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Characterization of MgATP-Driven H^+ Uptake into a Microsomal Vesicle Fraction from Rat Pancreatic Acinar Cells

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Summary. In microsomal vesicles, as isolated from exocrine pancreas cells, MgATP-driven H^+ transport was evaluated by measuring H^+-dependent accumulation of acridine orange (AO). Active H^+ uptake showed an absolute requirement for ATP with simple Michaelis-Menten kinetics (K_m for ATP 0.43 mmol/liter) with a Hill coefficient of 0.99. H^+ transport was maximal at an external pH of 6.7, generating an intravesicular pH of 4.8. MgATP-dependent H^+ accumulation was abolished by protonophores, such as nigericin (10^{-6} mol/liter) or CCCP (10^{-5} mol/liter), and by inhibitors of nonmitochondrial H^+ ATPases, such as NEM or NBD-Cl, at a concentration of 10^{-5} mol/liter. Inhibitors of both mitochondrial and nonmitochondrial H^+ pumps, such as DCCD (10^{-5} mol/liter) or Dio 9 (0.25 mg/ml), reduced microsomal H^+ transport by about 90%. Vanadate (2×10^{-3} mol/liter), a blocker of those ATPases, which form a phosphorylated intermediate, did not inhibit H^+ transport. The stilbene derivative DIDS (10^{-4} mol/liter), which inhibits anion transport systems, abolished H^+ transport completely. MgATP-dependent H^+ transport was found to be anion dependent in the sequence $Cl^- > Br^- >$ gluconate$^-$; in the presence of SO_4^{2-}, CH_3COO^- or NO_3^-, no H^+ transport was observed. MgATP-dependent H^+ accumulation was also cation dependent in the sequence $K^+ > Li^+ > Na^+ =$ choline$^+$. As shown by dissipation experiments in the presence of different ion gradients and ionophores, both a Cl^- and a K^+ conductance, as well as a small H^+ conductance, were found in the microsomal membranes. When membranes containing the H^+ pump were further purified by Percoll gradient centrifugation (ninefold enrichment compared to homogenate), no correlation with markers for endoplasmic reticulum, mitochondria, plasma membranes, zymogen granules or Golgi membranes was found.

The present data indicate that the H^+ pump located in microsomes from rat exocrine pancreas is a vacuolar- or "V"-type H^+ ATPase and has most similarities to that described in endoplasmic reticulum, Golgi apparatus or endosomes.

Key Words H^+ ATPase · endoplasmic reticulum · proton gradient · Cl^- transport · protonophores · acridine orange

Introduction

H^+-transporting ATPases (H^+ ATPases) have been shown to generate pH gradients in various cells and different intracellular compartments [for literature, *see* 1, 29]. Among these are proton pumps in plasma membranes of eukaryotic cells, which form a phosphorylated intermediate and are inhibited by vanadate ["P"-type ATPase, 26]. H^+ ATPases, which do not form a phosphorylated intermediate, can belong to the "F_0-F_1"-type including mitochondrial, bacterial and chloroplast H^+ pumps, or to a class of H^+ ATPases associated with membrane-bound organelles other than mitochondria. The latter type of proton pump is present in lysosomes, endosomes, secretory granules, Golgi elements, endoplasmic reticulum and also in yeast and plant vacuoles [1, 29] and has been shown to be inhibited by DCCD[1], NEM, and NBD-Cl [1, 20, 29]. Neither oligomycin at concentrations, at which mitochondrial H^+ ATPase is completely abolished ($1–5 \times 10^{-7}$ mol/liter), nor vanadate, a universal inhibitor of "P"-type ATPases, inhibit this vacuolar ("V"-type) H^+ ATPase [8, 24, 43].

To determine the characteristics of H^+ transport in microsomes from pancreatic acinar cells, we have used the acridine orange (AO) method [35]. Here we report the presence of an H^+-translocating, ATP-dependent pump in microsomes of pancreatic acinar cells that shows all features of a "V"-type H^+ pump. Neither vanadate nor oligomycin, but DCCD, NEM and NBD-Cl strongly inhibit this proton pump. Our data also show that ATP-driven H^+ transport is cation and anion dependent. As H^+

[1] *Abbreviations:* AO: acridine orange; ATP-γ-S: adenosine-5′-0-(3-thiotriphosphate); CCCP: carbonylcyanide-*m*-chlorophenylhydrazone; CK: creatine kinase; CP(Na): phosphocreatine (sodium salt); DCCD: N,N′-dicyclohexylcarbodiimide; DIDS: 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; HEPES: N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; MES: morpholinoethane sulfonic acid; MOPS: morpholinopropane sulfonic acid; Na_3VO_4: sodium vanadate; NBD-Cl: 7-chloro-4-nitrobenz-2-oxa-1,3-diazole; NMDG: N-methyl-D-glucamine; NEM: N-ethylmaleimide; PEP: phosphoenolpyruvate; TMA$^+$: tetramethylammonium$^+$.

transport does not copurify with marker enzymes for plasma membranes, endoplasmic reticulum, the Golgi apparatus or for zymogen granules of exocrine pancreas, its precise localization remains open. As potential candidates remain endosomes or condensing vacuoles [25], where H^+ transport has not yet been characterized.

Materials and Methods

Materials

Reagents were obtained from the following sources: phosphocreatine (CP, sodium salt), ATP (Tris-, magnesium- or dipotassium salt), CTP (disodium salt), GTP (disodium salt), ITP (trisodium salt), UTP (trisodium salt), MOPS, benzamidine, EDTA, Na_3VO_4, bovine serum albumin (lyophylized) and NBD-Cl from Sigma (Deisenhofen, FRG); antimycin A, oligomycin, NEM, HEPES, MES and the dye Serva Blue from Serva (Heidelberg, FRG). ATP-γ-S (tetralithium salt), PEP (monosodium salt), CK (350 U/mg at 25°C), trypsin inhibitor (from hen egg white) and CCCP were from Boehringer (Mannheim, FRG). DCCD, valinomycin, A 23187 and nigericin were from Calbiochem (Giessen, FRG). TMA^+ (as chloride or sulfate salt) was from Fluka (Buchs, Switzerland). Sodium azide, Tris and D-mannitol were purchased from Merck (Darmstadt, FRG). DIDS was from Pierce Chemical (Rockford, IL); Dio 9 from Gist-Brocades (Delft, Netherlands). Collagenase, type III (from *Clostridium histolyticum*, 139 U/mg at 37°C) was purchased from Worthington (Freehold, NJ). Percoll (23%, density, 1.129 g/ml, osmolality 17 mOsmol/kg H_2O) and Phadebas amylase test were from Deutsche Pharmacia (Freiburg, FRG). AO was from Eastman Kodak (Rochester, NY). All other reagents were of analytical grade.

