Biochimica et Biophysica Acta 937 (1988) 398–410
Elsevier

BBA 7 857

Proton ATPase in rat renal cortical endocytotic vesicles

Ivan Sabolić and Gerhard Burckhardt

Max-Planck-Institut für Biophysik, Frankfurt (Main) (F.R.G.)

(Received 14 September 1987)

Key words: Proton pump; ATPase, H^+-; Endocytotic vesicle; (Rat kidney)

To relate ATPase activity to the ATP-driven H^+-pump in rat renal endocytotic vesicles we applied an in vitro coupled optical test and a P_i-liberation assay. Endocytotic vesicles contain an ouabain-, vanadate- and oligomycin-insensitive ATPase. The ionophores for K^+ and H^+, valinomycin and carbonylcyanide *p*-chloromethoxyphenylhydrazone (CCCP), respectively, stimulated ATPase activity, indicating its relation to the electrogenic H^+-pump. This conclusion is supported by a similar distribution on a Percoll gradient of ATP-driven H^+ uptake into endosomes and ionophore-stimulated ATPase activity. Coupled optical and P_i-liberation assays were then used to characterize the H^+-ATPase with respect to the requirement for pH, nucleotides, anions, and mono- and divalent cations. The H^+-ATPase activity was decreased by widely used blockers: *N*-ethylmaleimide (NEM), dicyclohexylcarbodiimide (DCCD) and diethylstilbestrol (DES). Different sensitivities to these blockers proved that alkaline phosphatase and H^+-ATPase are separate entities. To investigate whether the NEM-, DCCD- and DES-sensitive ATPase activity is confined to intact endocytotic vesicles, cellular membranes from rat kidney cortex were separated on a Percoll density gradient. Surprisingly, endocytotic vesicles contain only a small fraction of the total NEM-, DCCD- and DES-sensitive ATPase activity. The majority of the blocker-sensitive ATPases belongs to membranes of as yet undefined cellular origin.

Introduction

A variety of organelles in eukaryotic cells wich serve in adsorptive and receptor-mediated endocytosis as well as exocytosis possess an acidic interior generated by an ATP-driven H^+-pump (for literature, see Ref. 1). At present, it is not clear whether the same H^+-pump is present in all of these organelles or a family of H^+-pumps exists with comparable properties. The characteristics of these H^+-pumps are basically similar. They are electrogenic and dependent on ATP. Permeant anions stimulate their activity whereas the carboxyl group reagent DCCD and the sulfhydryl group reagent *N*-ethylmaleimide strongly inhibit. On the contrary, ouabain, oligomycin and vanadate do not affect their activity. Thereby, these H^+-pumps are different from the oligomycin-sensitive mitochondrial H^+-ATPase and the vanadate-inhibitable H^+-pump present in gastric mucosa.

Most of the characteristics of the H^+-pumps in clathrin-coated vesicles, endosomes, lysosomes, endoplasmic reticulum and Golgi membranes have been investigating by studying ATP-driven H^+

Abbreviations: CCCP, carbonylcyanide *p*-chloromethoxyphenylhydrazone; DCCD, dicyclohexylcarbodiimide; H^+-ATPase, H^+ translocating ATPase (H^+-pump); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; levamisole, L-(−)-2,3,5,6-tetrahydro-6-phenylimidazo[2,1,*b*]thiazole hydrochloride; Mes, 4-morpholineethanesulfonic acid; TMA^+, tetramethylammonium (cation).

Correspondence: I. Sabolić, Faculty of Medicine, Department of Physiology, Salata 3, pp 978, 41000 Zagreb, Yugoslavia.

transport with fluorescent indicators rather than by measuring the ATPase activity. In a few cases, the ATPase activity in isolated organelles has been determined, but a good correlation between ATPase and ATP-driven H^+ transport has not been obtained [2–7].

In this contribution we used endocytotic vesicles from rat renal cortex which containe a well studied ATP-driven H^+-pump [3,8,9] to demonstrate and characterize the H^+-ATPase activity. Applying two different ATPase assays, a high degree of correlation between characteristics of ATP-driven H^+ uptake and ATPase activity has been found.

Materials and Methods

Preparation of endocytotic vesicles

Endocytotic vesicles were isolated from rat renal cortical homogenate by differential and Percoll gradient density centrifugation as described in detail previously [3,8], with modifications to yield a much higher enrichment of the H^+-pump activity [9]. As estimated from the initial rates of ATP-driven, Cl^--stimulated acridine orange quenching, vesicles were enriched in H^+-pump activity about 40-fold (38.6 ± 2.36, $n = 29$) compared to the homogenate. The enrichment factors for leucine arylamidase (1.63 ± 0.08, $n = 16$) and alkaline phosphatase (1.73 ± 0.03, $n = 6$), markers for luminal membranes, and for $(Na^+ + K^+)$-ATPase (0.45 ± 0.14, $n = 4$), a marker for contraluminal membranes, indicate that the preparations of vesicles were slightly contaminated with brush-border membranes, but not at all with basolateral membranes.

For the measurements of the H^+-pump and ATPase activities, the vesicles were prepared in the following buffer: 300 mM mannitol, 100 mM potassium gluconate, 5 mM $MgSO_4$, 5 mM Hepes-Tris (pH 7.0).

Assays

Protein was meausred by the method of Bradford [10], using bovine serum albumin as a standard. Leucine arylamidase (EC 3.4.11.2) and alkaline phosphatase activities (EC 3.1.3.1) were measured at 37 °C using commercial kits (Merckotests 3359 and 3344, respectively). $(Na^+ + K^+)$-ATPase activity (EC 3.6.1.3) was measured by the coupled optical assay described by Berner and Kinne [11]. ATP-driven, Cl^-stimulated H^+-pump activity was measured by the quench method of acridine orange fluorescence as described in detail previously [3,8,9].

For determination of ATPase activity in membrane vesicles by the coupled optical assay, a Shimadzu UV-300 double-beam/dual wavelength spectrophotometer with a chart recorder was used. Vesicles (50 μg protein) were added to 2.0 ml ATPase buffer I (330 mM mannitol, 110 mM potassium gluconate, 5.5 mM $MgSO_4$, 2.2 mM ouabain, 1.1 mM levamisole, 5.5 μg/ml oligomycin, 55 mM Hepes-Tris (pH 7.0)) or II (the same as the preceding but containing 110 mM KCl instead of potassium gluconate) and preincubated at 37 °C for 10 min in the absence (controls containing solvent) or presence of various ionophores and inhibitors added from stocks. The measurement of ATPase activity was started by addition of 0.2 ml substrate/enzyme buffer (5.5 mM phospho*enol*pyruvate, 55 mM ATP, 2.42 mM NADH, 105 U/ml pyruvate kinase, 195 U/ml lactate dehydrogenase, 5.5 mM Hepes-Tris (pH 7.0). The decrease in NADH absorbance was continuously monitored at 340 nm. During the measurement, the vesicles were constantly stirred. For calculations of ATPase activity we used the rates which were recorded between the 5th and the 6th minute (cf, Fig. 1). The ATPase activity, thus obtained, was linear up to 75 μg vesicle protein per assay (data not shown) and is expressed in nmol NADH split per min per mg protein (nmol/min per mg protein).

