a]

Phosphate liberation was determined with cholate-pretreated vesicles in the presence of 5 mM ATP and 5 mM of the indicated divalent cations. 15 min before the start of the reaction, the vesicles were exposed to 0 or 100 μm N-ethylmaleimide (NEM). Phosphate release is expressed in μmol/min · mg protein (first three columns). The last column shows the NEM-sensitive activity in % of the control (ATP). Each datum is the mean ± SD of 10–12 determinations (pooled from three preparations).
[a] $P < 0.001$ with respect to Mg^{2+}.

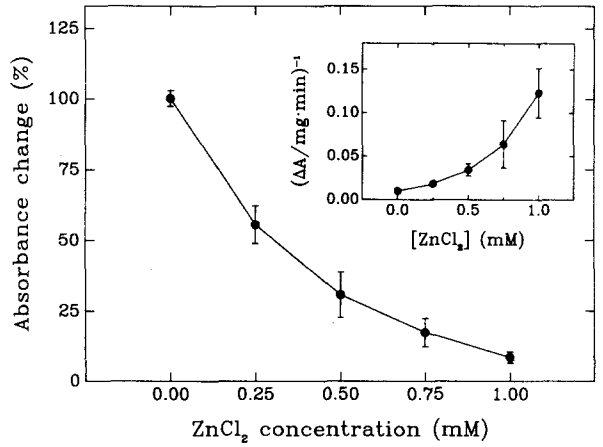

Fig. 4. Inhibition of ATP-driven H^+ uptake by Zn^{2+}. Two mM ATP was added to cholate-pretreated brush-border membrane vesicles (25 μg protein/assay) suspended in buffers containing the indicated concentrations of $ZnCl_2$. The absorbance decrease of acridine orange in the absence of Zn^{2+} was set to 100% for each of three preparations with duplicate determinations. The *inset* is a Dixon-plot of the same data

ATPase of porcine renal basolateral membrane vesicles (*data not shown*), attenuates ATP-driven H^+ uptake by 18% at 0.3 mM. These partial inhibitions reflect probably nonspecific effects on the H^+-pump.

Table 7 shows furthermore that 10 μM of the sulfhydryl-group reagent, *p*-chloromercuribenzene sulfonate (PCMBS), abolishes ATP-driven H^+ uptake suggesting that SH groups are of functional importance. A complete inhibition of H^+ uptake into cholate-pretreated vesicles is also seen in the presence of 10 μM of either N-ethylmaleimide (NEM) or 4-chloro-7-nitro-benzoxa-1,3-diazole (NBD-Cl), respectively.

Table 7. Sensitivity of the reoriented H^+-pump to inhibitors

Inhibitor	Concentration	H^+ uptake		*n*
		ΔA/mg · min	% of control	
A: Inhibitors for mitochondrial F_0F_1-ATPase				
Azide	0.5 mM	1.00 ± 0.16	73.1 ± 6.8[a]	6
Oligomycin	10 μg/ml	1.01 ± 0.13	77.6 ± 6.4	6
DCCD	20 μM	1.30 ± 0.15	82.3 ± 6.1	4
	200 μM	0.12 ± 0.06	7.3 ± 3.7[b]	4
B: Inhibitor for phosphorylating ATPases				
Vanadate	0.3 mM	1.14 ± 0.18	82.4 ± 6.1	6
C: Inhibitors for vacuolar ATPases				
PCMBS	10 μM	0	0	3
NEM	10 μM	0	0	3
NBD-Cl	10 μM	0	0	3

H^+ uptake into cholate-pretreated vesicles was measured with 2 mM ATP and 5 mM Mg^{2+} by the decrease of acridine orange absorbance. Inhibitors were added to vesicles 15 min prior to addition of ATP in H_2O (azide, vanadate, PCMBS) or ethanol (0.25% for oligomycin, NEM, NBD-Cl; 2% for DCCD). Controls without inhibitors received an equivalent amount of solvent. The absorbance decrease of these controls was 1.37 ± 0.14 (water); 1.31 ± 0.24 (0.25% ethanol); and 1.59 ± 0.15 (2% ethanol). Shown are means ± SD from *n* determinations (2–3 preparations).
[a] $P < 0.01$.
[b] $P < 0.001$ with respect to controls.