Methods

Preparation of Subcellular Fraction Enriched in Endoplasmic Reticulum

Pancreatic vesicles enriched in endoplasmic reticulum were prepared from 12 male Wistar rats (200–250 g), which had been fasted overnight. Acinar cells were isolated by collagenase digestion as described previously [15, 40]. Isolated cells were washed twice in an ice-cold homogenization buffer containing (in mmol/liter): mannitol 290, HEPES 5, KCl 10, $MgCl_2$ 1, benzamidine 1, adjusted with Tris/HCl to pH 7.0. Homogenization and subsequent fractionation steps were performed as described by Imamura and Schulz [15]. Briefly, the homogenate was centrifuged at 1000 × *g* for 13 min, and the supernatant was further centrifuged at 11,000 × *g* for 15 min in a Beckman Ti 60 rotor. The 11,000 × *g* pellet was composed of an upper whitish fluffy layer and a yellowish bottom layer, which were separated. The fluffy layer, which is enriched by about twofold in both plasma membranes and ER, was stored for further use. In some experiments, it was applied to a Percoll gradient for further purification. For that purpose 1 ml of vesicle suspension was mixed with 9 ml of Percoll solution containing (in mmol/liter): mannitol 270, HEPES 5, KCl 10, benzamidine 1, $MgCl_2$ 1 and 11% (wt/vol) Percoll, pH 7.0 adjusted with Tris/HCl. The resulting density was 1.035 g/cm³. A gradient of densities from 1.020 to 1.141 g/cm³ was generated by spinning the tubes at 41,000 × *g* for 40 min in a Beckman ultracentrifuge (Model L3-50) using a Beckman SW 27 swing-out rotor. Five 2-ml fractions were collected from the top to the bottom of the gradient. To separate vesicles from Percoll, the fractions collected were spun down at 100,000 × *g* for 60 min in a Beckman Ti 60 23.5° fixed-angle rotor. All vesicle fractions collected were taken up in standard homogenization buffer at a protein concentration of about 20 mg/ml. Vesicles were either used immediately for H^+ transport studies or kept frozen in liquid nitrogen until use.

Measurements of H^+ Pump Activity

The pH-sensitive dye AO was used to visualize the formation and dissipation of H^+ gradients [35]. The weak base AO is accumulated in vesicles, when the intravesicular pH is smaller than the extravesicular pH. The decrease of intravesicular pH can be monitored by either the quenched fluorescence of the accumulated dye or by change in absorbance due to AO uptake from the medium. We have chosen the latter method to monitor H^+ uptake into vesicles.

Membrane vesicle protein (600 μg) was preincubated at 25°C for 15 min in 2 ml of a medium containing basically (in mmol/liter): mannitol 300, KCl 100, HEPES 5, $MgSO_4$ 5, oligomycin 0.01 and test substance or solvent as indicated, adjusted to pH 7.0 with Tris/HCl, in a cuvette. AO was added to the medium at a final concentration of 6 μmol/liter. The difference in absorbance in the cuvette at 493–540 nm was measured in an Aminco DW-2 UV-Vis spectrophotometer (Silverspring, MD), and the signal was displayed continuously on a recorder unit. When the absorbance signal had reached a stable base line, H^+ transport was initiated by adding 1.25 mmol/liter of MgATP from a 0.5-mol aqueous stock solution titrated to pH 7.0 with Tris. When a steady-state H^+ gradient had been reached, the protonophores nigericin (10^{-6} mol/liter) or CCCP (10^{-5} mol/liter) were added to dissipate the pH gradient over the vesicle membrane.

The initial rate of acidification driven by the H^+ pump was estimated by drawing the tangent to the initial part of the absorbance recording and was expressed as absorbance change per min (ΔA/min). The initial rate of ATP-induced acidification was found to be linearly related to the vesicle protein concentration when assayed up to 600 μg/ml (Fig. 1). We have, therefore, used protein concentrations within this range for studying H^+ transport.

Measurement of Ion Conductance Pathways in the Vesicle Membrane

When intravesicular acidification due to passive ion fluxes was estimated, membrane vesicles were loaded with the respective ions by homogenizing isolated cells in a buffer in which KCl was replaced by the ions to be tested, but was otherwise the same as described above. Each following step for preparation was performed in the same buffer, so that in the final preparation vesicles were preloaded with the ions of interest. Vesicles were then preincubated for 5 min in a buffer containing AO and ions as

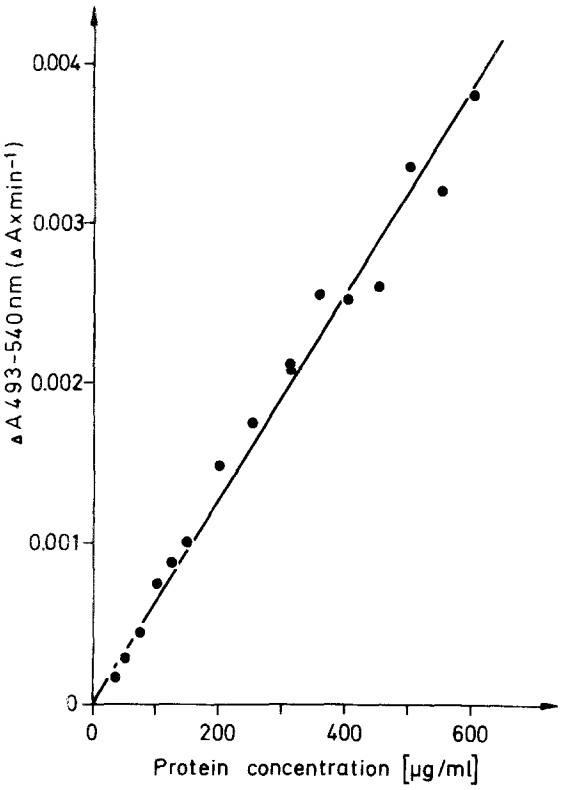

Fig. 1. Effect of protein concentration on the initial rate of MgATP-dependent H^+ uptake as measured by acridine orange (AO) absorbance change in pancreatic microsomal vesicles. Vesicles were prepared in homogenization buffer as described in Materials and Methods and incubated in a high KCl^- incubation medium containing 6 µM AO. TrisATP (1.25 mmol/liter) induced a decrease in absorbance-reflecting intravesicular acidification. The initial rate of the H^+ pump-driven acidification was estimated as described in Materials and Methods. Protein concentration was varied between 37 µg/ml and 600 µg/ml

indicated in the text and in the legends to the figures. Ionophores were subsequently added to monitor intravesicular acidification.

Assay for Determination of Protein, RNA and Marker Enzymes

Protein and RNA concentrations, activities of $(Na^+ + K^+)$-ATPase, NADPH cytochrome *c* reductase, alkaline phosphatase, glutamate dehydrogenase and cytochrome *c* oxidase in subcellular fractions were determined as previously described [3, 39]. Thiamin pyrophosphatase activity was determined according to Novikoff and Heus [23]. Amylase activity was measured using the Phadebas amylase test kit (Pharmacia).

Results

Nucleotide Specificity of the H^+ Pump

As shown in Fig. 2, addition of Tris-ATP (1.25 mmol/liter) to microsomal vesicles in a standard incubation medium with 100 mmol/liter KCl, resulted in a rapid decrease of absorbance due to uptake of AO. A "steady-state" was reached after about 15–20 min. That this uptake is due to intravesicular acidification was demonstrated by rapid discharge of accumulated protons in the presence of the electroneutral K^+/H^+ ionophore nigericin. Similarly, the electrogenic protonophore CCCP dissipated the H^+ gradient that had been developed in the presence of ATP (*see* Fig. 5, Table 1). ATP-driven intravesicular acidification was dependent on the presence of Mg^{2+}; without Mg^{2+} no H^+ transport occurred (*not shown*). Figure 2 also demonstrates that H^+ ion uptake was only obtained in the pres-

