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An ATP-Driven Proton Pump in Brush-Border Membranes from Rat Renal Cortex

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Summary. The rate of ATP hydrolysis in ATP-preloaded plasma membrane vesicles derived from the luminal membrane of renal cortical tubules, and the rate of H^+ secretion out of the same vesicles were investigated. Both were inhibited at low temperature, by the action of filipin, an antibiotic that complexes with cholesterol in plasma membranes, and by the action of blockers of mitochondrial F_o hydrogen channels, dicyclohexylcarbodiimide and Dio-9. Valinomycin in the presence of K^+ showed a stimulatory effect, the protonophor carbonyl-cyanid-*p*-trifluormethoxy-phenylhydrazone stimulated the intravesicular ATP hydrolysis and apparently abolished acidification of the extravesicular medium. Lowering of the pH of the extravesicular medium retarded ATP hydrolysis, while readjustment of extra- and intravesicular pH accelerated ATP hydrolysis again. These findings strongly support the assumption that an ATP-driven proton pump is located in the luminal membrane of renal cortical tubules.

Key words intravesicular ATP hydrolysis · H^+ secretion · anion-sensitive Mg^{2+}-ATPase · ATP-driven H^+ pump · renal brush-border membrane vesicles

Introduction

Although an electroneutral Na^+/H^+ exchange system has been well-established in brush-border membranes of renal cortical proximal tubule [27, 33], it appears likely that a substantial fraction of protons is extruded over the luminal membrane in a sodium-independent and electrogenic way [45] most probably using ATP hydrolysis as driving force. Since Skou described in 1957 the possible involvement of an ATPase in active sodium transport across biological membranes [40], an increasing number of ATPases has been speculated to act as ion pumps. Over the last years our interest has been focused on an anion-sensitive Mg^{2+}-ATPase which could be proven to be present in brush-border membranes of the proximal tubule [25, 26]. This ATPase has been tentatively hypothesized to be involved in proton transport. However, the direct demonstration of ATP-driven proton transport across the brush-border membrane was lacking hitherto.

This paper describes experiments with ATP-loaded membrane vesicles in which proton translocation and ATP hydrolysis was observed. They occurred simultaneously with a similar time-course and exhibited a similar sensitivity to various inhibitors. It therefore appears that the brush-border membrane contains an ATP-driven proton [1] pump.

Materials and Methods

Materials

Carbonyl-cyanide-*p*-trifluormethoxy-phenylhydrazon (CFCCP), phosphoenol pyruvate (PEP), pyruvate kinase, and valinomycin were obtained from Boehringer (Mannheim, Germany); oligomycin and Triton X-100 from Serva (Heidelberg, Germany); the luciferin-luciferase testkit from Abimed (Duesseldorf, Germany; distributor for Lumac, Florida) and TRIS-ATP from Sigma (St. Louis, Missouri). Filipin was kindly provided by the Upjohn Company (Kalamazoo, Michigan), as was Dio-9 from Gist and Brocades (Delft, The Netherlands). Dicyclohexylcarbodiimide (DCCD) and all other reagent were purchased from Merck (Darmstadt, Germany).

Preparation of Membrane Vesicle Fractions

A membrane fraction rich in brush-border membranes was prepared as recently described [26]. In principle, it is based on the homogenization of rat kidney cortex of male rats (body weights of about 200 g) in 6 volumes (wt/vol) of ST-buffer (250 mM sucrose and 10 mM triethanolamine-HCl, pH 8.0 at 20 °C) which is followed by several differential centrifugation steps in the above-described sucrose medium. In the case of preloading the membrane vesicles with ATP the sucrose medium included 3 mM TRIS-ATP, 5 mM PEP, 5 mM K_2SO_4, 6 mM $MgSO_4$, 500 μg oligomycin and about 400 units of pyruvate kinase per 10 ml homogenate.

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[1] We describe the ATP-driven movement of protons in one direction knowing that we cannot distinguish in this type of study between H^+ movement in one direction and OH^- movement in the opposite direction.

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For measurements of ATP hydrolysis the resulting membrane pellet was immediately homogenized 20 times at low speed in 1.5 ml of the sucrose medium, pH 8.0, with unchanged concentration of K^+, Mg^{2+} and oligomycin, plus additional 20 mM mannitol and 2 mM ouabain but in the absence of ATP, PEP and pyruvate kinase. The membrane vesicles suspension (about 6 mg protein/ml in average) was kept in ice and aliquots of 300 µl were taken at intervals for individual experimental setups.

For measurements of proton secretion the membrane pellet was suspended in 1.5 ml of a weakly buffered medium of the following composition: 130 mM KCl, 0.2 mM triethanolamine, 20 mM mannitol, 5 mM K_2SO_4, 6 mM $MgSO_4$, 2 mM ouabain, 50 µg oligomycin/ml, pH 8.0. The membrane suspension was kept in ice and aliquots of 200 µl were taken at intervals. The relative "age" (t_o) of the membrane vesicles was calculated from the time the pellet was suspended, this time being considered as $t_o = 0$ min.