ATPase activity measured by the P_i-liberation assay was also performed at 37 °C. Vesicles (50 μg protein) were added to 1.0 ml prewarmed ATPase buffer III (250 mM sucrose, 100 mM KCl, 5 mM $MgSO_4$, 2 mM ouabain, 1 mM levamisole, 5 μg/ml oligomycin, 5 mM ATP, 50 mM Hepes-Tris (pH 7.0)) which contained either solvent or various ionophores and inhibitors, without preincubation. The reaction was stopped 15 min later by addition of 0.1 ml ice-cold 20% (w/v) trichloroacetic acid. The liberated phosphate (P_i) was determined colorimetrically [12]. The ATPase activity was linear up to 75 μg vesicle protein per assay (data not shown) and is expressed in μmol liberated P_i per

15 min per mg protein (μmol P_i/15 min per mg protein).

Deviations from the above described specifications are indicated in the text or in the legends of the figures and tables.

Inhibitors and ionophores were added from water (vanadate, *N*-ethylmaleimide) or ethanol stocks (valinomycin, oligomycin, CCCP, DCCD, diethylstilbestrol, gramicidin) before addition of vesicles. The final concentrations of these compounds are indicated in the legends of the figures and tables. Controls contained an equivalent amount (1%) of ethanol.

Statistical evaluation of the data

The data were evaluated statistically by means of Student's *t*-test at the 5% level of significance.

Materials

Lactate dehydrogenase, pyruvate kinase (both from rabbit muscle, in glycerol solution), valinomycin, NADH (disodium salt), phospho*enol*pyruvate (tricyclohexylammonium salt), ATP, GTP, UTP, ITP, and CTP (all disodium salts) were from Boehringer (Mannheim, F.R.G.). Oligomycin, *N*-ethylmaleimide and diethylstilbestrol were from Serva (Heidelberg, F.R.G.). DCCD was from Calbiochem (Los Angeles, CA, U.S.A.), vanadate, ascorbic acid and L-cysteine from Merck (Darmstadt, F.R.G.), and levamisole from Sigma (St. Louis, MO, U.S.A.). Other chemicals were of analytical grade. A stock solution of vanadate was prepared in water at least 10 days before use.

Results

H^+-ATPase in endocytotic vesicles

Following addition of ATP to a suspension containing endocytotic vesicles and the components of the coupled optical assay (for details see Materials and Methods), a time-dependent decrease of NADH absorbance was observed (Fig. 1, gluconate, EtOH). This absorbance decrease indicates the presence of ATPase activity in endocytotic vesicles. In the presence of chloride (Cl^-, EtOH), and to a much greater extent in the presence of ionophores for K^+ and H^+, i.e., valinomycin and CCCP, respectively (Cl^- + Val + CCCP), the absorbance decrease proceeded faster, indicating the stimulation of ATPase. Since the NADH absorbance decrease started to be proportional to time after 4–5 min, the absorbance difference between the fifth and sixth minute was used to estimate the ATPase activity in all further experiments.

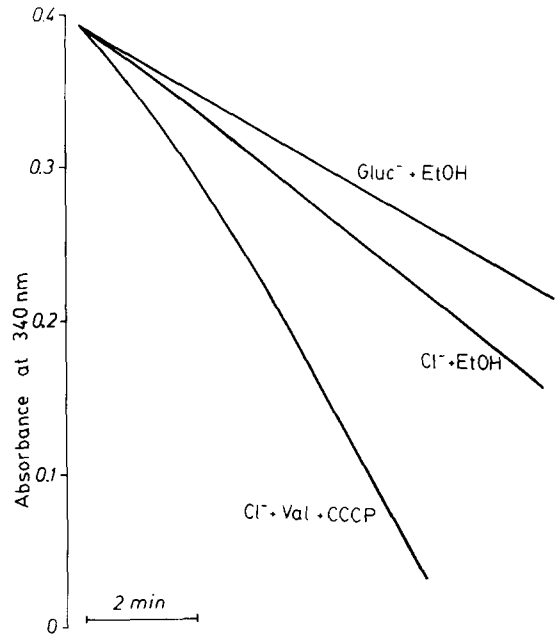

Fig. 1. ATPase activity in endocytotic vesicles as demonstrated by the coupled optical assay. Vesicles were added to ATPase buffer I (gluconate) or II (Cl^-) which contained ethanol (EtOH) or valinomycin plus CCCP (Val + CCCP). The reaction was started by adding substrate/enzyme buffer (for details see Materials and Methods). Before starting the reaction, the vesicles were preincubated with EtOH or ionophores at 37 °C for 10 min.

The ATPase activities in endocytotic vesicles as measured by the coupled optical assay, and, in addition, by a P_i-liberation assay, are listed in Table I. As found by the optical test, endocytotic vesicles exhibited an ouabain-, levamisole- and oligomycin-insensitive ATPase activity in the absence of permeant anions and ionophores (gluconate + ethanol). This ATPase was stimulated by Cl^- (chloride + ethanol) and further by valinomycin in the presence of K^+, i.e., by dissipation of the inside-positive membrane potential created by the electrogenic H^+-pump present in the vesicle membrane [3,13]. A bigger stimulation of ATPase activity was observed with the protonophore CCCP

TABLE I

EFFECTS OF CHLORIDE, IONOPHORES AND AMMONIA ON ATPase ACTIVITY IN ENDOCYTOTIC VESICLES, AS MEAUSRED BY THE COUPLED OPTICAL TEST AND P_i-LIBERATION ASSAYS

ATPase activity was measured in either ATPase buffer I (gluconate) or II (chloride) and in ATPase buffer III with the optical test and the P_i-liberation assays, respectively. The buffers contained the indicated additions in the following final concentrations: ethanol, 1%; valinomycin, 5 μM; CCCP, 10 μM; gramicidin, 10 μg/ml; NH_4Cl, 22.5 mM. Shown are means ± S.E., obtained with 4–9 different vesicle preparations. Statistically significant differences are indicated by * (vs. gluconate + ethanol) and ** (vs. chloride + ethanol). n.d., not determined.

Buffer	Additions	ATPase activity	
		Optical test (nmol/min per mg protein)	P_i liberation (μmol/15 min per mg protein)
Gluconate	ethanol	161.8 ± 10.2	n.d.
	valinomycin + CCCP	354.5 ± 28.5 *	n.d.
Chloride	ethanol	202.3 ± 11.3 *	2.09 ± 0.17
	valinomycin	212.1 ± 12.7	2.57 ± 0.25
	CCCP	262.2 ± 15.5 **	3.23 ± 0.19 **
	valinomycin + CCCP	408.4 ± 17.6 **	3.83 ± 0.20 **
	gramicidin	347.5 ± 9.9 **	3.00 ± 0.16 **
	NH_4Cl	369.3 ± 15.2 **	3.57 ± 0.26 **

and the cation ionophore gramicidin, i.e., compounds which dissipate proton gradients and membrane potential [13]. A high stimulation of ATPase activity was observed also with 22.5 mM ammonium buffer (NH_4Cl). Under this condition, NH_3 enters the vesicles by nonionic diffusion and binds a proton, leading to formation of ammonium ions. This results in the dissipation of the H^+ gradient, but not of the membrane potential [13]. The highest ATPase activity was recorded in the presence of valinomycin + CCCP, i.e., when both membrane potential and proton gradient are rapidly and completely abolished. Stimulation of ATPase by valinomycin + CCCP was present in both, gluconate- and chloride-containing buffers. The activity of the ATPase in the presence of valinomycin + CCCP was by 15% higher in the chloride buffer than in the gluconate buffer, indicating that chloride may have a small direct stimulatory effect on the ATPase activity.