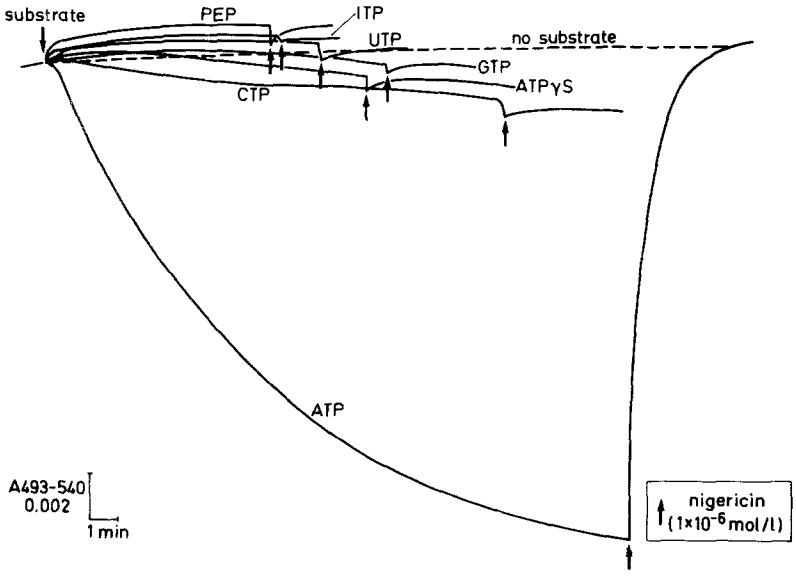

Fig. 2. Substrate specificity of the H^+ pump in pancreatic microsomal vesicles. Vesicles (*see* Materials and Methods) were incubated in a high K^+ incubation medium with AO. Substrates (1.0 mmol/liter) were added to the medium where indicated and intravesicular acidification was recorded. Arrows indicate addition of nigericin to dissipate the H^+ gradient generated

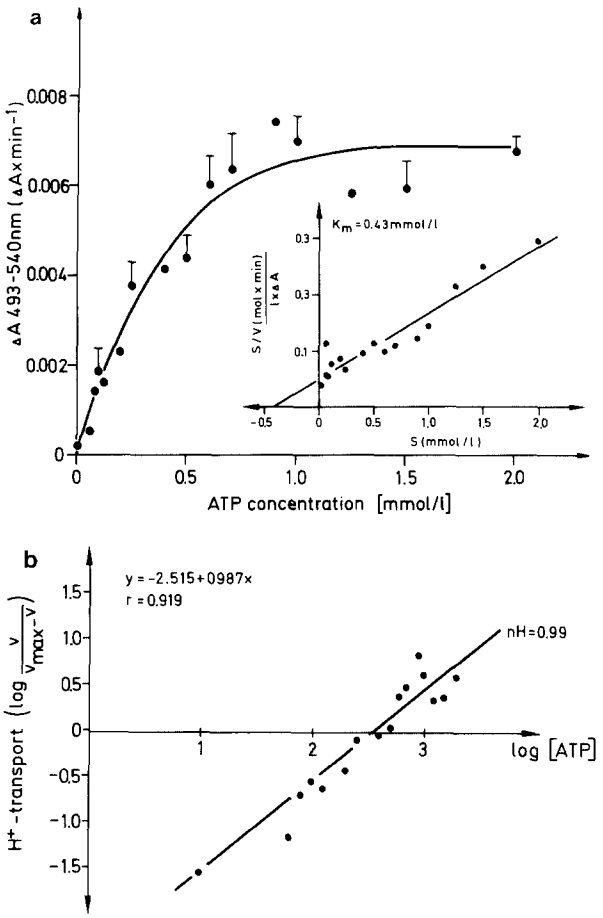

Fig. 3. (*a*) Dependence of H^+ transport into pancreatic microsomal vesicles on the ATP concentration. Vesicles were incubated in a high K^+ incubation medium with AO. TrisATP was added to the incubation medium at the indicated final concentration, and the initial rate of acidification was measured. Means ± SEM of two to eight different experiments are shown. The inset shows a Hanes-Woolf plot of the data. Half-maximal rate of acidification was obtained at an ATP concentration of 0.43 mmol/liter. (*b*) Hill plot of H^+ transport into microsomal vesicles from exocrine pancreas in dependence on ATP concentration. The graph was plotted using the initial rate of ATP-dependent acidification as V (Δ A/mg protein) *vs.* ATP concentration (μmol/liter). The data from Fig. 3*a* were used for calculation

ence of ATP and was not supported by CTP, GTP, UTP, ITP, ATP-γ-S or phosphoenolpyruvate.

The initial rates of H^+ uptake increased as a function of ATP concentration in the incubation medium and became maximal at 10^{-3} mmol/liter of ATP (Fig. 3*a*). The Hanes-Woolf plot (Fig. 3*a* inset) shows that H^+ uptake as plotted in dependence on ATP concentration follows simple Michaelis-Menten kinetics with an apparent K_m of 0.43 mmol/liter ATP. A Hill coefficient of 0.99 was calculated from a Hill plot of the data (Fig. 3*b*).

Dependence of ATP-Driven H^+ Uptake on Anions

Both the rate of H^+ uptake and the H^+ gradient generated by the H^+ pump into pancreatic microsomal vesicles were dependent on the presence of specific anions in the incubation medium. As shown in Fig. 4, following addition of 1.25 mmol/liter Tris-ATP to the medium, maximal H^+ transport was observed in the presence of Cl^-, whereas in the presence of Br^-, H^+ uptake was reduced by about 50%. Addition of nigericin to the medium resulted in a rapid dissipation of the H^+ gradient, which was maximal in the presence of Cl^- in the medium. When KCl was isosmotically replaced by other K^+ salts such as K-gluconate, K_2SO_4, $KCOOCH_3$, KNO_3, or KSCN (*not shown*), no significant H^+ uptake took place (Fig. 4). This indicates that Cl^- is necessary for maximal H^+ transport to occur.

Dependence of ATP-Driven H^+ Uptake on Cations

The rate of ATP-driven H^+ uptake was also dependent on the presence of specific cations in the medium, K^+ being most efficient for H^+ accumulation (Fig. 5). When KCl was replaced by Cl^- salts of Li^+, Na^+ or choline$^+$, the initial H^+ uptake rate was decreased to 69 ± 6, 45 ± 4 and 42 ± 5% of the KCl control, respectively (mean values ± SE of three to six experiments). The Δ pH generated by the H^+ pump was also maximal in the presence of K^+, as compared to the other cations tested, as can be seen from the dissipation signal induced by CCCP. Note that the dissipation rate of the H^+ gradient induced by the electrogenic protonophore CCCP was smaller than that induced by the electroneutral K^+/H^+ exchanger nigericin (*see* Figs. 2 and 4 as compared to Fig. 5). This indicates that the dissipation rate of protons in the presence of CCCP was limited by intrinsic ion conductances in the membrane, which account for charge compensation of the H^+ diffusion potential.

Dependence of H^+-Pump Activity on the External pH (pH_o)

The optimal extravesicular pH for H^+ pump activity was measured in the presence of different pH buffers adjusted between 4.5 and 8. As can be seen from Fig. 6, no H^+ transport took place below pH 5. With increasing extravesicular pH values higher than 5, MgATP-dependent H^+ uptake rate increased steeply, reached highest H^+ uptake rate at pH 6.7, and then declined again.

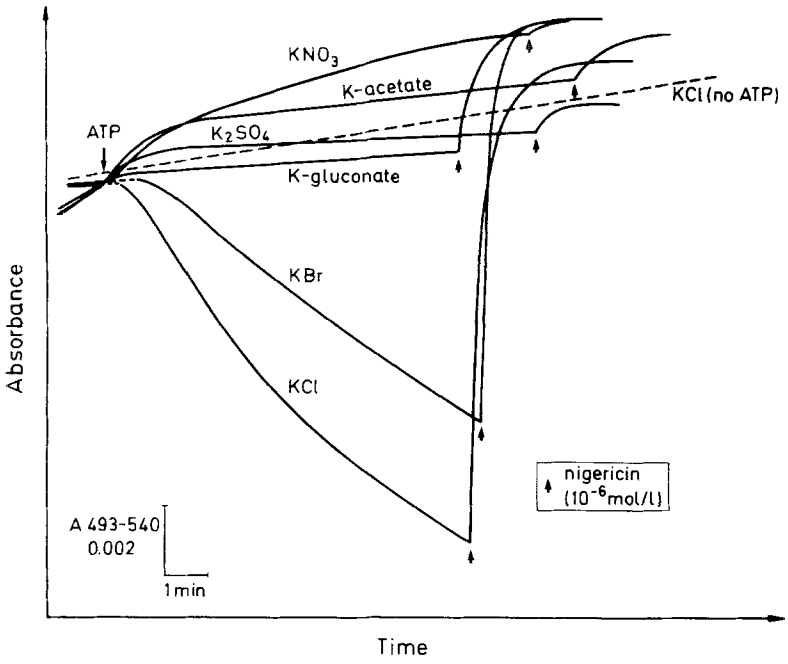

Fig. 4. Dependence of ATP-driven H^+ transport into pancreatic microsomal vesicles on the presence of different anions in the external medium. Pancreatic microsomal vesicles were prepared as described in Materials and Methods and preincubated at 25°C for 15 min in an AO medium with 100 mmol/liter K-salts where the anions were either 100 mmol/liter Cl^-, Br^-, gluconate$^-$, acetate$^-$, and NO_3^- or 67 mmol/liter SO_4^{2-}. Upon addition of TrisATP (1.25 mmol/liter), the acidification was recorded. Nigericin (1×10^{-6} mol/liter) was added, where indicated, to dissipate the H^+ gradient generated by the H^+ pump. Typical for three different experiments