In one experiment a preparative free-flow electrophoresis was carried out in the FF 48 Electrophoresis (Desaga, Heidelberg, Germany) as described in detail elsewhere [22].

Measurements of ATP Hydrolysis

Fifty µl of membrane vesicles kept at 0 °C were removed from a 300-µl batch, mixed for 30 sec with NRS® (Abimed, luciferin-luciferase testkit) to release ATP, then boiled for 2 min and centrifuged for 5 min in an Eppendorf centrifuge. The clear supernatant was analyzed for its ATP content. This value is being considered as the amount of ATP found in the intravesicular space at time 0. The membrane vesicle suspension was then brought to 37 °C and at given time intervals 50 µl samples were taken out, treated and analyzed as described above. Solutions of CFCCP, DCCD and valinomycin were prepared in ethanol. The final concentration of ethanol in the incubation medium was 0.5%. Filipin and Dio-9 were dissolved in N,N-Dimethylformamide (DMF); in the case of filipin the final DMF concentration was 1%, 2% in the case of Dio-9. Neither concentration of DMF showed an effect on ATP hydrolysis.

The analysis of ATP is based on the luciferin-luciferase assay. We used a testkit for nonmicrobial ATP available from Abimed. Measurements were taken in a Bioluminescence Analyzer XP 2000 (Scan AG., Switzerland).

Measurements of Change in pH of the Extravesicular Medium

A sealed vial was specially designed to monitor pH changes. It had a very small opening on the side allowing addition of medium or inhibitors. The temperature of the vial was kept at 37 °C, by means of an external temperature-controlled water circuit. The electrode (combination electrode model 5990-45, Cole-Palmer Instruments Company, Chicago, Illinois) was bathed in 600 µl of extravesicular medium previously pre-equilibrated in 100% N_2 so as to avoid any significant contamination with CO_2. This solution was well mixed with a magnetic stirrer. Whenever this medium was renewed by withdrawing the electrode from the vial, 10 to 15 min were necessary to arrive at a near steady state, characterized by an electrode drift not exceeding 1% per min. The electrode potential difference was monitored with a Radiometer electrometer (model PHM 64, Radiometer Instruments, Copenhagen, Denmark) and recorded on a Radiometer (Servograph REC 61, with a high sensitivity unit REA 112) strip chart recorder. When the state of low drift was achieved, 200 µl of membrane vesicle suspension were added to the 600 µl of buffer by the lateral sleeve and the pH was continuously monitored for the next 10 min. The observed ΔpH (in millivolts) was converted to nmol of hydrogen ion activity by calibrating the system with a rapid injection of 10 nmol HCl to the membrane vesicle preparation. Backtitration with 10 nmol NaOH gave essentially identical results.

Enzymatic Characterization of the Membrane Fraction and Protein Assay

The activity of alkaline phosphatase and Na^+–K^+-ATPase was determined according to standard procedures as described in [24]. The assay for succinic dehydrogenase activity was performed after preincubation in 0.1% desoxycholate for 30 min at 0 °C according to the method of Gibbs and Reimer [19]. Cytochrome *c* oxidase was determined according to the method of Cooperstein and Lazarow [8]. The optimal conditions for hexokinase activity were taken from Bergmeyer [3]. Protein was estimated after precipitation with 10% trichloroacetic acid in the cold and dissolution of the precipitate in 1 N NaOH according to Lowry et al. [29] with bovine serum albumin as standard.

Results

Loading of Brush-Border Membrane Vesicles with ATP

Haase et al. have shown that at least 85% of brush-border membranes vesiculate spontaneously into right-side-out vesicles [21]. Consequently, active sites catalyzing hydrolysis of ATP located *in vivo* at the cytoplasmic face, i.e. the inside of the vesicles, are not freely accessible to ATP added to the outside of the vesicles. In pilot experiments we were unable to load isolated brush-border membrane vesicles after their preparation without inducing leakiness of the vesicles. A reasonable loading of the membrane vesicles with ATP was, however, achieved if ATP and an ATP regenerating system were present during the initial homogenization. As shown in Table 1, brush-border membrane vesicles prepared in the absence of ATP and the regenerating system contain a very low amount of ATP. If ATP is added to the homogenization medium alone, no increase in ATP content of the final membrane preparation is observed. Al-

Table 1. Prerequisite for loading of brush-border membrane vesicles with ATP

Addition to the homogenization medium	ATP content of the final membrane vesicles preparation (ng/mg membrane protein)	
None	0.003	($n=3$)
ATP alone	0.003	($n=3$)
ATP plus regenerating system	0.56 ± 0.04	($n=12$)
ATP plus regenerating system after repeated washing	0.58	($n=2$)
ATP plus inactivated regenerating system	0.003	($n=2$)

though the membrane preparation is performed at 2–4 °C a rapid hydrolysis of ATP takes place, probably during tissue homogenization. In the presence of both, ATP and the regenerating system, an ATP content of about 0.56 ng/mg protein is found. If one uses an intravesicular volume of approximately 3 µl/mg protein [23] this corresponds to an ATP concentration of about 0.2 mM/liter.