Comparable data were obtained with the P_i-liberation assay. Here, however, we were not able to measure the ATPase activity in the gluconate-containing ATPase buffer I, as commercially available potassium gluconate contains P_i as an impurity. As shown in Table I, the ATPase activity measured by the P_i-liberation assay in the chloride-containing buffer (ATPase buffer III) was also slightly stimulated by valinomycin (K^+ was present in the buffer), more by CCCP, gramicidin and NH_4Cl, and most by valinomycin + CCCP. Stimulation of ATPase activity by the compounds which dissipate membrane potential or proton gradient, or both directly proves the presence of an electrogenic H^+-ATPase in endocytotic vesicles. In further experiments, the (valinomycin + CCCP)-stimulated portion of the ATPase activity was taken as a measure for the H^+-ATPase in endocytotic vesicles.

Effect of anions

As shown in our previous studies with the acridine orange fluorescence quench technique [3], the ATP-driven H^+ accumulation in endocytotic vesicles is stimulated by monovalent anions such as Cl^-, I^-, Br^- and SCN^-. Therefore, besides Cl^-, which has already been shown to stimulate ATPase activity in endocytotic vesicles (Table I), we tested the effect of various other anions. The ATPase activity was measured by the coupled optical test in buffers which contained 25 mM of various anions. The data are shown in Table II as specific and relative activities. To calculate relative activities, we used the activities observed in the gluconate-containing buffer as a reference. In complete accordance with the findings in acridine

TABLE II

ANION STIMULATION OF ATPase ACTIVITY IN ENDOCYTOTIC VESICLES

ATPase was measured by the coupled optical assay in a buffer which contained 300 mM mannitol, 5 mM $MgSO_4$, 2 mM ouabain, 1 mM levamisole, 5 μg/ml oligomycin, 50 mM Tris-Hepes (pH 7.0) and 25 mM indicated anions in the form of potassium salts. Relative activity vs. gluconate was calculated for each membrane preparation separately. Shown are means ± S.E. obtained with five different vesicle preparations. Statistically significant differences (vs. gluconate) are indicated by an asterisk.

Anion	ATPase activity	
	nmol/min per mg protein	relative activity
$Gluconate^-$	157.1 ± 11.2	100(± 4.96)
Cl^-	198.7 ± 12.5 *	126.5 ± 6.20 *
SCN^-	177.5 ± 10.2	113.1 ± 3.59 *
I^-	184.8 ± 9.6 *	117.6 ± 4.55 *
Br^-	185.5 ± 14.2	118.1 ± 4.13 *
F^-	159.1 ± 8.9	101.3 ± 1.57
NO_3^-	135.3 ± 8.6	86.1 ± 2.37 *
HCO_3^-	185.5 ± 14.2	118.1 ± 3.93 *
SO^{2-}	163.0 ± 12.5	103.8 ± 4.59
SO_3^{2-}	88.4 ± 3.3 *	56.3 ± 3.84 *
PO_4^{3-}	164.3 ± 10.6	102.6 ± 5.49

orange studies, not only Cl^-, but also SCN^-, I^- and Br^- stimulated the ATPase activity. In addition, a stimulation by 18% was measured with HCO_3^-. A slight inhibition (about 14%) of the activity was observed with NO_3^-, and a stronger inhibition (about 44%) with SO_3^{2-}. F^-, SO_4^{2-} and PO_4^{3-} had no effect.

Optimum pH

ATPase activity in endocytotic vesicles was measured at various pH values in chloride-containing buffers in nonstimulated (Fig. 2, EtOH) and ionophore-stimulated conditions (Fig. 2, Val + CCCP). As measured by both assays, the ATPase in endocytotic vesicles exhibited a peak of activity at pH values between 6.75 and 7.50. The (valinomycin + CCCP)-stimulated portion of the ATPase activity, i.e., the activity of the H^+-ATPase in endocytotic vesicles, showed an optimum at pH 7.0 (Fig. 2, triangles).

Nucleotide specificity

As the coupled optical assay may not operate equally effective with all nucleotides, the nucleo-

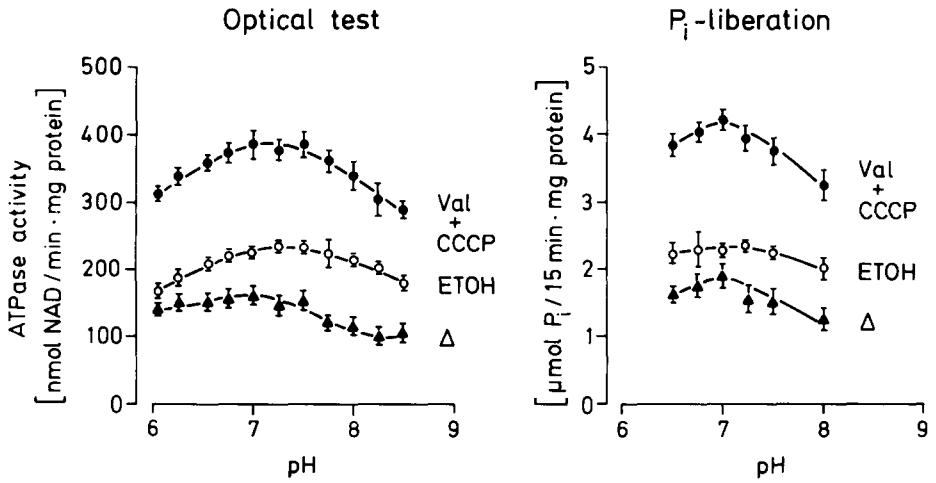

Fig. 2. Optimum pH of H^+-ATPase in endocytotic vesicles. Optical test: Vesicles (50 μg protein) were diluted into ATPase buffer II which was buffered with either 50 mM Mes-Tris (pH 6.05–6.56), 50 mM Hepes-Tris (pH 6.75–7.75) or 50 mM Tris-Hepes (pH 8.00–8.50) in the absence (1% ethanol, EtOH) or presence of 5 μM valinomycin + 10 μM CCCP (Val + CCCP). (Val + CCCP)-stimulated ATPase, which represents the difference in ATPase activity in the presence and absence of ionophores, is shown by Δ. Before starting the reaction with substrate/enzyme buffer, vesicles were preincubated with ethanol and ionophores at 37 °C for 10 min. P_i-liberation: Vesicles (50 μg protein) were diluted into ATPase buffer III buffered with 50 mM Tris-Hepes (pH 6.50–8.00) in the absence (EtOH) and presence of valinomycin + CCCP (the concentrations see above). The measurements were started without preincubation by the addition of vesicles. The (Val + CCCP)-stimulated portion of ATPase activity is shown by Δ. Data are mean ± S.E. obtained with three vesicle preparations.