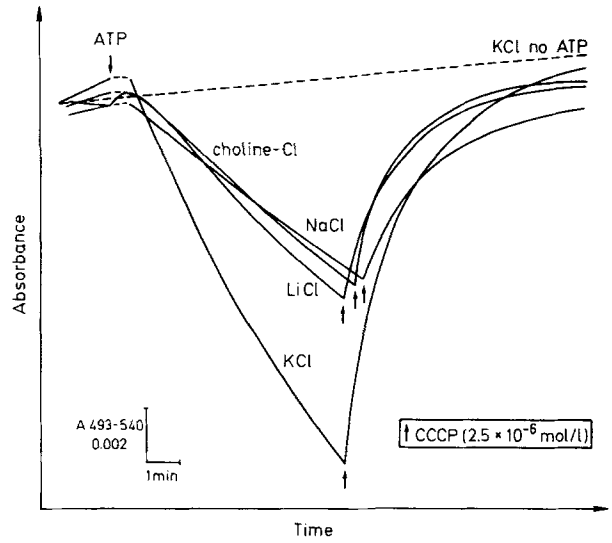

Fig. 5. Dependence of ATP-driven H^+ transport into pancreatic microsomal vesicles on the presence of different monovalent cations in the incubation medium. KCl (100 mmol) was isosmotically replaced by either LiCl, NaCl or choline Cl. ATP-driven H^+ uptake was initiated by addition of 1.25 mmol/liter TrisATP to the AO incubation medium and the acidification was measured. CCCP (2.5×10^{-6} mol/liter) was added where indicated to dissipate the H^+ gradient. Typical for three different experiments

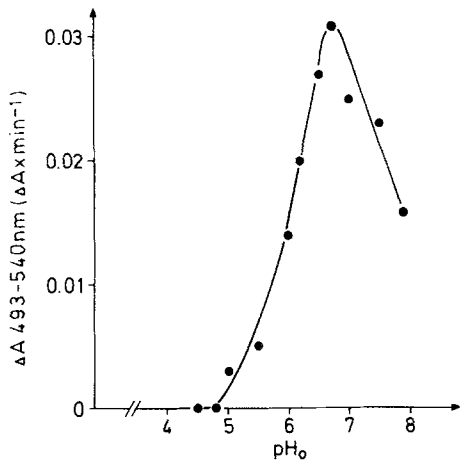

Fig. 6. Effect of extravesicular pH (pH_o) on MgATP-driven H^+ transport into pancreatic microsomal vesicles. Isolated vesicles were prepared as described in detail in Materials and Methods and incubated at different pH_o at 25°C for 15 min in AO incubation medium. The pH range, 4.5–6.8, was adjusted with MES (5 mmol/liter); the pH range, 7.0–8.0, with MOPS (5 mmol/liter). The initial rate of intravesicular acidification after addition of MgATP (1.25 mmol/liter) to the medium is shown on the ordinate. The curve is representative for three different experiments

Estimation of Intravesicular pH (pH_i) Generated by the H^+ Pump

In order to estimate the pH gradient that can be established by the H^+ pump, intravesicular pH was determined by "zero point titration" after a steady state of H^+ uptake had been reached. As shown in Fig. 7*a*, MgATP-dependent H^+ ion uptake was monitored at an optimal medium pH value of 6.7 until a steady state of H^+ uptake was reached. The H^+ gradient that was established over the microsomal vesicles was dissipated by CCCP (Fig. 7*a*) or