Evidence that the ATP present in the final membrane fraction is indeed in the intravesicular space can be derived from two different experiments. First, repeated washing of the membrane vesicle suspension does not decrease the ATP amount (Table 1). Second, in another series of experiments, ATP-loaded membrane vesicles were incubated at 37 °C in the presence of hexokinase and glucose. Any ATP present at the outside of the vesicles would have entered the reaction with glucose and hexokinase and thereby would have been hydrolyzed. As shown in Fig. 1, the ATP content of the membrane vesicles is not affected by the addition of glucose and hexokinase unless Triton X-100 is added, a detergent that opens the vesicles.

The amount of ATP found in the final membrane preparation decreases with time, even in the presence of oligomycin, ouabain and even when the membranes were kept at low temperature. Actually, the intravesicular ATP content declined from 100% measured at time 0 to 85% after 1 hr, and 60% after 2 hr, respectively.

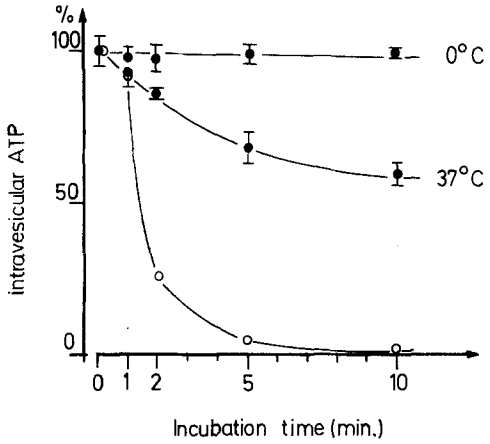

Fig. 1. Intravesicular ATP hydrolysis in ATP-preloaded membrane vesicles. a) Effect of temperature: (●—●) the vesicles were incubated at 0 °C, (●—●) or the vesicles were incubated at 37 °C. Mean values of 4 experiments are shown. b) Effect of D-glucose and hexokinase present in the extravesicular meidum: after incubation for 1 min, Triton X-100 was added to one batch (○—○). The final concentration of Triton X-100 was 0.5%. Mean values of three experiments are shown. In this Figure, as well as in the following ones, the amount of ATP measured at the given time points is expressed as percent of the amount of ATP present in the intravesicular space at time zero of any individual experiment = 100%

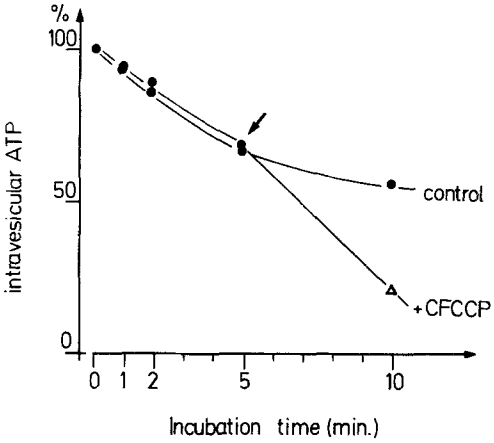

Fig. 2. Effect of CFCCP on intravesicular ATP hydrolysis in ATP-preloaded membrane vesicles. (●—●) ATP hydrolysis in the absence of CFCCP (carbonyl-cyanid-*p*-trifluormethoxy-phenylhydrazone). (●—△) addition of 5 µg CFCCP after 5 min of incubation (*see arrow*). Mean values of three experiments are shown

Intravesicular ATP Hydrolysis

When membrane vesicles loaded with ATP and the regenerating system were incubated at 37 °C an intravesicular ATP hydrolysis was observed (Fig. 1). The apparent rate of ATP hydrolysis by the membranes, however, is not constant. It is higher within the first minutes (0.17 ng/5 min/mg protein) and decreases in the later period of the experiment (0.06 ng/5 min/mg protein). The ATP hydrolysis is also temperature dependent. As shown in Fig. 1 incubation of the membranes at 0 °C inhibits the hydrolysis almost completely indicating that an enzymatic process is involved in the hydrolysis.