TABLE III

NUCLEOTIDE DEPENDENCE OF H^+-ATPase ACTIVITY IN ENDOCYTOTIC VESICLES

ATPase activity was measured in ATPase buffer III which contained 2 mM nucleotides and either 1% ethanol or 5 μM valinomycin + 10 μM CCCP. The (valinomycin + CCCP)-stimulated part of the ATPase activity is given by Δ. Shown are means ± S.E. of ATPase activities of three membrane preparations. Statistically significant differences vs. ethanol are indicated by the asterisk.

Nucleotide	ATPase activity; P_i liberation (μmol P_i/15 min per mg protein)		
	ethanol	valinomycin + CCCP	Δ
ATP	2.46 ± 0.15	3.48 ± 0.18 *	1.02 ± 0.05
GTP	1.47 ± 0.08	1.68 ± 0.25	0.21 ± 0.03
UTP	1.45 ± 0.21	1.58 ± 0.21	0.12 ± 0.03
ITP	1.70 ± 0.23	1.79 ± 0.14	0.09 ± 0.02
CTP	1.06 ± 0.29	1.17 ± 0.23	0.10 ± 0.03

tide specificity was tested with a P_i-liberation assay only (Table III). In the absence of ionophores (ethanol) endocytotic vesicles contain a triphosphatase activity which is highest with ATP, and 30–40% lower with other nucleotides. Valinomycin plus CCCP stimulated the triphosphatase activity strongly in the presence of ATP, and to a much smaller extent with GTP, UTP, ITP, or CTP. Therefore, the preparations of endocytotic vesicles contain a H^+-ATPase which is specific for ATP and, in addition, a nonspecific triphosphatase. The preference of the H^+-ATPase for ATP is in complete agreement with previous acridine orange studies in which the H^+ accumulation in endocytotic vesicles was highest with ATP [3].

Dependence on divalent cation

Table IV displays the ATPase activities as functions of various divalent cations. The data obtained by the coupled optical assay indicate no significant ATPase activity in the absence of divalent cation (None) and in the presence of 5 mM Ba^{2+}, Zn^{2+}, Cu^{2+} or Ca^{2+}. High enzyme activities and stimulation by valinomycin plus CCCP were recorded only with Mg^{2+} and Mn^{2+}. However, the apparent absence of the ATPase activity with some divalent cations, as observed in this assay, can be due to an interference of the cations with the optical system. Thus, pyruvate kinase, which is

TABLE IV

DIVALENT CATION DEPENDENCE OF H^+-ATPase IN ENDOCYTOTIC VESICLES

ATPase activity was meausred in ATPase buffer II and ATPase buffer III with the coupled optical assay (A) and the P_i-liberation assay (B), respectively. The buffers contained 5 mM of divalent cations as chloride salts and either 1% ethanol or 5 μM valinomycin + 10 μM CCCP. The (valinomycin + CCCP)-stimulated part of the ATPase activity is given by Δ. Shown are means ± S.E. of ATPase activities obtained with three membrane preparations. Statistically significant differences vs. ethanol are indicated by the asterisk.

Divalent cation	ATPase activity		
	ethanol	valinomycin + CCCP	Δ
A. Coupled optical assay (nmol/min per mg protein)			
None	15.5 ± 2.1	8.5 ± 2.1	0
Mg^{2+}	284.1 ± 13.4	537.2 ± 14.1 *	238.3 ± 3.5
Mn^{2+}	270.7 ± 56.4	568.2 ± 19.7 *	297.5 ± 36.7
Ba^{2+}	8.5 ± 2.1	8.5 ± 2.1	0
Zn^{2+}	28.2 ± 2.1	26.1 ± 0.0	0
Cu^{2+}	15.5 ± 2.1	13.4 ± 0.0	0
Ca^{2+}	17.6 ± 2.1	17.6 ± 2.1	0
B. P_i liberation (μmol P_i/15 min per mg protein)			
None	0.38 ± 0.15	0.90 ± 0.22	0.52 ± 0.09
Mg^{2+}	2.56 ± 0.34	4.03 ± 0.45 *	1.48 ± 0.11
Mn^{2+}	3.38 ± 0.43	5.48 ± 0.71 *	2.10 ± 0.29
Ba^{2+}	0	0.24 ± 0.20	0.24 ± 0.20
Zn^{2+}	0	0.09 ± 0.04	0.09 ± 0.04
Cu^{2+}	0.18 ± 0.13	0.42 ± 0.18	0.24 ± 0.07
Ca^{2+}	1.31 ± 0.12	1.68 ± 0.16	0.19 ± 0.08

present as a part of the coupled optical system, requires Mg^{2+} or Mn^{2+} for full activity [14]. The possibility that some of the tested cations inhibited the activity of the enzymes present in the system could not be excluded. Therefore, we have tested the effect of divalent cations also by the P_i-liberation assay. As shown also in Table IV, very small or no ATPase activity was observed in buffers containing no divalent cations and in the presence of Ba^{2+}, Zn^{2+} or Cu^{2+}. A significant activity was recorded with 5 mM Ca^{2+}, indicating the presence of a Ca^{2+}-dependent ATPase in the preparation of endocytotic vesicles. Ca^{2+}-stimulated ATPase was not further investigated. The highest ATPase activities were recorded with Mg^{2+} and Mn^{2+}. A significant stimulation of the ATPase activity by ionophores was observed only in the presence of Mg^{2+} or Mn^{2+}. Therefore, Mg^{2+} or Mn^{2+} are

necessary to support the activity of H^+-ATPase in endocytotic vesicles.

Dependence on monovalent cation

As the activity of pyruvate kinase in a coupled optical test depends on K^+ [14], the effects of monovalent cations on ATPase activity in endocytotic vesicles were investigated only by the P_i-liberation assay. Neither of the tested monovalent cations affected the activity of ATPase in the absence of ionophores (Table V, ethanol). Moreover, the potency of valinomycin plus CCCP to stimulate the ATPase activity was equal in the presence of any of the listed monovalent cations, indicating that the H^+-ATPase requires no specific monovalent cation for the activity. These data agree completely with the observations in acridine orange studies, which showed the ATP-driven H^+ accumulation in endocytotic vesicles to be independent of monovalent cations [3].

Effect of inhibitors

In previous studies [3], we found that the ATP-driven H^+ uptake into endocytotic vesicles can be inhibited by *N*-ethylmaleimide and DCCD. Vanadate, an inhibitor of various ATPases which are phosphorylated during the catalytic cycle, did not inhibit ATP-driven H^+ uptake. The same was found for levamisole, an inhibitor of alkaline phosphatase. In the present experiments, these and other putative inhibitors of H^+-pumps were tested on ATPase activity in endocytotic vesicles. In order to exclude further alkaline phosphatase as an enzyme which may have contributed to ATP splitting in our experiments, we have tested also various inhibitors on this enzyme.

