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Characterization of proton transport in bone-derived membrane vesicles

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ATP-dependent proton transport in membrane vesicles prepared from the medullary bone of egg-laying hens, a source rich in osteoclasts, was characterized. Proton transport was abolished by bafilomycin A_1 (10 nM) and *N*-ethylmaleimide (50 μM), but not by oligomycin (15 μg/ml), vanadate (100 μM) or SCH 28080 (100 μM), thereby differentiating this H^+-ATPase from the F_1F_0- and phosphorylated-type of ATPases. Preincubation of the membrane vesicles at 0°C for 1 h in the presence of KCl (0.3 M) and Mg-ATP (5 mM) resulted in almost complete loss of H^+-transport activity (cold-inactivation). Preventing the formation of a membrane potential by voltage clamp ($K^+_{in} = K^+_{out}$ + valinomycin) increased both the rate of H^+-transport and the equilibrium ΔpH, suggesting an electrogenic proton transport mechanism. Thus, the H^+-ATPase in this bone-derived membrane vesicle preparation shows the characteristics of a vacuolar H^+-ATPase in its inhibitor- and cold-sensitivity and its electrogenic mechanism. The anion sensitivity of the H^+-ATPase was investigated by varying the intra- and/or extra-vesicular salt composition. The H^+-ATPase had no absolute requirement for any specific anion, but membrane permeable anions were found to stimulate proton transport activity, presumably by acting as charge compensators for the electrogenic hydrogen ion transport. However, some anions, such as sulfate, acetate and nitrate were directly inhibitory to the ATPase. The results are in agreement with the recently proposed mechanism of osteoclast acidification: a vacuolar H^+-ATPase working in parallel with a Cl^--channel resulting in electroneutral HCl secretion.

Introduction

The cell responsible for bone resorption, the osteoclast, secretes acid into the compartment formed between its highly folded plasma membrane, the ruffled border, and the bone surface [1]. Recently, evidence has been presented that a H^+-ATPase, functionally and immunologically similar to the vacuolar type of proton pumps, is polarized to the ruffled border and is responsible for osteoclast acid secretion [2–7].

The electrogenic and bafilomycin-sensitive proton pumps, the vacuolar H^+-ATPases, are a distinct class of cation pumps, different from the P- and F_1F_0-type of ATPases [8]. As indicated by the name, this class of ATPases is found in fungi and plant vacuoles [9,10] and also in eukaryotic intracellular membrane compartments such as lysosomes [11], chromaffin granules [12] and clathrin-coated vesicles [13]. However, there are exceptions to an intracellular localization. For example, a vacuolar H^+-ATPase is found in the plasma membrane of renal intercalated cells, where it is thought to be involved in the process of urinary acidification [14]. These proton pumps, however, are thought to originate from intracellular membrane vesicles which, under stimulation, are fused with the plasma membrane. A similar mechanism has been suggested for osteoclast acidification [3].

The magnitude of the pH gradient formed by vacuolar H^+-ATPases is believed to be regulated mainly by the membrane potential and the requirement for anions [12]. Anions have been shown to affect the activity of the vacuolar type of H^+-ATPases, both indirectly by acting as charge compensators for the electrogenic H^+-transport and also directly through anion sensitive regulatory sites on the H^+-ATPase [12–20]. The reported variation in anion sensitivity among vacuolar H^+-ATPases could suggest that anions might be involved in the cellular regulation of these widely distributed enzymes.

In the present study, we have characterized the ATP-dependent proton transport activity found in membrane vesicles prepared from a source rich in osteoclasts, the medullary bone of egg-laying hens, and report on the inhibitor-, divalent cation-, cold- and

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anion-sensitivity and electrogenicity of the H^+-ATPase. This has extended previously published characterizations of this H^+-ATPase in osteoclast- and bone-derived membrane vesicles [2–7].

Materials and Methods

Materials. All chemicals were purchased from Sigma, USA, except Acridine orange, which was obtained from Merck (Germany). SCH 28080 was synthesized by Astra Hässle AB. Bafilomycin A_1, isolated from *Streptomyces* sp., was purified by Astra Hässle AB, Mölndal, Sweden.