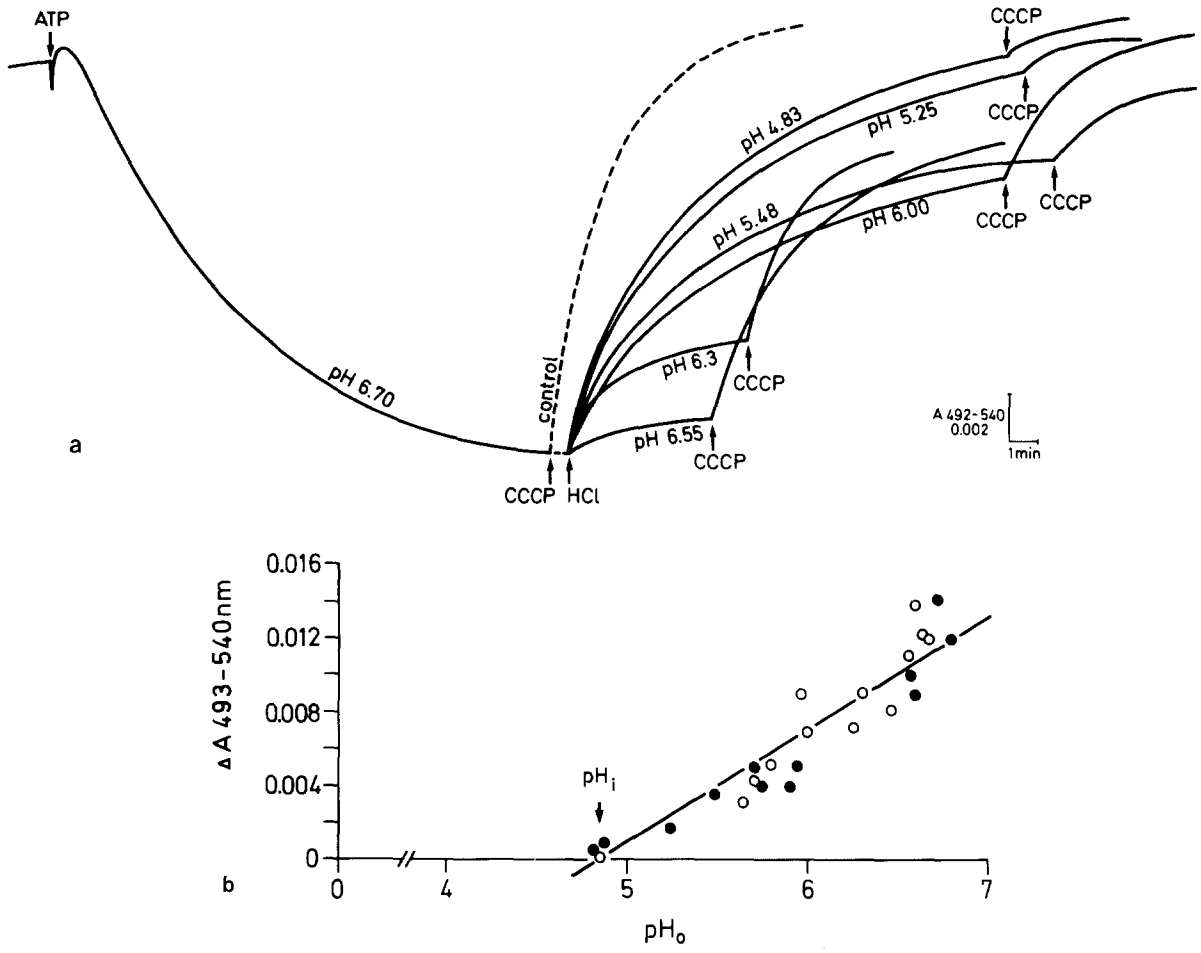

Fig. 7. (*a*) "Zero point titration" experiment to determine intravesicular pH (pH_i). Vesicles were prepared as described in Materials and Methods and incubated in a standard AO incubation medium, adjusted to pH 6.70 with Tris/HCl. Upon addition of ATP to the medium, the H^+ pump was allowed to acidify the intravesicular space until a steady state was reached. Then, CCCP (5×10^{-6} mol/liter) was added to dissipate the pH gradient generated, or HCl (0.5 N) was added to titrate the external pH (pH_o) to different values of pH_o. Addition of CCCP resulted in a signal of varying magnitude, depending upon the Δ pH across the vesicular membrane. At a pH_o of 4.8, CCCP did not induce any change in absorbance, indicating that Δ pH was zero and that pH_i was also 4.8. Typical for two experiments. (*b*) "Zero point titration" curve to estimate intravesicular pH (pH_i). pH_o is plotted against the magnitude of the change in absorbance induced by addition of CCCP (5×10^{-6} mol/liter) to the medium. Values of two different preparations are shown (filled and open symbols). The intercept of the linear regression line with the abscissa ("zero point") reflects the internal pH of the H^+ gradient generated by the H^+ pump of pancreatic microsomal vesicles

by nigericin (*not shown*). The degree of protonophore-induced change in absorbance was an estimate of the H^+ gradient present over the vesicle membrane. When the medium was acidified by addition of HCl (the pH of the medium being determined with a pH electrode), absorbance change was reversed due to the decrease in H^+ gradient over the microsomal membrane (*see* Fig. 7*a*). Consequently, CCCP-induced signal, reflecting dissipation of the H^+ gradient, was smaller at acidic pH_o than at more alkaline pH_o. When pH_o is identical to the pH_i, no H^+ gradient is present and no CCCP signal should be observed. As shown in Fig. 7*a*, following CCCP addition, no change in the absorbance signal was seen at a pH_o of 4.8. A plot of CCCP-induced absorbance changes at different pH_o values from two different vesicle preparations demonstrates that the regression line crosses the X-axis at pH_o 4.8, where no CCCP-induced absorbance changes are observed, and, therefore, this value reflects the intravesicular pH, which could be established by the H^+ pump (Fig. 7*b*). This corresponds

Table 1. Effect of inhibitors and ionophores on MgATP-dependent acidification into membrane vesicles from pancreatic microsomes, as measured by acridine orange absorbance changes at 493 and 540 nm

Compound	Concentration	H^+ transport (% of control)	(n)
No	—	100	(32) —
Na_3VO_4	1.0×10^{-3} mol/liter	91 ± 6	(14) NS
DCCD	1.0×10^{-3} mol/liter	0	(1) —
	1.0×10^{-5} mol/liter	14 ± 3	(4) $P < 0.001$
	1.0×10^{-6} mol/liter	64 ± 14	(3) NS
	1.0×10^{-7} mol/liter	95 ± 3	(3) NS
NEM	5.0×10^{-4} mol/liter	0	(1) —
	1.0×10^{-5} mol/liter	3 ± 3	(5) $P < 0.001$
	1.0×10^{-6} mol/liter	48 ± 8	(3) $P < 0.025$
	1.0×10^{-7} mol/liter	102 ± 10	(3) NS
NBD-Cl	1.0×10^{-5} mol/liter	0	(1) —
	5.0×10^{-6} mol/liter	0	(1) —
	1.5×10^{-6} mol/liter	26	(2) —
	1.0×10^{-6} mol/liter	43 ± 7	(5) $P < 0.005$
	1.0×10^{-7} mol/liter	86	(1) —
Dio 9	0.250 mg/ml	8 ± 8	(5) $P < 0.001$
	0.025 mg/ml	83	(2) —
DIDS	1.0×10^{-4} mol/liter	0	(4) $P < 0.001$
	1.0×10^{-5} mol/liter	14	(1) —
	1.0×10^{-6} mol/liter	17	(1) —
	1.0×10^{-7} mol/liter	50	(1) —
Oligomycin	1.0×10^{-5} mol/liter	61 ± 8	(4) $P < 0.02$
CCCP	1.0×10^{-5} mol/liter	0	(15) $P < 0.005$
Nigericin	1.0×10^{-6} mol/liter	0	(17) $P < 0.005$
A 23187	1.0×10^{-5} mol/liter	0	(1) —
Valinomycin	1.0×10^{-6} mol/liter	91 ± 6	(13) NS

Microsomal vesicles (600 μg) were preincubated for 15 min at 25°C in 2 ml of acridine orange medium containing 100 mmol/liter KCl and test compounds or their carrier substance at a volume not exceeding 1% of total incubation medium. H^+ transport was started by addition of MgATP (1.25 mmol/liter) to the medium, as described in Materials and Methods. Results are calculated as a percentage of respective control (100% is equivalent to 0.0148 ± 0.001 absorbance U/min). The number of experiments is given in brackets.

to a 79-fold increase in intravesicular H^+ concentration at an external pH where the pump is operating maximally.

Effect of Inhibitors on ATP-Dependent H^+ Uptake

Inhibitors of different H^+ pumps were tested on ATP-driven H^+ uptake into membrane vesicles from pancreatic microsomes. Although there are no specific inhibitors that allow an unequivocal determination of the type of H^+ pump under study, the use of several types of inhibitors at various concentrations can provide valuable information to identify the different types of H^+ ATPases [1], as well as to characterize some of their properties. Vanadate, an inhibitor of ATPases, which form a phosphorylated intermediate in their enzymatic reaction pathway [7, 17, 22, 26], did not affect H^+ uptake in our studies (*see* Table 1). NEM and NBD-Cl, which inhibit nonmitochondrial H^+ ATPases at micromolar concentrations [8, 9–12, 14, 24, 38], abolished intravesicular acidification at a concentration of about 10^{-5} mol/liter (*see* Table 1). DCCD and Dio 9, which both inhibit mitochondrial, as well as nonmitochondrial H^+ ATPases [2, 37], inhibited H^+ uptake in our preparation. The mitochondrial-inhibitor oligomycin inhibited H^+ uptake into vesicles by about 40% at a high concentration of 10^{-5} mol/liter,

suggesting that the vesicles are contaminated with mitochondrial inside-out vesicles, or that the H^+ pump under study is partially sensitive to oligomycin. We also examined the effect of the stilbene derivative DIDS, an inhibitor of anion transport systems [5, 6, 16], on ATP-dependent H^+ transport. DIDS, at a concentration of 10^{-4} mol/liter, abolished H^+ transport completely (*see* Table 1).

Effect of Ionophores on H^+ Uptake

The H^+ gradient across the microsomal vesicle membrane established by the H^+ pump can be dissipated by protonophores. Ionophores, which exchange H^+ ions for other cations in a neutral way, can dissipate H^+ gradients if these cations are present at the *trans* side of the vesicles, irrespective of intrinsic ion permeabilities of the membrane. Electrogenic protonophores, such as CCCP, lead to breakdown of the H^+ gradient only, if the membrane is permeable to either a cation for which H^+ can exchange or for an anion that can passively follow H^+ flux. The degree and rate of H^+ gradient dissipation by electrogenic protonophores can, therefore, be informative for the presence of other ion conductances in the membrane.