Prior to these measurements, we checked whether the above-mentioned inhibitors interact with the coupled optical system, e.g., by inhibiting the enzymes present in the test. For this purpose, the activity of the optical system was measured in the absence of vesicles. In this case, the assay contained 3 mM ADP instead of 5 mM ATP, and the concentration of pyruvate kinase was decreased from 9.55 U/ml to 10 mU/ml. Under these condition, the rate of NADH consumption was limited by low activity of pyruvate kinase. The reaction was started by addition of PK to the buffers, with or without ionophores and inhibitors. The concentrations of the tested compounds were the same or higher than those used in the

TABLE V

EFFECT OF MONOVALENT CATIONS ON H^+-ATPase ACTIVITY IN ENDOCYTOTIC VESICLES

ATPase activity was measured in ATPase buffer III which contained 100 mM indicated monovalent cations in the form of chloride salts and either 1% ethanol or 5 μM valinomycin + 10 μM CCCP. The (valinomycin + CCCP)-stimulated portion of the ATPase activity is given by Δ. Shown are means ± S.E. for the enzyme activities in four membrane preparations. Statistically significant differences vs. ethanol are indicated by an asterisk.

Cation	ATPase activity (μmol P_i/15 min per mg protein)		
	ethanol	valinomycin + CCCP	Δ
TMA^+	2.45 ± 0.09	4.63 ± 0.73 *	2.18 ± 0.64
K^+	2.40 ± 0.11	4.56 ± 0.78 *	2.16 ± 0.68
Na^+	2.45 ± 0.14	4.68 ± 0.79 *	2.23 ± 0.68
Li^+	2.44 ± 0.09	4.72 ± 0.84 *	2.29 ± 0.75
Rb^+	2.48 ± 0.11	4.59 ± 0.78 *	2.11 ± 0.67
Cs^+	2.43 ± 0.13	4.48 ± 0.77 *	2.06 ± 0.65

TABLE VI

INTERACTION OF VARIOUS IONOPHORES WITH THE COUPLED OPTICAL ASSAY SYSTEM

The reactions were measured at 37 °C. The assay mixture contained 300 mM mannitol, 100 mM KCl, 5 mM $MgSO_4$, 2 mM ouabain, 50 mM Hepes-Tris (pH 7.0), 0.22 mM NADH, 3 mM ADP, 0.5 mM phospho*enol*pyruvate, 6 U/ml lactate dehydrogenase, and ionophores or inhibitors in the concentrations as indicated. The reactions were started by the addition of 10 mU/ml pyruvate kinase. The decrease of NADH absorbance was continuously recorded at 340 nm. The rates of absorbance changes between the 5th and the 6th minute of measurement were used to calculate the relative activities. Each datum is the mean of two or three measurements.

Additions (concentration)	Relative activity (%)
None	100
Ethanol (1%)	105.9
Oligomycin (5 μg/ml)	99.2
Valinomycin (5 μM) + CCCP (10 μM)	105.2
Vanadate (1 mM)	74.7
N-Ethylmaleimide (1 mM)	63.1
DCCD (1 mM)	100.8
Diethylstilbestrol (50 μM)	89.8
Levamisole (50 mM)	102.9
Zn^{2+} (1 mM)	2.9
Ascorbic acid (1 mM)	99.4
L-Cysteine (10 mM)	102.3

preceding experiments. The rates of NADH absorbance decrease were recorded at 37°C. The rates observed between the 5th and the 6th minute of the measurement, i.e., in the interval used to estimate the ATPase activity in all other experiments, were used also to calculate the relative ADPase activities. The data are summarized in Table VI. In comparison with the activity in the absence of any ionophore or inhibitor (none), the activity of the coupled optical system was not affected by 1% ethanol, 5 μg/ml oligomycin, 5 μM valinomycin + 10 μM CCCP, 1 mM DCCD, 50 mM levamisole, 1 mM ascorbic acid or 10 mM L-cysteine. Vanadate, *N*-ethylmaleimide, Zn^{2+} (1 mM each), 50 μM diethylstilbestrol inhibited the rate of NADH absorbance decrease by 25, 37, 97 and 10%, respectively. Thus ATPase activities in the presence of inhibitors which interfered with the coupled optical system were tested by the P_i-liberation assay. DCCD and L-cysteine were tested with both assays.

Table VII lists the effects of various inhibitors on ATPase activity in endocytotic vesicles. As recorded in the coupled optical test, levamisole (25 mM) and L-cysteine (10 mM) did not change the rate of ATPase activity in the absence of ionophores (ethanol), whereas 1 mM DCCD inhibited by 84%, and 0.5 mM ascorbic acid by 40%. In the presence of ionophores (valinomycin + CCCP), the ATPase was strongly stimulated in the absence of inhibitors (none), much less with levamisole, ascorbic acid and L-cysteine, and not at all with DCCD. Consequently, the H^+-ATPase in endocytotic vesicles (Δ) was inhibited by 62% with levamisole, 58% with ascorbic acid, 91% with L-cysteine, and completely with DCCD. In the assay of P_i-liberation, 1 mM vanadate and 10 mM L-cysteine did not inhibit ATPase activity in control experiments (ethanol). A partial inhibition (up to 50%) was observed with *N*-ethylmaleimide, DCCD and Zn^{2+} (1 mM each) and with 50 μM diethylstilbestrol. With ionophores (valinomycin + CCCP) we found a strong stimulation of P_i formation in the absence of inhibitors (none), and in the presence of vanadate. A much smaller stimulation was measured in the presence of L-cysteine, and

TABLE VII

EFFECT OF VARIOUS INHIBITORS ON H^+-ATPase ACTIVITY IN ENDOCYTOTIC VESICLES AS MEASURED BY THE COUPLED OPTICAL AND P_i-LIBERATION ASSAYS

The assays were performed with the coupled optical test and P_i-liberation assays, respectively. ATPase buffer II and III contained the indicated concentrations of inhibitors plus either 1% ethanol or 5 μM valinomycin + 10 μM CCCP. In the optical assay, vesicles were preincubated with inhibitors and ionophores at 37°C for 10 min before starting the reaction with substrate/enzyme buffer. In the assay of P_i liberation, the reaction was started by the addition of vesicles, without preincubation. Δ represents the (valinomycin + CCCP)-stimulated part of the ATPase activity. Shown are means ± S.E. for the number of membrane preparations indicated by *n*. Statistically significant differences are indicated by one (vs. ethanol) or two asterisks (vs. none).

Inhibitor (concn.)	ATPase activity		
	ethanol	valinomycin + CCCP	Δ
A. Coupled optical assay (nmol/min per mg protein) ($n = 4$)			
None	153.9 ± 11.9	306.0 ± 42.1 *	152.1 ± 32.8
Levamisole (25 mM)	157.6 ± 11.3	216.1 ± 16.1 *	58.5 ± 5.9 **
DCCD (1 mM)	24.5 ± 4.6	25.5 ± 5.0	1.1 ± 0.8 **
Ascorbic acid (0.5 mM)	92.9 ± 20.2	156.6 ± 27.0	63.7 ± 22.7 **
L-Cysteine (10 mM)	162.6 ± 15.9	175.7 ± 17.8	13.9 ± 7.0 **
B. P_i liberation (μmol P_i/15 min per mg protein) ($n = 5$)			
None	1.98 ± 0.15	3.17 ± 0.13 *	1.19 ± 0.08
Vanadate (1 mM)	2.16 ± 0.22	3.23 ± 0.19 *	1.07 ± 0.06
N-Ethylmaleimide (1 mM)	1.39 ± 0.20	1.36 ± 0.27	0
DCCD (1 mM)	1.07 ± 0.18	0.80 ± 0.12	0
Diethylstilbestrol (50 μM)	1.70 ± 0.26	1.88 ± 0.27	0.18 ± 0.07 **
L-Cysteine (10 mM)	2.11 ± 0.11	2.76 ± 0.14 *	0.65 ± 0.09 **
Zn^{2+} (1 mM)	1.24 ± 0.11	1.24 ± 0.11	0