Effect of Protonophores, pH Gradient and Valinomycin. The time course of ATP hydrolysis observed in the brush-border membrane vesicles is similar to the time course observed with submitochondrial particles. In the case of mitochondrial particles the ever-increasing inhibition of ATP hydrolysis was attributed to the generation of an electrochemical potential difference for protons across the membrane [28]. In order to test whether a similar situation prevailed in the brush-border membrane vesicles the protonophor CFCCP was added to the membranes. Indeed, it was found (Fig. 2) that CFCCP stimulated the intravesicular ATP hydrolysis suggesting that the rate of ATP hydrolysis under 'nonmodified' conditions had been

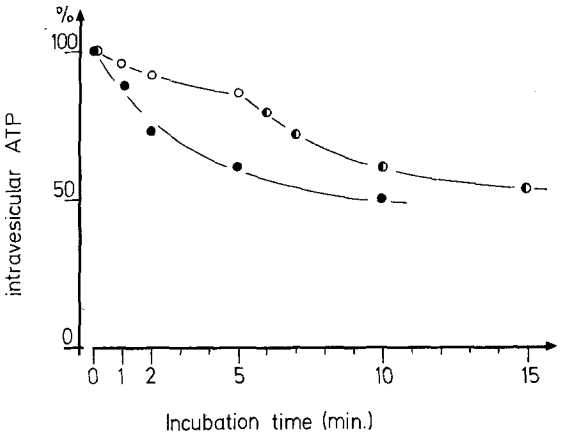

Fig. 3. Effect of transmembranal pH gradient on intravesicular ATP hydrolysis in ATP-preloaded membrane vesicles. (●—●) control run at pH 8.0; (○—○) ATP hydrolysis at an extravesicular pH of 6.5; (◐—◑) ATP hydrolysis after readjustment of the extravesicular pH to 8.0. Mean values of two experiments are shown

Table 2. Effect of valinomycin on intravesicular ATP hydrolysis

Incubation time (min)	Amount of intravesicular ATP							
	In the absence of valinomycin				In the presence of valinomycin			
	No. of experiment			$\bar{X}$	No. of experiment			$\bar{X}$
	#1	#2	#3	(in %)	#1	#2	#3	(in %)
0	0.48	0.64	0.52	100%	0.48	0.64	0.52	100%
5	0.33	0.49	0.35	72%	0.29	0.43	0.33	65%
10	0.28	0.42	0.32	62%	0.25	0.36	0.29	56%

The experiments were performed in the absence and in the presence of 1 µl valinomycin (=10 µg). The control contained an equivalent amount of ethanol. 15 mM K^+ were present in the intravesicular as well as in the extravesicular medium. The intravesicular ATP content of each individual experiment is given in ng per mg of membrane protein. The mean values of the three experiments are expressed as percent of the amount of ATP present in the intravesicular space at time zero =100%.

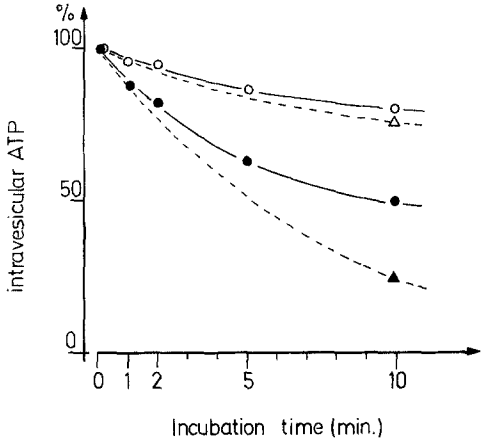

Fig. 4. Effect of filipin and DCCD (dicyclohexylcarbodiimide) on intravesicular ATP hydrolysis in ATP-preloaded membrane vesicles. (●—●) ATP hydrolysis in a control run, i.e. in the absence of any drug; (●---▲) ATP hydrolysis in a control run with 5 µg CFCCP present during the entire incubation time. (○—○) ATP hydrolysis in the presence of either 0.5 mg filipin or 0.1 mM DCCD (both compounds showed a similar inhibitory potency). (○--△) ATP hydrolysis in the presence of either 0.5 mg filipin or 0.1 mM DCCD *and* 5 µg CFCCP. Mean values of three experiments are shown

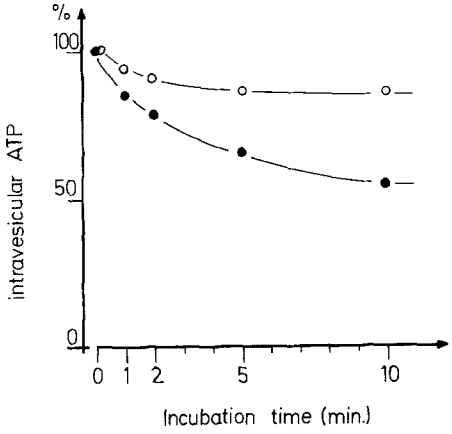

Fig. 5. Effect of Dio-9 on intravesicular ATP hydrolysis in ATP-preloaded membrane vesicles. (●—●) ATP hydrolysis in the absence of Dio-9; (○—○) ATP hydrolysis in the presence of 0.5 mg Dio-9. Mean values of two experiments are shown