As shown in Table 1, the electroneutral K^+/H^+ exchanger, nigericin, and the Ca^{2+}/H^+ exchanger, A23187, completely dissipated the H^+ gradient. In the presence of CCCP, H^+ ions were also released but the rate of dissipation was smaller as compared to nigericin (*compare* Figs. 4 and 5), indicating the presence of other ion conductances in the membrane, which are smaller than those induced by nigericin.

In the presence of the K^+-ionophore valinomycin and a vesicle inward-directed K^+-gradient ($K_o > K_i$), ATP-driven H^+ uptake was not significantly changed.

Permeability Properties of the Microsomal Membrane

As mentioned above, ionophores are useful tools to obtain information about the intrinsic permeability pathways of a membrane. The membrane appears to be relatively tight for H^+ ions since an H^+ gradient of about two pH units can be established by the H^+ pump (Fig. 7b). As the electrogenic protonophore CCCP, when added at steady state, dissipated the H^+ gradient generated by the H^+ pump, the vesicle membrane must be permeable to another anion or cation.

Anion Conductances in the Vesicle Membrane

The Cl^- dependency of H^+ transport shown in Fig. 4 could be explained by ATP-dependent HCl cotransport, or Cl^- could function as charge compensator for electrogenic ATP-driven H^+ uptake. In the latter case, either a Cl^- conductance or a Cl^- cation cotransporter (e.g., a KCl cotransporter together with a conductance pathway for the cotransported cation) should be present in the vesicle membrane. We estimated relative anion conductances by measuring the dependence of H^+ ion efflux on the accompanying anions in vesicles containing either Cl^- or SO_4^{2-}. Vesicles were loaded with these anions by homogenizing them in a buffer containing either (in mmol/liter) KCl 100, mannitol 100, or K_2SO_4 50, mannitol 150, and with otherwise identical composition as the standard homogenization buffer. To monitor H^+ uptake, preloaded vesicles were diluted 60-fold into a standard AO incubation buffer, which contained 100 mmol/liter N-methyl-D-glucamine (NMDG)-cyclamate instead of 100 mmol/liter KCl and no ATP. The K^+ gradient ($K_i^+ > K_o^+$) was used to load vesicles with protons by subsequent addition of valinomycin and CCCP; the inside-negative electrical potential difference generated by valinomycin and $K_i^+ > K_o^+$ being the driving force for H^+ uptake.

As shown in Fig. 8, when valinomycin was added to the medium, a slow intravesicular acidification occurred that was more rapid in vesicles preloaded with K_2SO_4 as compared to vesicles preloaded with KCl. Since the electrical potential difference across the vesicle membrane should be more negative inside the vesicles with valinomycin and an impermeant anion as compared to a permeant anion, an increased rate of H^+ uptake into SO_4^{2-} vesicles, as compared to Cl^- vesicles, indicates a higher conductance for Cl^- than for SO_4^{2-}. When valinomycin and CCCP were added together, rapid acidification occurred in both types of vesicles. However, acidification was somewhat smaller in Cl^- vesicles as compared to SO_4^{2-} vesicles. When CCCP was added about 2 min after valinomycin, H^+ ion influx was even smaller in Cl^- vesicles. When CCCP was added after about 10 min, acidification was minimal in KCl vesicles. This indicates that in the presence of valinomycin, KCl efflux from the vesicles took place due to an intrinsic Cl^- conductance. Consequently, the K^+ gradient decreased with time in vesicles preloaded with KCl. In K_2SO_4 vesicles, however, an interval of about 2 or 10 min between additions of valinomycin and CCCP did not reduce H^+ ion influx markedly, indicating that

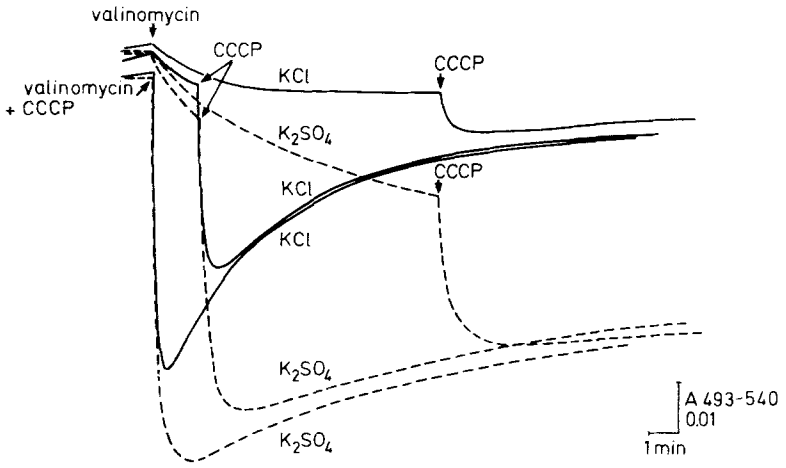

Fig. 8. Presence of Cl^- and H^+ conductances in microsomal vesicles from exocrine pancreas. Vesicles were preloaded with either 100 mmol/liter KCl (——), or 50 mmol/liter K_2SO_4 (----) and preincubated in an AO medium with 100 mmol/liter NMDG-cyclamate for 5 min in the cuvette. Addition of the electrogenic K^+-ionophore valinomycin (1×10^{-6} mol/liter) induced an inside negative diffusion potential resulting in intravesicular acidification. The electrogenic protonophore CCCP (1×10^{-5} mol/liter), when added together with or after valinomycin, increased the H^+ permeability of the membrane, and rapid acidification occurred, when valinomycin was also present in the medium. Typical for three experiments

the K^+ gradient was maintained when SO_4^{2-} was the accompanying anion. Similarly, dissipation of the H^+ gradient, as seen by the upward-directed curve, was more rapid in Cl^- vesicles as compared to SO_4^{2-} vesicles.

Cation Conductances in the Vesicle Membrane

K^+ conductance was estimated by measuring the dependence of H^+ ion influx in vesicles containing either K-gluconate, Na-gluconate, or NMDG-gluconate. Vesicles were preloaded with either 100 mmol/liter K-gluconate, 100 mmol/liter Na-gluconate, or 100 mmol/liter NMDG-gluconate, as described in the previous section, and diluted into a standard AO incubation buffer, which contained 100 mmol/liter TMA-gluconate and no ATP. If an endogenous K^+ conductance was present in the medium, the inside-to-outside directed K^+ gradient and the resulting inside negative electrical potential difference should derive H^+ into the vesicles in exchange for K^+; in the presence of a less permeant cation, H^+ uptake should be reduced or absent. As shown in Fig. 9, upon addition of CCCP to increase maximally H^+ conductance across the membrane, intravesicular acidification occurred in the presence of K-gluconate, whereas, in vesicles preloaded with either Na-gluconate or NMDG-gluconate, CCCP-induced acidification was absent. This indicates that a K^+ conductance is present in the microsomal membrane.

An intrinsic *H^+ conductance* of vesicle membranes could be demonstrated by inducing an inside-negative diffusion potential with valinomycin and a vesicle inward-to-outward directed K^+ gradient. As shown in Fig. 8, when vesicles were prepared in the presence of 50 mmol/liter K_2SO_4 and transferred to a medium with NMDG-cyclamate, addition of valinomycin caused H^+ ion uptake as a result of a vesicle inside negative diffusion potential.