negligible stimulation in the presence of diethylstilbestrol. No stimulation of ATP splitting was obtained in the presence of *N*-ethylmaleimide, DCCD and Zn^{2+}. The data from these experiments indicate that vanadate does not inhibit H^+-ATPase in endocytotic vesicles, whereas *N*-ethylmaleimide, DCCD, diethylstilbestrol, and Zn^{2+} are strong inhibitors. Besides Zn^{2+}, also some other inhibitors of alkaline phosphatase (levamisole, ascorbic acid, L-cysteine), used in concentrations known to inhibit the activity of this enzyme in various tissues [15–18], inhibit partially the H^+-ATPase activity. As meausred by the acridine orange fluorescence quench method, the same compounds inhibit also the H^+-pump in the vesicles (data not shown).

The inhibition of the H^+-ATPase (H^+-pump) in endocytotic vesicles by compounds which inhibit alkaline phosphatase activity suggests that this enzyme may be responsible for the observed ATPase and H^+-pumping activity. Such a possibility is further indicated by the finding of a relatively high specific activity of alkaline phosphatase in endocytotic vesicles; the vesicle preparations are always enriched in the activity of this enzyme between 1.5- and 2.5-times compared with the homogenate. To investigate further a possible relationship between alkaline phosphatase and H^+-ATPase, we tested the effect of the above used inhibitors also on alkaline phosphatase activity in endocytotic vesicles.

As shown in Table VIII, alkaline phosphatase activity is completely inhibited with 1 mM levamisole, i.e., at a concentration 25-fold smaller than that used to inhibit H^+-ATPase activity. A complete inhibition of the enzyme activity was obtained also with 10 mM L-cysteine and 1 mM Zn^{2+}. Vanadate (1 mM) inhibited 86%, whereas 0.5 mM ascorbic acid inhibited weakly, *N*-Ethylmaleimide, DCCD, and diethylstilbestrol, in concentrations which inhibited H^+-ATPase completely, did not affect the activity of alkaline phosphatase. The experiment clearly shows that alkaline phosphatase and H^+-ATPase are two separate entities with different sensitivities to inhibitors.

Although these experiments rule out the possibility that alkaline phosphatase plays a role as a H^+-ATPase (H^+-pump), all our buffers for ATPase measurements contained 1 mM levamisole, to diminish ATP splitting by the alkaline phoshatase.

TABLE VIII

EFFECT OF VARIOUS INHIBITORS ON ALKALINE PHOSPHATASE ACTIVITY IN ENDOCYTOTIC VESICLES

Shown are means ± S.E. of alkaline phosphatase activity measured at pH 9.8 with three different membrane preparations. The vesicles were preincubated with the indicated inhibitors at 37 °C for 10 min before starting the reaction by the addition of substrate. Control (none) contained 1% ethanol. Statistically significant differences vs. 'none' are indicated by the asterisk.

Inhibitor (concentration)	Alkaline phosphatase activity (μmol/min per mg protein)
None	2.23 ± 0.36
Levamisole (1 mM)	0.10 ± 0.01 *
Ascorbic acid (0.5 mM)	1.82 ± 0.15
L-Cysteine (10 mM)	0.02 ± 0.01 *
Zn^{2+} (1 mM)	0.09 ± 0.00 *
Vanadate (1 mM)	0.31 ± 0.03 *
N-Ethylmaleimide	2.22 ± 0.31
DCCD (1 mM)	2.23 ± 0.35
Diethylstilbestrol (50 μM)	2.25 ± 0.30

Distribution of various ATPases along a Percoll gradient

The preceding experiments showed the presence of a (valinomycin + CCCP)-stimulated ATPase (H^+-ATPase) in endocytotic vesicles, the activity of which can be strongly inhibited by *N*-ethylmaleimide, DCCD and diethylstilbestrol. In order to determine whether the ATPase activity with a similar inhibitory pattern is present in other cellular membranes, we measured the distribution of ATPases along a 16% (w/w) Percoll gradient. The gradient has been used in previous [3,8,9] and present experiments to separate endocytotic vesicles from other cellular membranes. The gradient was fractionated in 1-ml portions and in each fraction we measured proteins and the activity of leucine arylamidase, the total ATPase (in absence of inhibitors), ($Na^+ + K^+$)-ATPase, oligomycin-sensitive and insensitive ATPases, DCCD-, *N*-ethylmaleimide-, and diethylstilbestrol-sensitive ATPases as well as the H^+-pump activity.

As described previously [3,9], luminal membranes distribute with two peaks along the gradient (Fig. 3A, leucine arylamidase). Total protein