inhibited by the $\Delta\mu H^+$ generated. Further evidence for this assumption was obtained from experiments where a pH gradient was established across the membranes. If the extravesicular pH was set to pH 6.5, the rate of intravesicular ATP hydrolysis was markedly inhibited (Fig. 3). If the extravesicular pH was then adjusted to pH 8.0 (as the pH inside the vesicles) the intravesicular ATP hydrolysis was again accelerated and proceeded at almost the same rate as in the control membranes incubated at pH 8.0. It was found in addition that under conditions where valinomycin should effectively 'short circuit' the membrane (Table 2), the rate of intravesicular ATP hydrolysis increased consistently although only to a small extent.

All these results taken together suggest that the intravesicular ATP hydrolysis is accompanied by the generation of an electrochemical potential difference for protons across the membrane that in turn inhibits the ATPase reaction.

Effect of Various Inhibitors on Intravesicular ATP Hydrolysis. In Figs. 4 and 5 the effect of the cholesterol-complexing antibiotic filipin, and of the blockers of mitochondrial F_o hydrogen channels, DCCD and Dio-9 on intravesicular ATP hydrolysis are compiled. Filipin has been shown in previous studies to inhibit

Table 3.

Time (min)	ΔpH_1 (mV)	Initial J_{H+} (nM/min mg protein)	ΔpH_2 (mV)
0	−5.6	5.3	7.8
44	+4.8	3.2	5.2
90	+8.3	2.2	0.3
122	+11.0	1.3	0.2

ΔpH_1 is the immediate shift in pH occurring within 30 sec following addition of the vesicle preparation to the incubation vessel. Depending upon the experiment (and presumably how matched were the pH of the intra- and extravesicular medium) it could be first negative (i.e. indicating alkalinization of the extravesicular medium). J_{H+} is the initial rate of proton ejection and ΔpH_2 the maximum acidification reached (7 min after the injection) by those vesicles with reference to the level achieved after ΔpH_1. In the presence of membrane protein 6 mV correspond to about 0.1 pH unit.

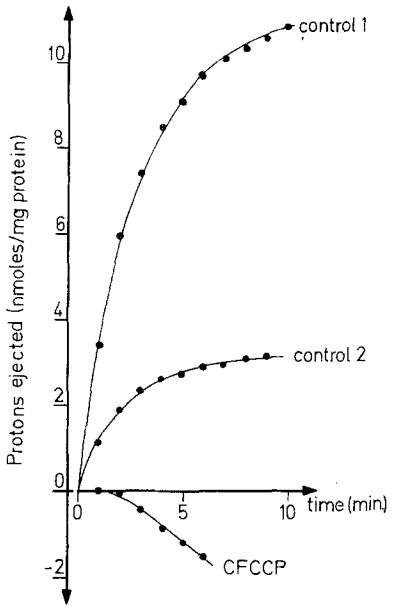

Fig. 6. Proton secretion out of ATP-preloaded membrane vesicles. Control 1 resembles the measurement of proton secretion using a vesicle preparation with a relative age of $t_o=0$ min; control 2 resembles a preparation with a t_o of 76 min. The effect of 5 µg CFCCP was tested using vesicles with a t_o of 24 min. The significance of the slight alkalinization observed in the presence of CFCCP (as well as of filipin, Dio-9 and DCCD) is not entirely clear. When followed over a longer time period, ranging from 12 to 25 min, the pH reached a stable value. This is probably related to the buffering capacity of the membrane protein since the same phenomenon was observed with membrane vesicles which had been preincubated with 0.5% Triton X-100. As a result, the true ΔpH_2 is slightly underestimated

exclusively the Mg^{2+}-ATPase activity in brush-border membranes [26], and DCCD and Dio-9 elicit in addition to their action in mitochondria [7, 20, 39] an inhibition of plasma membrane-bound proton transport processes [5, 10, 13]. All three agents inhibit

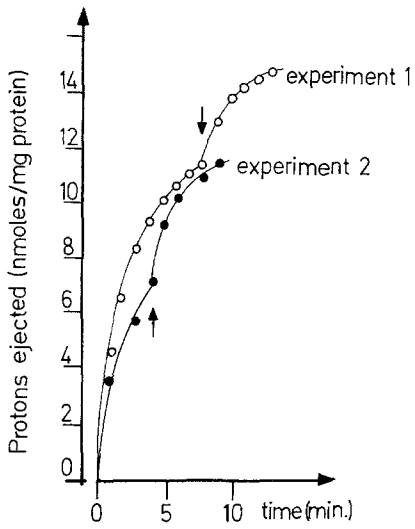

Fig. 7. Proton secretion out of ATP-preloaded membrane vesicles. At different time points as indicated by the arrows, 10 µg valinomycin were added to the reaction chamber. Experiment 1 resembles a vesicle preparation with a relative age of $t_o=0$ min, experiment 2 resembles a preparation with a t_o of 35 min

intravesicular ATP hydrolysis; their inhibitory action is most pronounced when the hydrolysis has been stimulated by the addition of CFCCP (Fig. 4).