Since at low concentrations NEM and NBD-Cl can be considered as typical inhibitors of vacuolar ATPases [11, 23, 28], we tested the kinetics of inhibition. Figure 5 shows that, after 15 min preincubation, 5 μM NEM abolishes ATP-driven H^+ uptake. The apparent K_i as determined from a Dixon plot (*inset*) is 0.77 μM. Using the same preincubation time, NBD-Cl proves an even more potent inhibitor

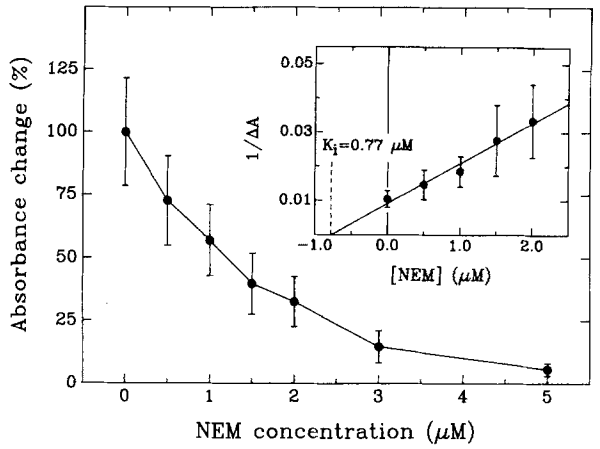

Fig. 5. Inhibition of ATP-driven H^+ uptake by N-ethylmaleimide (NEM). Cholate-pretreated vesicles (50 μg protein/assay) were incubated for 15 min at room temperature with various NEM concentrations in 150 mM KCl, 5 mM $MgCl_2$, 50 mM HEPES/Tris, pH 7, 6 μM acridine orange, prior to addition of 2 mM ATP. The absorbance decrease of the ΔpH indicator upon addition of ATP to vesicles not treated with NEM was set to 100% for each of three membrane preparations. Each datum is the mean of six determinations ± SD. The *inset* is the Dixon plot of the same data

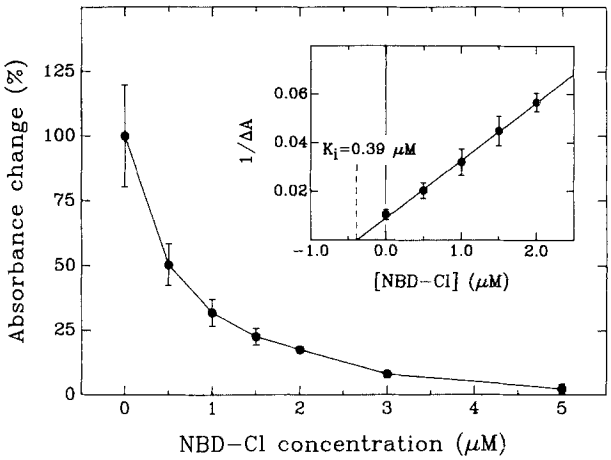

Fig. 6. Inhibition of ATP-driven H^+ uptake into cholate-pretreated vesicles by 4-chloro-7-benzoxa-1.3-diazole (NBD-Cl). The experimental conditions are the same as those described in the legend to Fig. 5 except that NBD-Cl was used as inhibitor

with an apparent K_i of 0.39 μM (Fig. 6). These experiments document the high affinity of the H^+-pump for these reagents which is typical for vacuolar H^+-ATPases.

Discussion

ATPases in Porcine Renal Brush-Border Membrane Vesicles

Intact porcine renal brush-border membrane vesicles hydrolyze ATP by an N-ethylmaleimide (NEM)-insensitive enzyme. Permeabilization of these vesicles by Triton X-100 decreases NEM-insensitive ATP hydrolysis by roughly two-thirds and, at the same time, exposes NEM-sensitive ATPases.