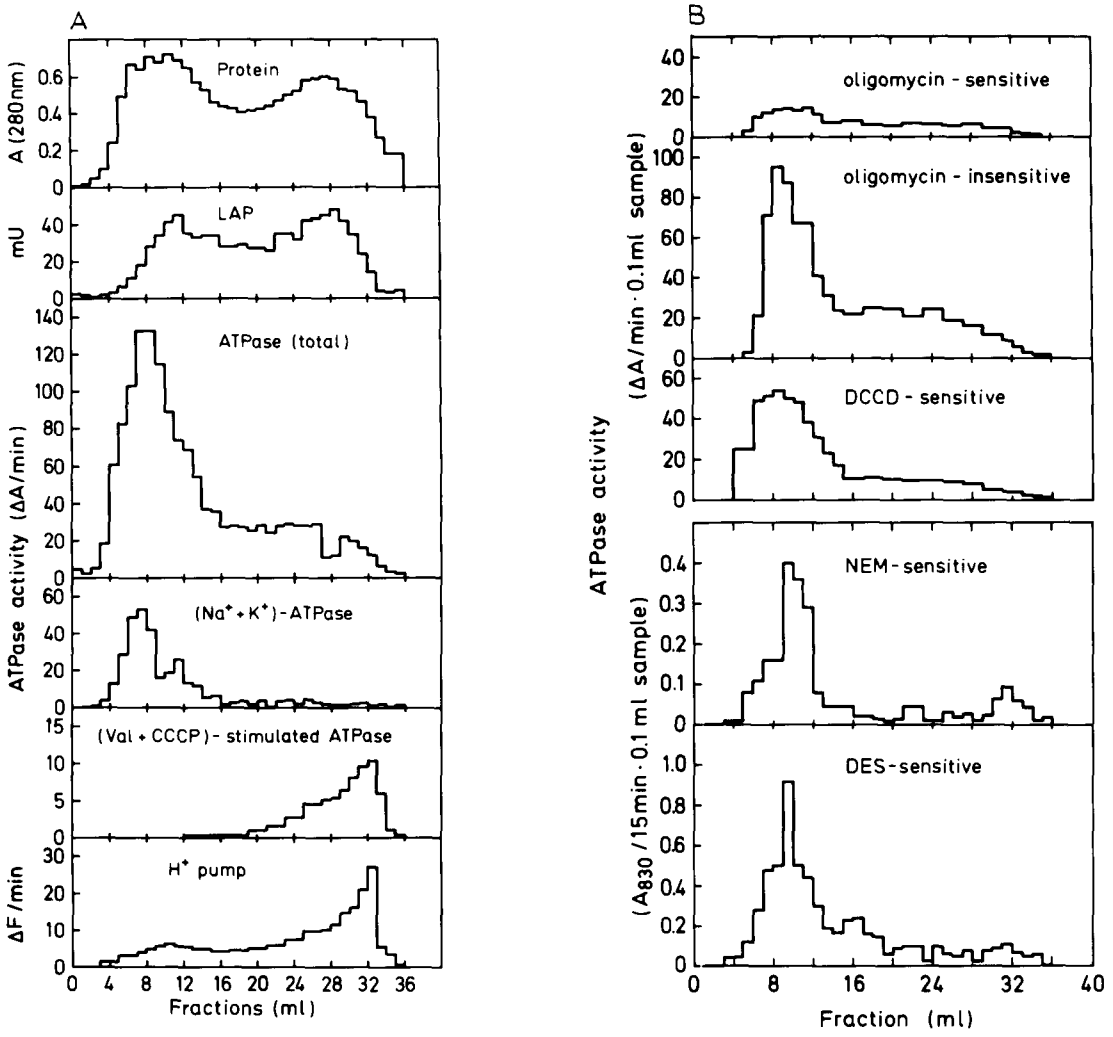

Fig. 3. Distribution of protein, leucine arylamidase activity (LAP) and the activity of H^+ pump and various ATPases along a Percoll gradient. The gradient was divided into 1-ml fractions. Fraction 1 denotes the top of the gradient. Protein was measured after diluting 50 μl of fraction sample into 1 ml water by testing the absorbance at 280 nm. Leucine arylamidase (LAP) activity was measured as described in Materials and Methods using 50 μl of fraction sample. 1 unit of enzyme activity corresponds to the splitting of 1 μmol leucine-*p*-nitroanilide at 37 °C per min. Total ATPase, ($Na^+ + K^+$)-ATPase, oligomycin-sensitive and insensitive ATPases, DCCD-sensitive ATPase and (Val + CCCP)-stimulated ATPase were measured by the coupled optical assay using 50–100 μl of fraction sample. Total ATPase was measured in ATPase buffer II, which was devoid of ouabain and oligomycin. ($Na^+ + K^+$)-ATPase is the difference between the total ATPase and the ATPase activity in the presence of 2 mM ouabain. Oligomycin-sensitive ATPase is the difference between the total ATPase and the ATPase activity in the presence of 5 μg/ml oligomycin. Oligomycin-insensitive ATPase was recorded in the presence of 2 mM ouabain + 5 μg/ml oligomycin. (Val + CCCP)-stimulated ATPase was measured in a complete ATPase buffer II and is shown as the difference in ATPase activity in the absence (1% ethanol) and presence of 5 μM valinomycin + 10 μM CCCP. H^+-pump activity was measured by fluorescence quenching of acridine orange and expressed as the initial rate of ATP-driven, Cl^--stimulated fluorescence quenching (ΔF/min). DCCD-sensitive ATPase was measured in ATPase buffer II and is shown as the difference of ATPase activities in the absence and presence of 1 mM DCCD. Before starting the reaction with substrate/enzyme buffer (ATPase measurements) or ATP (H^+-pump), vesicles were preincubated with indicated ionophores and inhibitors at 37 °C for 10 min. *N*-ethylmaleimide (NEM)- and diethylstilbestrol (DES)-sensitive ATPases were measured by the P_i-liberation assay in ATPase buffer III using 50 μl of fraction samples. The results are the differences of ATPase activities in the absence (1% ethanol) and presence of 1 mM inhibitors. The reactions were started without preincubation by the addition of vesicles.

shows a similar distribution. The total ATPase shows a high peak in the fractions at the top of the gradient and a decreasing activity toward the bottom. $(Na^+ + K^+)$-ATPase, a marker for basolateral membranes, exhibited a peak of activity also at the top of the gradient, and a negligible activity in the bottom fractions. On the contrary, the (valinomycin + CCCP)-stimulated ATPase, which corresponds to the H^+-ATPase in endocytotic vesicles, comigrates at the bottom of the gradient with the peak of the H^+-pump activity measured by the acridine orange fluorescence quench method. This experiment indicates unequivocally that both (valinomycin + CCCP)-stimulated ATPase and the ATP-driven, Cl^--stimulated intravesicular proton uptake reflect the same system, the H^+-ATPase (H^+-pump) in endocytotic vesicles.

Fig. 3B shows the distribution of ATPases sensitive to various inhibitors. The indicator for mitochondrial membranes, the oligomycin-sensitive ATPase, distributed all over the gradient, with a relatively broad peak at the top. Furthermore, the distribution of DCCD-, *N*-ethylmaleimide, and diethylstilbestrol-sensitive ATPases indicates that only a small part of the ATPase activity inhibitable by these compounds is present at the bottom of the gradient, where the peak of H^+-ATPase (H^+-pump) is localized. The highest activities of DCCD-, *N*-ethylmaleimide, and diethylstilbestrol-sensitive ATPases are found at the top of the gradient and copurify with $(Na^+ + K^+)$-ATPase.

Discussion

By using two different assays we demonstrated the presence of an ATPase activity in endosomes prepared from rat kidney cortex. As all measurements have been performed in the presence of ouabain, oligomycin and levamisole, this ATPase activity cannot be due to the presence of $(Na^+ + K^+)$-ATPase, mitochondrial H^+-ATPase and alkaline phosphatase, respectively. Stimulation of ATPase activity by ionophores that dissipate membrane potential and ΔpH rather indicates the presence of an electrogenic H^+-ATPase in the vesicle membrane.

Interestingly, valinomycin in the presence of K^+, and permeant anions (Cl^-, Br^-, I^-, SCN^-), which all dissipate membrane potential [13], stimulated ATPase activity only weakly. In contrast, NH_4^+, which solely dissipates ΔpH, and gramicidin or valinomycin plus CCCP, which dissipate both membrane potential and ΔpH [13], exhibited much stronger stimulation. Thus, the H^+-ATPase activity in endosomes is affected more by the ΔpH than by the membrane potential built up during ATP-driven H^+ transport. The systematic survey of the characteristics of H^+-ATPase as described here, and of ATP-driven H^+ uptake described in our previous studies [3,8,9], reveals a complete agreement of properties. H^+-ATPase and H^+ pumping are insensitive to ouabain, oligomycin and vanadate, require Mg^{2+} or Mn^{2+} but no specific monovalent cation, are specific for ATP, and are inhibited by Zn^{2+}, DCCD, *N*-ethylmaleimide, and diethylstilbestrol. The independence of H^+-ATPase on K^+ and vanadate rules out that the endosomal H^+-pump is similar to the gastric $(K^+ + H^+)$-ATPase. Furthermore, the failure of Na^+ to stimulate ATPase activity is in accordance with our previous finding that rat renal endocytotic vesicles do not possess an Na^+–H^+ exchanger in parallel to a H^+-pump [8].