Localization of MgATP-Dependent Proton Uptake

In an attempt to localize H^+ transport and to discriminate between intracellular organelles, which could be responsible for H^+ ion uptake, microsomes used for H^+ uptake studies were further subfractionated on a Percoll gradient. Markers for different organelles were determined in the microsomal fraction and the five fractions collected from the Percoll gradient and correlated with H^+ pump activity. As shown in Table 2, maximal enrichment of H^+ transport (2.93-fold as compared to the starting material) was found in the fourth fraction from the top of the gradient. The marker enzymes for plasma membranes, $(Na^+ + K^+)$-ATPase and alkaline phosphatase, were decreased about 10- and fivefold, respectively, in this vesicle fraction. Markers for endoplasmic reticulum, RNA and NADPH-cytochrome-*c*-reductase, were also reduced by 20- and threefold, respectively. The marker enzymes for mitochondria, glutamate dehydrogenase and cytochrome-*c*-oxidase, were decreased by about twofold in this vesicle fraction. Amylase and thiamin pyrophosphatase activities, which are the respective markers for zymogens and *trans*-Golgi membranes [19], were slightly reduced (83 and 65% of starting activities, respectively). Correlation of ATP-driven H^+ transport in the fractions obtained by Percoll gradient centrifugation with the activity

Table 2. Distribution of marker enzymes, H^+ uptake and protein in subcellular vesicle fractions from exocrine pancreas separated by Percoll gradient centrifugation

		11,000 × *g* Fluffy layer	P1	P2	P3	P4	P5	*r*
H^+ uptake	Spec. act.	18.8 ± 6.4(9)	1.7 ± 0.6(9)	5.8 ± 1.0(9)	22.6 ± 6.2(9)	54.9 ± 21.4(9)	22.5 ± 12.5(9)	
	Enrichment	1.00	0.09	0.31	1.20	2.93	1.20	
	Recovery	100.0%	1.4%	27.3%	31.1%	18.7%	6.0%	
$(Na^+ + K^+)$-ATPase	Spec. act.	39.0 ± 11.4(5)	118.2 ± 26.3(5)	32.2 ± 11.4(5)	13.4 ± 4.0(5)	3.0 ± 1.4(5)	0.5 ± 0.3(5)	0.67(6)
	Enrichment	1.00	3.03	0.83	0.34	0.09	0.01	
	Recovery	100.0%	61.4%	40.8%	8.4%	1.0%	1.0%	
AP	Spec. act.	33.6 ± 3.6(5)	63.0 ± 7.3(5)	28.2 ± 4.4(5)	14.2 ± 2.4(5)	5.8 ± 0.5(5)	3.0 ± 0.7(5)	0.73(6)
	Enrichment	1.00	1.88	0.84	0.42	0.17	0.09	
	Recovery	100.0%	34.6%	45.6%	10.6%	2.4%	2.4%	
RNA	Spec. act.	33.0 ± 6.7(5)	20.8 ± 5.1(5)	36.2 ± 9.7(5)	34.2 ± 18.3(5)	1.5 ± 0.9(5)	2.0 ± 2.0(4)	0.64(6)
	Enrichment	1.00	0.63	1.10	1.04	0.05	0.06	
	Recovery	100.0%	12.0%	62.2%	20.8%	2.2%	5.2%	
NADPH cyt-*c*-reductase	Spec. act.	6.5 ± 0.6(5)	4.2 ± 0.2(5)	6.2 ± 0.5(5)	4.6 ± 0.5(5)	2.2 ± 0.3(4)	1.3 ± 0.2(4)	0.58(6)
	Enrichment	1.00	0.63	0.94	0.70	0.34	0.20	
	Recovery	100.0%	12.6%	56.0%	18.4%	4.2%	5.0%	
Glutamate dehydrogenase	Spec. act.	83.0 ± 20.0(5)	25.0 ± 9.0(5)	50.0 ± 15.0(5)	63.0 ± 21.0(5)	52.0 ± 14.0(4)	14.0 ± 5.0(4)	0.16(6)
	Enrichment	1.00	0.30	0.60	0.76	0.63	0.17	
	Recovery	100.0%	6.4%	32.8%	18.8%	6.6%	2.8%	
Cyt-*c*-oxidase	Spec. act.	53.0 ± 9.0(5)	16.0 ± 2.0(5)	37.0 ± 6.0(5)	76.0 ± 11.0(5)	30.0 ± 5.0(4)	4.0 ± 1.0(4)	0.00(6)
	Enrichment	1.00	0.30	0.70	1.43	0.57	0.08	
	Recovery	100.0%	13.8%	39.8%	37.8%	6.6%	4.4%	
Amylase	Spec. act.	21.6 ± 1.2(5)	31.5 ± 5.8(5)	15.2 ± 2.4(5)	14.9 ± 4.4(5)	18.0 ± 2.9(5)	30.4 ± 3.4(5)	0.31(6)
	Enrichment	1.00	1.46	0.70	0.69	0.83	1.41	
	Recovery	100.0%	15.2%	38.4%	16.2%	6.6%	14.8%	
Thiamin-pyrophosphatase	Spec. act.	1031.5 ± 187.0(5)	2528.9 ± 343.5(5)	677.3 ± 119.6(5)	301.1 ± 84.7(5)	410.7 ± 197.8(5)	49.6 ± 49.6(5)	0.47(6)
	Enrichment	1.00	2.45	0.66	0.29	0.40	0.05	
	Recovery	100.0%	25.8%	37.6%	6.4%	2.3%	0.6%	
Protein	Yield	92.2 ± 8.1(14)	11.2 ± 1.7(14)	48.5 ± 3.7(14)	22.2 ± 0.9(14)	8.7 ± 1.4(14)	11.2 ± 2.4(14)	—
	Recovery	100.0%	12.0%	53.0%	24.0%	9.0%	12.0%	

The procedure for subcellular fractionation is described in Materials and Methods. Results are given as mean ± SEM of 4 to 14 preparations. ATP-dependent H^+ uptake is expressed in mA/mg protein, enzyme activities in mU/mg protein, except for amylase (U/mg) and thiamin pyrophosphatase (nmol/h · mg protein). RNA is expressed in μg/mg protein and protein yield in mg. *r* = correlation coefficient of H^+ uptake with respective enzyme activities or RNA content.

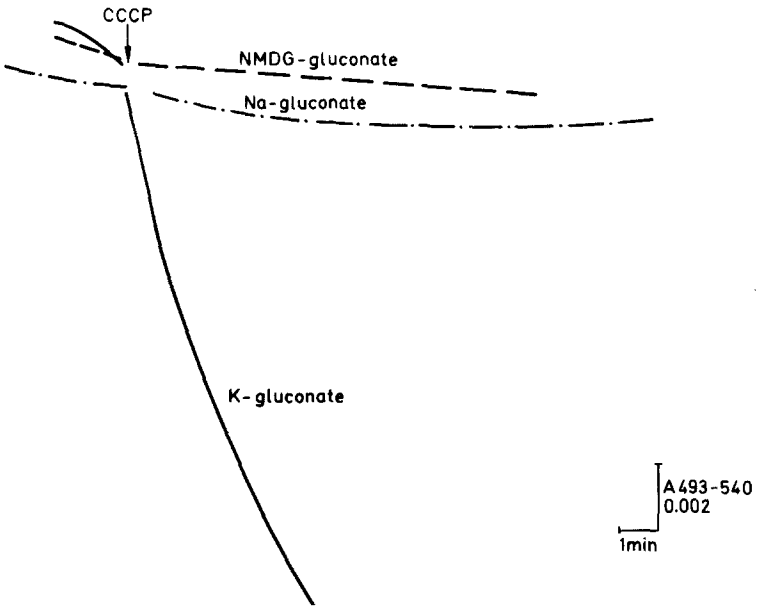

Fig. 9. Presence of a K^+ conductance in microsomal vesicles from exocrine pancreas. Vesicles were preloaded with either 100 mmol/liter K-gluconate (——), 100 mmol/liter NMDG-gluconate (----), or 100 mmol/liter Na-gluconate (-·-·-) and preincubated in an AO medium with 100 mmol/liter TMA-gluconate for 5 min in the cuvette. Addition of the electrogenic protonophore CCCP (1×10^{-6} mol/liter) increased the H^+ permeability of the membrane maximally. An endogenous K^+ conductance induced an inside negative diffusion potential resulting in H^+ ion influx. Typical for three experiments

of either marker enzyme did not yield any statistical significant difference of the correlation coefficient *r* from 0, indicating that H^+ transport under study was neither localized in mitochondria, nor in endoplasmic reticulum, plasma membranes, Golgi apparatus or in zymogen granules.