These results are very similar to data obtained with brush-border membrane vesicles from rat kidney which revealed the presence of NEM-insensitive ATPases at the outside (extracellular side) and NEM-sensitive ATPases at the inside (cytoplasmic side) of the membrane [31]. Although it has not been proven we assume that porcine renal brush-border membrane vesicles are oriented right side out similar to respective vesicles of rat kidney [9] and conclude that the NEM-sensitive ATPases are located at the cytoplasmic face of the brush-border membrane.

Since endosomes also contain NEM-sensitive ATPases some of the enzyme activity may arise from contamination with these membranes. We consider this possibility, however, unlikely, because endosomes enriched from pig kidney cortex exhibit a much smaller NEM-sensitive ATPase activity than purified brush-border membranes (*data not shown*).

After transient exposure of porcine renal brush-border membrane vesicles to cholate, NEM-insensitive ATP splitting is considerably reduced. In contrast, NEM-sensitive ATPase activity is quantitatively recovered in cholate-pretreated vesicles and is now detectable in the absence of Triton X-100. Addition of Triton increases NEM-sensitive ATP hydrolysis only by 14%. We conclude from these findings that transient exposure of vesicles to cholate (*i*) inactivates largely NEM-sensitive ecto-ATPases and (*ii*) reorients nearly all NEM-sensitive ATPases from the inside (in intact membranes) to the outside.

Inside-Out H^+-Pumps in Cholate-Pretreated Vesicles

If some or all of the reoriented NEM-sensitive ATPases are involved in ATP-driven H^+ transport, cholate-pretreated vesicles should be capable of ATP-driven H^+ uptake. Indeed, cholate-pretreated vesicles, but not intact brush-border membranes, exhibit intravesicular acidification in the presence of ATP. These findings indicate that, similar to NEM-sensitive ATPases, ATP-driven H^+-pumps have been relocated from the inside of intact vesi-

cles to the outside in cholate-pretreated membranes. Since these pumps are functional, a denaturation has probably not occurred during transient exposure to cholate, and vesicles formed during cholate removal by dialysis are tight for protons and thus allow for creation of a transmembrane ΔpH.

It may be argued that H^+-pumps from other cellular membranes than brush-border membranes have been incorporated into vesicles forming during cholate removal. Such a possibility cannot be dismissed with absolute certainty but it seems remote for the following reasons. First, intact brush-border membranes do not show detectable H^+ uptake in the presence of external ATP ruling out the presence of significant amounts of contaminating membranes with H^+-pumps located at their outer face. Second, ATPase activities in intact brush-border membranes and in cholate-pretreated vesicles are similar. Thus, it is unlikely that a minor contaminant containing NEM-sensitive H^+-ATPases was enriched in cholate-pretreated vesicles. Third, cholate-pretreated vesicles showed enrichments for marker enzymes (leucine aminopeptidase (LAP), alkaline phosphatase, γ-glutamyltranspeptidase) similar to those in the intact brush-border membranes. Fourth, when purified brush-border membrane vesicles were subjected to a Percoll density gradient, LAP activity and ATP-driven H^+ uptake measured after cholate treatment comigrated in the same fractions (*data not shown*). Therefore, if cholate itself does not alter the characteristics, the results obtained with cholate-pretreated vesicles reflect most likely the properties of ATP-driven H^+-pumps of proximal tubular brush-border membranes.

Characteristics of ATP-Driven H^+ Uptake

The relocation of H^+-ATPases during cholate treatment enabled us to study ATP-driven H^+ uptake in much greater detail than has hitherto been possible. As opposed to previous loading of ATP into brush-border membrane vesicles and monitoring of H^+ efflux [19, 20, 31], all experimental conditions can be kept constant since the effectors are added to the external incubation medium. Moreover, the detection of ATP-driven intravesicular acidification by acridine orange allows recording of large, reproducible signals with relatively small amounts of protein.