In extension to our previous studies we demonstrate here that the H^+-ATPase works optimally at pH 7.0 and is affected by some well known inhibitors of alkaline phosphatase, such as Zn^{2+}, ascorbic acid, L-cysteine and levamisole. By investigating in more detail the inhibitor sensitivity of H^+-ATPase and alkaline phosphatase, we found that L-cysteine and levamisole are moderate inhibitors of the H^+-ATPase (at high concentrations) and strong inhibitors of alkaline phosphatase activity. Unlike the H^+-ATPase, alkaline phosphatase is strongly inhibited by vanadate, slightly by ascorbic acid and not at all by DCCD, *N*-ethylmaleimide, and diethylstilbestrol, demonstrating that H^+-ATPase and alkaline phosphatase are two different enzymes.

As shown in our experiments with various nucleotides, the preparations of endocytotic vesicles exhibit, in addition to H^+-ATPase, a nonspecific triphosphatase. Besides ATP, it splits GTP, UTP, CTP, and ITP with equal or slightly lower rates. The activity of this triphosphatase cannot be stimulated by ionophores which dissipate membrane potential or proton gradients, indicating that this enzyme does not serve as a proton trans-

locator. Furthermore, the experiments with divalent cations indicated the presence of a Ca^{2+}-stimulated ATPase in endocytotic vesicles. Also, this ATPase cannot account for the proton-pumping activity in the vesicles as it was not stimulated by ionophores. At present, it is not clear whether the nonspecific triphosphatase and the Ca^{2+}-dependent ATPase activities belong to endocytotic vesicles or to some other, contaminating membranes. A Ca^{2+}-stimulated ATPase has been found in basolateral membranes [20–22] and endoplasmic reticulum [19] of the renal proximal tubular cells, i.e., in the membranes which slightly contaminate our preparations of endocytotic vesicles [8,9].

The distribution of ATPases along a Percoll gradient demonstrated that the DCCD-, *N*-ethylmaleimide- and diethylstilbestrol-sensitive H^+-ATPase activity in intact endocytotic vesicles accounts for only a small part of the total oligomycin-insensitive ATPase. The majority of DCCD-, *N*-ethylmaleimide, and diethylstilbestrol-sensitive ATPases was found on the top of the gradient, whereas intact endosomes migrate at the bottom of the gradient. As DCCD- and diethylstilbestrol-sensitive ATPases were measured in the presence of ouabain and oligomycin, they do not reflect the activities of either $(Na^+ + K^+)$-ATPase or mitochondrial H^+-ATPase. Both of these ATPases are sensitive to DCCD [23,24], whereas diethylstilbestrol is a potent inhibitor of the mitochondrial H^+-ATPase [25]. On the other hand, neither of these ATPases is sensitive to *N*-ethylmaleimide, a potent inhibitor of the H^+-pump in various intracellular organelles (for literature, see Refs. 1 and 3). Therefore, the comigration of the high activities of DCCD-, *N*-ethylmaleimide-, and diethylstilbestrol-sensitive ATPases on the top of a Percoll gradient can be attributed to some membranes which may contain an H^+-ATPase, but are not able to accumulate protons. Although the exact origin of these membranes has to be investigated, our unpublished data indicate that these membranes cannot be basolateral membranes from the proximal tubule. Basolateral membrane vesicles isolated from rat renal cortex exhibit a high ATPase activity in the presence of ouabain, oligomycin and levamisole wich is, however, stimulated neither by Cl^- nor by valinomycin plus CCCP. It is strongly inhibited by DCCD and diethylstilbestrol, but insensitive to *N*-ethylmaleimide (data not shown), and thus is dissimilar to H^+-ATPase in endosomes. In accord with this finding is our previous observation of limited H^+ pumping activity in basolateral membrane vesicle preparations due to contaminating endosomes [8]. Further possible candidates could be broken endocytotic vesicles or unsealed Golgi or lysosomal membrane or the membranes from endoplasmic reticulum.

Acknowledgements

The authors thank Drs. I. Schulz, K.J. Ullrich and H. Murer for valuable discussions.

References

1 Mellman, I., Fuchs, R. and Helenius, A. (1986) Annu. Rev. Biochem. 55, 663–700.
2 Kaunitz, J.D., Gunther, R.D. and Sachs, G. (1985) J. Biol. Chem. 260, 11567–11573.
3 Sabolić, I. and Burckhardt, G. (1986) Am. J. Physiol. 250, F817–F826.
4 Van Dyke, R.W., Steer, C.J. and Scharschmidt, B.F. (1984) Proc. Natl. Acad. Sci. USA 81, 3108–3112.
5 Van Dyke, R.W., Hornick, C.A., Belcher, J., Scharschmidt, B.F. and Havel, R.J. (1985) J. Biol. Chem. 260, 11021–11026.
6 Van Dyke, R.W., Scharschmidt, B.F. and Steer, C.J. (1985) Biochim. Biophys. Acta 812, 423–436.
7 Xie, X.S., Stone, D.K. and Racker, E. (1983) J. Biol. Chem. 258, 14834–14838.
8 Sabolić, I., Haase, W. and Burckhardt, G. (1985) Am. J. Physiol. 248, F835–F844.
9 Sabolić, I. and Burckhardt, G. (1988) Methods Enzymol., in press).
10 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
11 Berner, W. and Kinne, R. (1976) Pfluegers Arch. 361, 269–278.
12 Fiske, C.H. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375–388.
13 Burckhardt, G., Moewes, B. and Sabolić, I. (1987) in Molecular Nephrology-Biochemical Aspects of Kidney Function. (Kovačević, Z. and Guder, W.G., eds.), pp. 57–62, Walter De Gruyter and Co., Berlin.
14 Bergmeyer, H.U. (1970) in Methoden der Enzymatischen Analyse, Band I (Bergmeyer, H.U., ed.), p. 471, Verlag Chemie, Weinheim.
15 Lopez, V., Stevens, T. and Lindquist, R.N. (1976) Arch. Biochem. Biophys. 175, 31–38.
16 Miggiano, G.A.D., Mordente, A., Martorana, G.E., Meucci, E. and Castelli, A. (1984) Biochim. Biophys. Acta 789, 343–346.

17 Van Belle, H. (1972) Biochim. Biophys. Acta 289, 158–168.
18 Vengesa, P.B. and Hopfer, U. (1979) J. Histochem. Cytochem. 27, 1231–1235.
19 Thevenod, F., Streb, H., Ullrich, K.J. and Schulz, I. (1986) Kidney Int. 29, 695–702.
20 Ghijsen, W., Gmaj, P. and Murer, H. (1984) Biochim. Biophys. Acta 778, 481–488.
21 Gmaj, P., Murer, H. and Carafoli, E. (1982) FEBS Lett. 144, 226–230.
22 Kinne-Saffran, E. and Kinne, R. (1975) J. Membr. Biol. 17, 263–274.
23 Moriyama, Y., Takano, T. and Ohkuma, S. (1986) Biochim. Biophys. Acta 854, 102–108.
24 Schoner, W. and Schmidt, H. (1969) FEBS Lett. 5, 285–287.
25 McEnery, M.W. and Pedersen, P.L. (1986) J. Biol. Chem. 261, 1745–1752.