Preparation of membrane vesicles. Bone-derived membrane vesicles were prepared as described by Väänänen et al. [4], with some modifications. Briefly, medullary bone scraped from the long bones of egg-laying hens was homogenized with a polytron homogenizer in isolation buffer (5 mM Hepes-Tris (pH 7.4), 250 mM sucrose, 1 mM EGTA). The membrane vesicles were obtained by differential centrifugation (1000 $\times g$ for 10 min, 10000 $\times g$ for 20 min and 40000 $\times g$ for 1 h). The final pellet was resuspended in isolation buffer, rapidly frozen and stored at −70°C. To load the vesicles with potassium salts, the membrane vesicles were further homogenized in isolation buffer containing 150 mM potassium with the indicated anion as counter-ion, centrifuged at 100000 $\times g$, resuspended in the same solution and then rapidly frozen and stored at −70°C.

Proton transport measurements. Proton transport was measured fluorometrically in a Shimadzu RF-5000 spectrofluorimeter (λ_{ex} 492 nm, λ_{em} 528 nm) using the fluorescent weak base Acridine orange (AO) as described previously [6]. The experimental conditions for each experiment are described in detail in the legends to the figures and tables. The rate of acidification was taken to be the maximum rate of decrease of AO fluorescence. Up to the maximum used in these experiments, the rate of acidification was observed to be proportional to the amount of vesicles added.

ATPase activity measurements. ATPase activity in the bone-derived membrane vesicles were measured as inorganic phosphate (P_i) release from ATP. Membrane vesicles (30 μg of protein, final concentration) were incubated at 37°C for 5 min in a medium containing 5 mM Hepes-Tris (pH 7.4), 150 mM KCl, 3 mM $MgSO_4$, 0.5 μM valinomycin in the presence or absence of the inhibitors. Reactions were initiated by the addition of 2 mM ATP (pH 7.4 with Tris) and incubated for another 15 min at 37°C. The amount of P_i released was analyzed according to Yoda and Hokin [23].

Protein determination. Protein was determined according to Bradford [24], using the Bio-Rad assay procedure and bovine γ-globulin as a standard.

Results

A protein-dependent Acridine orange quenching of fluorescence was observed upon addition of ATP to bone-derived membrane vesicles (Fig. 1). The quenching was rapidly reversed by the addition of the H^+/K^+-exchanger nigericin, confirming that a pH gradient had been formed. These results indicate that the Acridine orange fluorescence quenching represents proton transport by an H^+-ATPase. To characterize this H^+-ATPase further, several properties of the proton transport activity were investigated.

Inhibitor sensitivity

The effects of substances known to inhibit different types of ATPases were investigated. As shown in Table I, ATP-dependent H^+-transport was virtually abolished by *N*-ethylmaleimide (50 μM), but was insensitive or only partly inhibited by oligomycin (15 μg/ml), vanadate (100 μM) and SCH 28080 (100 μM), differentiating this H^+-ATPase from the F_1F_0- and phosphorylated-type of ATPases [8,22]. Moreover, and most conclusively, bafilomycin A_1, recently shown to be a specific inhibitor of vacuolar H^+-ATPases in nanomolar concentrations [20, 21], inhibited H^+-transport with