Measurements of Proton Secretion

In Table 3 a representative experiment is depicted in which the acidification of the extravesicular medium is recorded that occurred following injection of 200 µl of brush-border membrane vesicles into the reaction chamber. Immediately after the injection a change of medium pH occurred (ΔpH_1), then a slower acidification was observed (J_{H+}) that led to the establishment of a pH gradient above ΔpH_1. This gradient is called ΔpH_2. All three parameters changed as a function of the time elapsed between the final vesicle preparation and the pH measurements. As detailed in this Table, ΔpH_1 increased whereas the initial J_{H+} and ΔpH_2 decreased with time. The increase in ΔpH_1 is probably due to proton transfer accompanying the slow ATP hydrolysis shown above to occur in the membrane vesicles during storage at low temperature, the decrease in J_{H+} and ΔpH_2 might be related to the ΔpH present across the membrane and/or the decrease in intravesicular ATP content. In the following, the baseline was corrected for ΔpH_1 and, due to this time dependence, two control measurements were performed when the effects of various agents were tested, one before (control 1) and one after (control 2) the measurement of proton secretion in the presence of a drug.

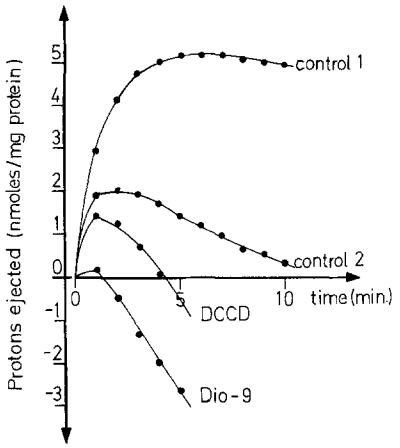

Fig. 8. Proton secretion out of ATP-preloaded membrane vesicles. Control 1 resembles the measurement of proton secretion out of a vesicle preparation with a relative age of $t_o=0$ min; control 2 resembles a preparation with a t_o of 88 min; the effect of 0.5 mg Dio-9 was tested using vesicles with a t_o of 34 min; the effect of 0.1 mM DCCD was tested using vesicles with a t_o of 54 min

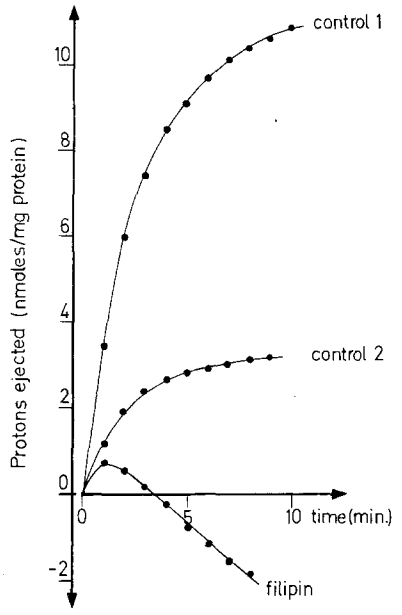

Fig. 9. Proton secretion out of ATP-preloaded membrane vesicles. Control 1 resembles the measurement of proton secretion using a vesicle preparation with a relative age of $t_o=0$ min; control 2 resembles a preparation with a t_o of 76 min. The effect of 0.5 mg filipin was tested using vesicles with a t_o of 59 min

Effect of Protonophores and Valinomycin on Proton Secretion. As shown in Fig. 6 the addition of CFCCP abolished acidification of the extravesicular medium. This finding suggests that during the incubation protons are transferred across the brush-border membrane which in the presence of CFCCP reenter the vesicle. In the presence of K^+ in the intra- and extravesicular space the addition of valinomycin immediately stimulates proton ejection from the vesicles (Fig. 7). The stimulation can be explained by the assumption that an electrical potential difference is generated across the membrane due to the action of an electrogenic proton pump. Valinomycin shortcircuits the membrane and thus diminishes the inhibitory effect of the electrical potential difference.

Effect of Various Inhibitors on Proton Secretion. Proton movement across the brush-border membrane was inhibited by the same components inhibiting the intravesicular ATP hydrolysis. In the presence of DCCD and Dio-9 (Fig. 8) as well as filipin (Fig. 9) no acidification of the incubation medium was observed. Dio-9 acted instantaneously after its addition to the membranes; for filipin and DCCD the time course of inhibition was not further investigated.