Summarizing the results obtained with cholate-pretreated vesicles we found that ATP-driven H^+ uptake was electrogenic, stimulated by ATP (apparent K_m, 93 μM) > GTP, ITP > UTP ≫ CTP, and required the presence of a divalent cation (Mg^{2+} > Mn^{2+}, but not Ca^{2+}). Potent inhibitors were ADP (K_i, 24 μM), NEM (apparent K_i, 0.77 μM) and 4-chloro-7-nitro-benzoxa-1,3-diazole (NBD-Cl, apparent K_i, 0.39 μM). N,N'-dicyclohexylcarbodiimide (DCCD) inhibited completely at 200 μM concentration. At concentrations sufficient to block F_0F_1, or phosphorylating ATPases, respectively, oligomycin, azide, and vanadate caused a partial inhibition of ATP-driven H^+ uptake into cholate-pretreated vesicles.

Similar results were obtained when ATP-driven H^+ uptake was measured in the presence of valinomycin and equal K^+ concentrations inside and outside the vesicles (150 mM; *data not shown*). With valinomycin, K^+ efflux can compensate for H^+ uptake diminishing the dependence of the electrogenic H^+-pump on chloride. Hence, the data summarized above reflect the properties of the H^+-pump rather than the characteristics of the transport system for chloride.

Comparison to Other Renal H^+-ATPases

Our results match the characteristics of the ATPase located at the cytosolic side of native renal brush-border membrane vesicles [31]. Together with previous H^+ efflux studies from ATP-loaded vesicles this finding underscores the involvement of NEM-sensitive ATPases in ATP-driven H^+ translocation across rat renal brush-border membranes. The characteristics of the H^+-pump in cholate-pretreated brush-border membrane vesicles resemble also closely those of ATP-driven H^+ transport [24, 26] and of H^+-ATPase activity in renal endosomes [25] including comparable apparent K_m values for ATP, 73 μM in endosomes [24] and 93 μM in cholate-pretreated vesicles (this study).

ATP-driven H^+ uptake and NEM-sensitive ATPase activity in cholate-pretreated brush-border membrane vesicles also share characteristics with the H^+-ATPase that has been purified from a microsomal fraction from bovine kidney medulla [13, 14]. The medullary ATPase hydrolyzes ATP (apparent K_m, 150 μM) ≈ ITP > UTP > GTP > CTP, requires $Mn^{2+} \approx Mg^{2+} \gg Ca^{2+}$ and is inhibited by DCCD (K_i, 5–8 μM), NEM (K_i, 22 μM), PCMBS, and NBD-Cl (K_i of 15 μM). Preference for ATP, Mg^{2+} and Mn^{2+} and inhibition by micromolar concentrations of NEM and NBD-Cl indicate that H^+-ATPases from rat and pig kidney cortex and bovine kidney medulla belong to the vacuolar class of H^+-ATPases. This conclusion is supported by studies with antibodies raised against three subunits of the bovine medullary ATPase [7]. These antibodies located immunologically related H^+-ATPases to the brush-border membrane and endosomes of proximal tu-

bule cells and, in collecting ducts, to apical vesicles in intercalated cells, type A.

Present evidence is therefore in favor of a similar type of H^+-ATPases in proximal tubule and collecting ducts and makes the existence of an other type of H^+-ATPase, as suggested earlier [20], unlikely. However, although belonging to the same class, the H^+-ATPases from cortical brush-border membrane vesicles, endosomes and medullary microsomes need not be identical. As will be discussed below, the nucleotide specificities of these ATPases differ with ITP being nearly as effective as ATP with the medullary enzyme [13], whereas it stimulates the brush-border enzyme half (this study) and the endosomal enzyme less than a tenth [25] as compared to ATP. Likewise, DCCD inhibited the medullary H^+-ATPase more efficiently than NEM and NBD-Cl [13]. In cholate-pretreated vesicles considerably higher concentrations of DCCD than of NEM and NBD-Cl were required for full inhibition. It will be interesting to relate differences of H^+-ATPases to their sorting to and location in plasma membranes (brush-border) and intracellular membranes (endosomes, microsomes).