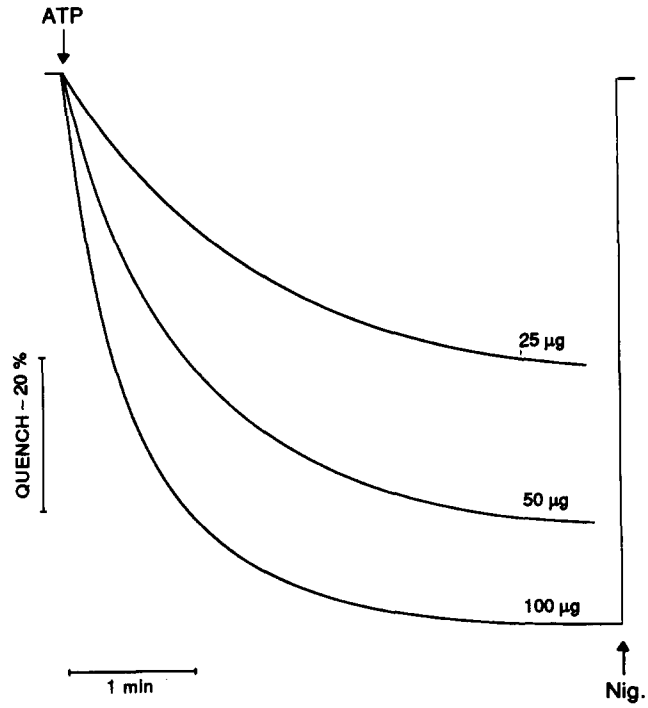

Fig. 1. ATP-dependent proton transport in bone-derived membrane vesicles. Membrane vesicles (20, 50 or 100 μg protein/ml, final concentration) were added to a medium containing 5 mM Hepes-Tris (pH 7.4), 150 mM KCl, 3 mM $MgSO_4$, 1 μM Acridine orange and 1 μM valinomycin. After 10 min preincubation reactions were initiated by the addition of 1 mM Tris-ATP (pH 7.4), and proton transport was measured as described in Materials and Methods. At the end of the experiment, nigericin (1 μg/ml, final concentration) was added. Typical traces out of several experiments are shown.

TABLE I

Inhibitor sensitivity of ATP-dependent H^+-transport in bone-derived membrane vesicles

Membrane vesicles (50 μg protein/ml final concentration) were added to a medium containing 5 mM Hepes-Tris (pH 7.4), 3 mM $MgSO_4$, 150 mM KCl, 1 μM valinomycin, 1 μM AO and ethanol (control) or the indicated concentrations of substances. After 10 min preincubation reactions were initiated by the addition of 1 mM ATP (pH 7.4 with Tris). The initial rate of acidification was measured as described in Materials and Methods. Values are expressed as the percentage of the initial rate of a control, measured in the same experiment, and are the mean ± S.E. for two or three determinations on separate preparations. The control rates were (in (% AO quench/min per mg) × 10^2) 12 ± 1 (*N*-ethylmaleimide and oligomycin), 15 ± 0 (vanadate) and 9 ± 3 (SCH 28080).

Inhibitor	Initial rate of acidification (% of control)
N-Ethylmaleimide	
1 μM	65 ± 7
10 μM	13 ± 4
50 μM	5 ± 1
Oligomycin	
1 μg/ml	92 ± 7
10 μg/ml	74 ± 10
15 μg/ml	51 ± 5
Vanadate	
10 μM	95 ± 10
100 μM	87 ± 8
SCH 28080	
10 μM	109 ± 4
100 μM	80 ± 1

an $IC_{50} = 0.4 \pm 0.1$ nM (mean ± S.E., $n = 3$ experiments) (Fig. 2). Thus, these results strongly suggest that a vacuolar H^+-ATPase is responsible for the proton transport activity in the medullary bone-derived membrane vesicles. As reported for the inhibition of other vacuolar H^+-ATPases [21], the inhibition by bafilomycin A_1 was related to the amount of protein in the assay (Fig. 2, inset).

Despite almost total inhibition of proton transport by both NEM and bafilomycin A_1, neither of these inhibitors had any significant effect on the rate of ATP-hydrolysis (Table II). This result could be explained by a low degree of coupling between ATPase activity and H^+-translocation in the enzyme or by the fact that the inhibitors uncouple the ATPase activity. However, both NEM and bafilomycin have been shown to inhibit the ATPase activity of other vacuolar H^+-ATPases [8,21]. Thus, the most likely explanation is that the vacuolar H^+-ATPase activity and/or protein only represents a small part of the total ATPase activity and/or protein in this preparation. The amount of vacuolar H^+-ATPase protein present in the preparation can be estimated from the inhibition of proton transport by bafilomycin A_1. The IC_{50} for this inhibition was, as shown above, 0.4 nM/50 μg protein or 8 pmol/mg. If it is assumed that one inhibitor molecule is needed to inhibit one H^+-ATPase molecule and the molecular mass of the enzyme is taken to be 575 kDa, which is an approximate average of the molecular masses published for vacuolar H^+-ATPases [8], a vacuolar H^+-ATPase protein content of no more than ~ 0.009 mg/mg of protein (or 0.9%) is obtained. Thus, the vacuolar H^+-ATPase may constitute a very small part of the total protein content in this preparation.