Discussion

Studies of ATP-dependent processes in brush-border membrane vesicles are complicated by the fact that the membranes are predominantly oriented right-side-out. Therefore, the former cytoplasmic face is not easily accessible for ATP added to the extravesicular medium. The vesicles have to be either turned around or the vesicles have to be loaded with ATP. In a variety of preliminary experiments using sonication, proteases, and polyethyleneglycol we were unable to reverse the orientation of brush-border membrane in the vesicles (Schaefer and Kinne, *unpublished*). Thus we embarked on the route of preloading. In a first attempt we tried to preload already isolated membrane vesicles. But even though ATP and a regenerating system were offered over a long incubation time, the resulting intravesicular ATP concentration was insufficient for further experiments. We tend to explain this failure by the large molecular size of pyruvate kinase for which the vesicle membrane seems to be an impermeable barrier. As it turned out we had to have ATP and the regenerating system present already during the first homogenization of the cortical tissue. During the disruption of the cells the future intravesicular space of the microvilli was transiently accessible for solutes in the extravesicular space. But even then, the impeded diffusion of pyruvate kinase had to be taken into consideration. Concentrations of pyruvate kinase which *in vitro* are sufficient for an optimal regeneration of ATP had to be raised eight- to 10-fold to provide the regenerating system with sufficient pyruvate kinase units of activity per volume to result in an intravesicular ATP concentration high enough to work with.

The process of loading brush-border membrane vesicles with ATP includes, besides ATP and its regen-

erating system, K^+ and Mg^{2+} in a quite high molarity for optimal pyruvate kinase activity. This shift in ionic strength of the isolation medium affected the behavior of the membranes during differential centrifugation. Homogenization and isolation in the unmodified sucrose/triethanolamine medium had produced plasma membrane layers that were extremely fluffy and therefore could be separated easily from the more tightly packed mitochondrial pellets. Now, in the presence of 5 mM K^+ and 6 mM Mg^{2+} the former fluffy layers also became more packed. Consequently, the yield in plasma membrane protein was lower and the enrichment of alkaline phosphatase as a marker enzyme for brush-border membranes was slightly decreased. The degree of mitochondrial contamination could be kept at the usual low level. The specific activity of succinic dehydrogenase and cytochrome *c* oxidase was about one-tenth and less of the activity found in the homogenate.

Evidence that we were indeed dealing with processes occurring in brush-border membranes was provided by one experiment in which ATP-preloaded membrane vesicles were further purified using the free-flow electrophoresis. Although this additional procedure led to the already described time-dependent decrease in the intravesicular ATP concentration, the resulting highly purified brush-border membrane vesicles showed essentially the same reactions as seen with the membrane fraction used otherwise.

With respect to the ATP regenerating system, it is evident that it is not sufficient to maintain the ATP level constant when the membrane vesicles are incubated at 37 °C. This decrease in ATP concentration, here referred to as intravesicular ATP hydrolysis, represents the action of ATP hydrolyzing enzymes which is partially compensated by the regenerating system. Thus, no enzymatic activities can be deduced from the apparent rate of intravesicular hydrolysis. Likewise, estimates of the stoichiometry of proton translocation across the membrane versus ATP hydrolysis give only fictitious numbers. In an experiment in which ATP breakdown and proton secretion were measured simultaneously, a number of 36 protons per ATP was obtained. This is about twelve times higher than the coupling ratio reported recently for turtle bladder [12]. With regard to the ATP hydrolyzing enzymes we favor the view that most of the activity is related to ATP hydrolase activities and not to protein kinase activity since the ATPase activity in the membrane preparation is about 60 μmoles/hr/mg protein, whereas the protein kinase activity amounts only to about 2.4 μmoles/hr/mg protein.

The presence of the regenerating system and the ATP hydrolysis occurring inside the vesicles raise another problem related to the proton release observed with the pH electrode. At pH 8.0, ATP hydrolysis is associated with proton generation. Thus, a proton gradient might be established across the membrane and the proton secretion observed might be linked only indirectly with the ATPase activity. Two arguments speak against this possibility. Experiments were carried out in which the pH of a reaction mixture containing the regenerating system and ATP was followed during hydrolysis of ATP by a kidney cortex homogenate. Although the ATP content of the reaction mixture decreased to one-tenth of the initial value, no change in the pH of the solution was observed. This is in accordance with the initial aim of the study, namely to use a solution inside the vesicle with a buffer capacity strong enough to maintain the intravesicular pH constant. The other argument deals with the effect of CFCCP on the proton release. If a pH gradient would exist across the membrane, an increased proton efflux rather than a decreased efflux would have been expected to occur. We therefore suppose a causal coupling between intravesicular ATP hydrolysis and proton transfer across the membrane.