Comparison with Extrarenal H^+-ATPases

Vacuolar H^+-ATPases from plants, fungi and from organelles in mammalian cells (for reviews *see* Refs. [11, 23, 28]) are composed of three (plants) or more subunits (organelles), are relatively insensitive to inhibitors of F_0F_1-ATPases, oligomycin, azide, and DCCD, and to an inhibitor of phosphorylating *P*-ATPases, vanadate, but exquisitely sensitive to NEM and NBD-Cl. The latter two compounds block enzyme activity at low micromolar concentrations and interact with side groups close to the nucleotide binding site of the *V*-ATPases. Thus, these enzymes exhibit the same characteristics as the renal H^+-ATPases discussed above.

Here we would like to focus on two aspects with regard to stimulation by anions and specificity for nucleotides. Enhancement of ATP-driven H^+ uptake by chloride has been found in this study as well as in many earlier experiments on renal [16, 18, 24] and nonrenal membranes such as hepatic endosomes and multivesicular bodies [12, 27, 33], microsomes from pancreatic acinar cells [29], clathrin-coated vesicles [1, 34], chromaffin granules [21], and vacuoles from *Neurospora crassa* [5], oat roots [3, 35], and yeast [32]. A marked difference exists, however, with respect to nitrate and thiocyanate. Nitrate stimulated ATP-driven H^+ uptake in cholate-pretreated vesicles slightly, and thiocyanate markedly, as compared to chloride (this study), whereas they did not stimulate or even inhibit nonrenal H^+-ATPases. The reason for this discrepancy is not clear. Moriyama and Nelson [21] noted that nitrate and thiocyanate inhibited the chromaffin granule H^+-ATPase only from the inside of the granules. In our experiments, nitrate and thiocyanate were offered together with ATP from the outside and may not have reached their inhibitory site at the inside of the vesicles. Thereby the stimulatory effect of these anions was not masked by a direct inhibition of the ATPase.

Some differences between our results and those obtained with renal and nonrenal membranes also exist with respect to the nucleotide specificity of the H^+-pump. Here we show that GTP and ITP support one-third and UTP one-tenth of H^+ uptake as compared to ATP. It seems that the pump is not completely specific for ATP and can utilize these nucleotides although at lower efficiency. In accordance with this intepretation is the inhibition of H^+ uptake exerted by GTP, ITP, UTP and also CTP in the presence of ATP: these nucleotides seem to compete with ATP for the pump. Their relatively slow hydrolysis (*cf.* Table 4) leads to a smaller rate of H^+ uptake. No strict preference for ATP was also seen in liver lysosomes [15] and vacuoles from yeast [17, 32], whereas H^+-pumps from clathrin-coated vesicles [34, 36], pancreatic microsomes [29], and liver endosomes [12, 27] utilized exclusively ATP. It needs to be established whether these nucleotide specificities reflect differences in the nucleotide binding domain of these H^+-ATPases.

A potentially important finding of this study is the inhibition of ATP-driven H^+ uptake by ADP. Kinetic experiments revealed a competitive inhibition suggesting an interaction of ADP with the ATP-binding site (product inhibition). The K_i (24 μM) for ADP was approximately one-fourth of the apparent K_m for ATP indicating a high affinity of the H^+-pump for ADP. An inhibition by ADP was found already for vacuolar H^+-ATPases from other sources such as corn and radish roots [30, 35], and yeast [32]. The high affinity of the brush-border H^+-pump for ADP may render this enzyme susceptible to regulation by the concentrations of both, ATP and ADP, within the microvillus. Since in proximal tubule cells the mitochondria are mostly located at the opposite cell pole, the concentration of ATP may be relatively low and that of ADP high in the core of the microvilli tending to inhibit the H^+-pump. The physiological role of such a possible regulation needs to be established.

Taken together, we have reoriented ATP-driven H^+-pumps in renal brush-border membrane vesicles by transient cholate exposure. This method allowed a detailed characterization of both H^+-

ATPase and H^+ transport. All data indicate that a vacuolar or *V*-type H^+-ATPase is located in the brush-border membrane of proximal tubule cells and may be responsible for a part of H^+ secretion into the lumen of the proximal tubule.

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