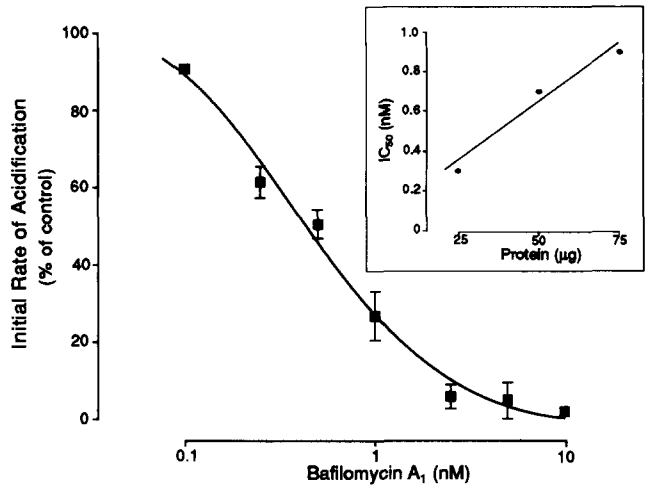

Fig. 2. Effect of bafilomycin A_1 on ATP-dependent H^+-transport in bone-derived membrane vesicles. The effect of bafilomycin A_1, dissolved in DMSO, on proton transport in medullary bone-derived membrane vesicles (50 μg protein/ml) was measured as described for other substances in the legend to Table I. Values are the means ± S.E. for three determinations on separate preparations. Inset: Dependence of bafilomycin A_1 inhibition on the amount of protein (25, 50 or 75 μg protein) in the assay. A representative experiment from one preparation is shown.

Cold inactivation

Treatment of vacuolar H^+-ATPases in the cold under certain conditions has been shown to induce release of the hydrophilic catalytic sector of the enzyme and, thereby, inhibition of proton transport activity [20]. It was therefore of interest to investigate whether

TABLE II

Inhibitor sensitivity of ATPase activity in bone-derived membrane vesicles

The effect of the indicated inhibitors on the ATPase activity in the bone-derived membrane vesicles was measured as described in Materials and Methods. Values are the mean ± range of two determinations on separate preparations. The mean control ATPase activity was 27 ± 1 μmol P_i/mg per h.

Inhibitor	ATPase activity (% of control)
N-Ethylmaleimide (100 μM)	86 ± 2
Bafilomycin A_1 (10 nM)	96 ± 0
Oligomycin (10 μg/ml)	81 ± 0

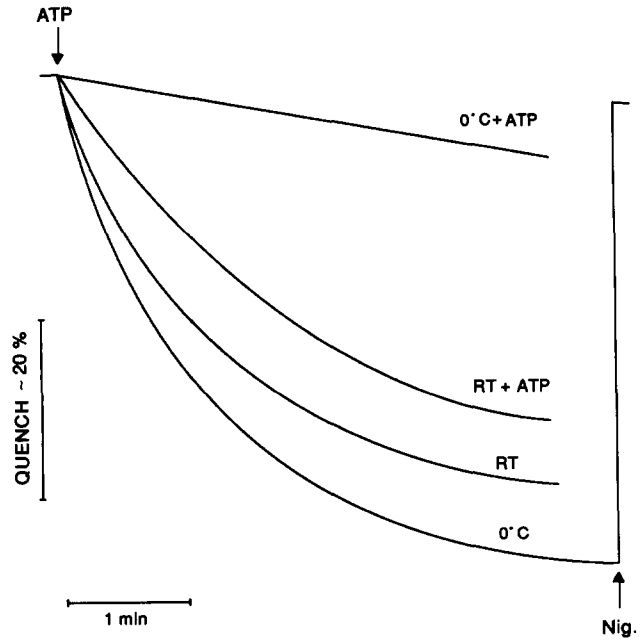

Fig. 3. Effect of cold treatment on ATP-dependent H^+-transport in bone-derived membrane vesicles. Membrane vesicles (1 mg protein/ml, final concentration) were suspended in an incubation medium containing 5 mM Hepes-Tris (pH 7.4), 5 mM $MgSO_4$, 0.3 M KCl with or without 5 mM ATP and incubated on ice or at room temperature for 1 h. Aliquots (50 μg protein/ml, final concentration) were then taken, and proton transport was measured as described in the legend to Fig. 1. A representative experiment of three is shown.