Table 4. Effect of various maneuvers on intravesicular ATP hydrolysis and proton secretion by brush-border membrane vesicles preloaded with ATP

Maneuver	Intravesicular ATP hydrolysis	Proton secretion
Low temperature	Inhibited	Inhibited
Filipin	Inhibited	Inhibited
DCCD	Inhibited	Inhibited
Dio-9	Inhibited	Inhibited
CFCCP	Stimulated	Inhibited
Valinomycin	Stimulated	Stimulated
pH gradient ($pH_o < pH_i$)	Inhibited	ND

Table 4 summarizes the effect of various maneuvers on the intravesicular ATP hydrolysis in ATP-preloaded membrane vesicles and on the proton secretion of these vesicles. The stimulation of ATP hydrolysis by CFCCP on one hand and the inhibition of proton secretion on the other hand is in accordance with the action of a proton pump establishing an electrochemical potential difference for protons across the membrane that is subsequently dissipated in the presence of a protonophor.

Compounds like DCCD and Dio-9 that show a clear inhibition of either tested systems, have become known first in connection with studies with mitochondria giving way to the impression that they are totally specific for mitochondria. That this is not imperative has been shown for instance in yeast and fungi [4, 10, 13], and in our recent work with renal brush-border membranes [26]. Of course, it does not mean that we did not pay attention to the small amount

of mitochondria in the final membrane preparation. Oligomycin was used in all experiments to suppress mitochondrial processes. The most powerful tool hitherto in discriminating between plasma membrane-bound or mitochondrial processes is the sterol specific antibiotic filipin. This compound exhibits an inhibition curve with a partial inhibition of the Mg^{2+}-ATPase located in the luminal membrane and of the intravesicular ATP hydrolysis for low concentration (=complexing action), followed by a restoration of the enzyme activity and diminishing influence of ATP hydrolysis with increasing concentration (= protonophore action). The inhibition of proton secretion out of ATP-preloaded membrane vesicles was studied using filipin concentrations that inhibit the ATPase activity, i.e. below ionophoretic action of filipin.

So far, we were able to demonstrate the existence of an ATPase in the isolated membrane vesicles with the most tempting feature being sensitive towards anions. We were also able to demonstrate ATP hydrolysis and synchronous proton secretion out of these vesicles. What remains to be done is the demonstration that the ATP hydrolysis is catalyzed by the plasma membrane-bound ATPase leading to active proton pumping, as has been done already for proton transport across plasma membranes of fungi [37; 38, 41, 42], across the luminal membrane of the turtle urinary bladder [12], and across plasma membrane vesicles derived from gastric mucosa [35]. In these terms, the inhibitory action of DCCD on ATP hydrolysis as well as on proton secretion is of particular interest. DCCD interacts with a proteolipid component of the F_o basepiece of mitochondrial ATPase designated as subunit 9 in yeast mitochondrial ATPase complex. This subunit obviously plays an important role in proton translocation through the mitochondrial membrane [32]. An analogous protolipid, sensitive to DCCD, has been demonstrated by several investigators in the F_o basepiece of the proton translocating bacterial ATPase [1, 15, 34]. No such proteolipid component has, however, been shown in yeast and fungi plasma membrane ATPases, which have been solubilized, purified, and isolated [4, 14, 30, 36]. Unlike mitochondria [31] they seem to form vanadate-sensitive phosphorylated intermediates [2, 9, 46], but on the other hand, they are still sensitive towards DCCD and Dio-9; in the absence of a proton channel, the mechanism of action of those drugs most probably involves direct binding to the catalytic unit. Clearly this problem is not yet solved but it might be added that cytochrome *c* oxidase which also functions as a proton pump is also inhibited by DCCD [6].

In the turtle bladder, proton pumping has also been shown to be electrogenic [44], sensitive to DCCD, Dio-9, vanadate, but not to oligomycin [43], and most probably related to a plasma membrane ATPase located in the apical membrane [12]; it therefore resembles yeast and fungi proton pumps. With the exception of vanadate not yet tested, the proton pump under investigation in the present study exhibits striking similarities to those plasma membrane ATPases.

A final point of discussion should be the electrogenic nature of the pump. Our results obtained with valinomycin on ATP hydrolysis and on proton secretion suggest a stimulation of the proton pump under conditions when the membrane has been 'electrically short circuited', an indication for an electrogenic component. The effects observed are, however, small and certainly require further investigation.

The present demonstration of the existence of a brush-border proton pump provides a strong basis for several physiological observations not well-explained if only an antiport mechanism would exist: electrogenicity of HCO_3^- transport [16, 17], partial insensitivity to ouabain [18, 45], and variable stoichiometry with sodium [11]. The relative physiological significance of those two systems remains however to be clarified as well as their possible modulation by different stimuli. It appears reasonable to hypothesize that the antiport is associated with the bulk transfer of proton, although the relative importance of this contribution is likely to vary between species. The ATP-driven proton pump could serve as a mechanism for altering the stoichiometry between Na^+ and H^+ or could serve the purpose of very fine adjustment of intracellular hydrogen ion activity.

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