Discussion

We studied the characterisics of ATP-driven H^+ uptake in microsomes from rat pancreatic acinar cells. It shares many properties of the vacuolar ("*V*"-type) H^+ ATPases described in several nonmito-

chondrial organelles, such as the endoplasmic reticulum, lysosomes, endosomes, clathrin-coated vesicles, secretory granules and Golgi vesicles [29, 41]. This will be discussed in the following section.

Localization of MgATP-Dependent H^+ Transport Under Study

The microsomes used for our studies have been previously shown to be enriched in markers for endoplasmic reticulum such as NADPH-cytochrome-*c*-reductase and ribonucleic acid (RNA) by about two or threefold [3, 15, 39]. They are contaminated by mitochondria by about 10–20% and enriched in plasma membranes by about 1.5-fold. Nevertheless, we assume that H^+ transport neither occurs in mitochondria nor in plasma membranes. Most of the experiments were done in the presence of the inhibitor of mitochondrial H^+ ATPase oligomycin. As shown in Table 1, a high concentration of oligomycin (10^{-5} mol/liter) inhibited H^+ uptake into microsomal vesicles by about 40%, which could be due to mitochondrial contamination. As mitochondrial H^+ transport is already abolished at the low concentrations of $1–5 \times 10^{-7}$ mol/liter oligomycin [8, 11, 43], the inhibition of H^+ uptake observed at 10^{-5} mol/liter indicates that the H^+ pump of microsomal vesicles is sensitive to high concentrations of oligomycin.

It is also unlikely that H^+ transport occurs in plasma membranes, since it did not correlate with plasma membrane marker enzymes (Table 2). Furthermore, plasma-membrane-located H^+ pumps so far studied belong to the "*P*"-type ATPases, which form a phosphorylated intermediate as part of their reaction cycle [13, 18, 26, 32] and are inhibited by vanadate [26]. Insensitivity to vanadate indicates that our H^+ pump differs from the "*P*"-type ATPases, such as the gastric K^+/H^+ ATPase [31].

The sensitivity of the H^+ pump to low concentrations of NEM and NBD-Cl and to higher concentrations of DCCD and Dio 9 suggests that it belongs to the vacuolar or "*V*"-type ATPases [1, 26, 29]. H^+ ATPases of the "*V*"-type are associated with membrane-bound organelles other than mitochondria. They are found in "vacuoles" such as lysosomes, endosomes, clathrin-coated vesicles, endoplasmic reticulum, hormone storage or secretory granules, and Golgi vesicles [1, 4, 20, 29, 41]. In an attempt to localize H^+ transport, this microsomal fraction was subfractionated on a Percoll gradient. As shown in Table 2, maximal H^+ uptake was found in a fraction that was neither enriched in the markers for endoplasmic reticulum, nor in those for plasma membranes, mitochondria, or *trans*-Golgi membranes [19, 20]. H^+ transport is not located in zymogen granules either since isolated zymogen granules did not show ATP-driven H^+ uptake (*unpublished observations*).

It, therefore, appears that the H^+ transport under study occurs into another compartment, e.g., lysosomes, endosomes, condensing vacuoles derived from Golgi [25], or a yet unrecognized organelle.

Functional Characterization of MgATP-Dependent H^+ Transport Under Study

The H^+ transport system found in this study is specific for ATP only. A similar nucleotide specificity has been described for hepatic Golgi membranes [11] and endoplasmic reticulum [28], and for bovine brain clathrin-coated vesicles [38]. H^+ pumps in endocytotic vesicles [30], lysosomes [14, 24, 34] and liver clathrin-coated vesicles [9, 43], however, can also utilize to a smaller extent CTP, GTP, UTP, and ITP.

In contrast to all "*V*"-type H^+ pumps described so far, for which no specific cation is necessary for the optimal H^+ pump activity [9, 10, 24, 30, 43, 44], MgATP-driven H^+ transport into pancreatic microsomes is stimulated by K^+. In the presence of Na^+, Li^+ or weakly permeant cations such as choline$^+$, or TMA^+ and NMDG (*not shown*), H^+ uptake is markedly reduced (*see* Fig. 5). The K^+ dependency, however, cannot be attributed to the presence of a K^+/H^+ ATPase, as described in gastric vesicles [31], which is inhibited by vanadate and forms a phosphorylated intermediate. However, a direct effect of K^+ on the H^+ ATPase could explain the stimulatory effect of K^+ on H^+ uptake and cannot be excluded from the present studies.

H^+ ATPases located in mitochondria, fungal plasma membranes and vacuoles are stimulated by Cl^- [20]. The requirement for Cl^- is attributed to charge compensation of electrogenic ATP-driven H^+ uptake and could occur via a Cl^- conductance present in the membrane [11]. Evidence for the presence of a Cl^- conductance in pancreatic microsomal membranes was demonstrated in the experiments of Fig. 8. Furthermore, a Cl^- conductance has been found in membranes from the same fraction by patch-clamp methods [33]. It should be emphasized, however, that the Cl^- conductance found in our membrane vesicle preparation, may not reside in the same membrane as the H^+ ATPase. Since the vesicle population is heterogenous, it might be also possible that coupling of H^+ uptake to ion fluxes is different in different vesicles. For instance, dependence of H^+ uptake on Cl^- might be due to the presence of a Cl^- conductance in some membranes, whereas in others, a K^+ conductance together with a KCl cotransporter could result in a

net uptake of Cl^- ions to accompany electrogenic H^+ uptake. As long as we are not able to separate different vesicle populations that contain the vacuolar-type H^+ ATPase and to demonstrate ion fluxes associated with H^+ uptake in different vesicles, these possibilities remain speculative. The anion dependency of H^+ transport observed is quite different from that found in endocytotic vesicles, where the lipophilic anion SCN^- stimulates ATP-driven H^+ uptake even more than Cl^- [30]. If the anion is only necessary for charge compensation of electrogenic H^+ transport, lipophilic anions, such as SCN^- or NO_3^- should be more effective for H^+ transport than Cl^-. However, SCN^- or NO_3^- could directly inhibit our H^+ ATPase, as has been demonstrated for the H^+ ATPase of liver multivesicular bodies [42] and for the H^+ ATPase of chromaffin granules in the reconstituted system [21]. SCN^- also dissipates the H^+ gradient (*not shown*) probably by formation of the membrane permeant HSCN [27]. It also appears that Cl^- is absolutely necessary for H^+ transport under study. Replacement of Cl^- by anions other than Br^- abolishes H^+ transport (Fig. 4). It, therefore, remains to be investigated, if Cl^- is coupled to H^+ transport or influences the H^+ pump in any other positive way.

Using a "zero point-titration" method, an internal pH of 4.8 was determined at steady state in our membrane vesicles (*see* Fig. 7*b*). A low pH of about 5.0 has been measured in lysosomes [24], and a pH between 5 and 6 has been measured in clathrin-coated vesicles, endosomes, secretory granules and Golgi vesicles [1, 9, 10]. Recently, Orci, Ravazzola and Anderson found highest densities of monoclonal anti-DNP binding sites, reflecting low pH compartments, in lysosomes and condensing vacuoles of exocrine pancreas and parotid gland [25], but not in endoplasmic reticulum or Golgi structures [25, 36].

Taken together, our H^+ pump could be localized in lysosomes, endosomes, or condensing vacuoles and has similarities but is not identical to "*V*"-type H^+ ATPases described so far.

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