the ATP-dependent H^+-transport activity in this preparation was also affected by 'cold treatment'. As shown in Fig. 3, incubation of the vesicles at 0°C for 1 h in the presence of KCl (0.3 M), Mg^{2+} (5 mM) and ATP (5 mM) almost totally abolished proton transport activity. The inhibition was dependent on the presence of ATP, and at room temperature the inhibition was much less. Hence, the H^+-ATPase present in this bone-derived membrane vesicle preparation was found to be sensitive to 'cold treatment'.

Divalent cation sensitivity

The result of substituting Mg^{2+} with other divalent cations on H^+-transport is shown in Table III. The order of effectiveness was $Mn^{2+} = Mg^{2+} \gg Ca^{2+} > Zn^{2+}$. In the presence of Zn^{2+}, Mg^{2+} could not support acidification (Table III), indicating that Zn^{2+} acts as a direct inhibitor of the ATPase. It has earlier been shown that vacuolar H^+-ATPases are sensitive to certain divalent cations, e.g., Zn^{2+} and Cu^{2+} [13].

Electrogenicity

All vacuolar H^+-ATPases investigated so far are electrogenic. Thus, H^+-transport by a V-type H^+-ATPase into membrane vesicles will generate both an intravesicular positive membrane potential ($\Delta\psi$) and a

TABLE III

Effect of divalent cation substitution on ATP-dependent H^+-transport in bone-derived membrane vesicles

ATP-dependent proton transport in membrane vesicles (50 μg of protein/ml, final concentration) was measured as described in the legend to Fig. 1 except that $MgSO_4$ was substituted with 3 mM $MgCl_2$ (control) or 3 mM of the chloride salt of the indicated divalent cations or 3 mM $MgCl_2$ plus 1 mM of the indicated divalent cation. Values are the mean ± range for two determinations on separate preparations. The initial H^+-transport in the presence of Mg^{2+} (control) was 13 ± 4 (% AO quench/min per mg) × 10^2.

Divalent cation	Initial rate of H^+-transport (% of control)
Mg^{2+}	100
Mn^{2+}	100 ± 12
Ca^{2+}	12 ± 4
Zn^{2+}	0
$Mg^{2+} + Mg^{2+}$	102 ± 16
$Mg^{2+} + Ca^{2+}$	97 ± 3
$Mg^{2+} + Zn^{2+}$	0

pH gradient (ΔpH). In order to test whether the present H^+-ATPase was electrogenic, membrane vesicles were prepared in the presence of potassium salts, and proton transport was measured in a potassium-containing medium in the presence (voltage clamp) or absence of valinomycin. Preventing the formation of $\Delta\psi$ by voltage clamp consistently increased both the rate of H^+-transport (Table IV) and the equilibrium ΔpH (data not shown), indicating that proton transport in this preparation is an electrogenic process.

Anion sensitivity

An important feature of vacuolar H^+-ATPases is their anion sensitivity, which might play a role in the

TABLE IV

Anion sensitivity of ATP-dependent H^+-transport in bone-derived membrane vesicles

Membrane vesicles (20–50 μg protein/ml, final concentration), loaded with the indicated potassium salt as described in Materials and Methods, were added to a cuvette containing 5 mM Hepes-Tris (pH 7.4), 3 mM $MgSO_4$, 1 mM ATP, 1 μM Acridine orange, ethanol or 1 μM valinomycin, 150 mM K^+ with the indicated anion as counterion and sucrose to maintain isosmolarity. The initial rate of proton transport was calculated as described in Materials and Methods. Values are mean ± S.E. for 4–9 determinations on 2–4 separate preparations. The rates in the presence of K_2SO_4 and K-acetate inside the vesicles were compared to the rate with KCl as the inside salt using a paired Student's t-test (* $P < 0.05$).

Salt inside	Salt outside	Initial rate of H^+-transport (% AO quench/min per mg) × 10^2	
		+ ethanol	+ valinomycin
KCl	KCl	4 ± 1	20 ± 8
K_2SO_4	KCl	6 ± 1	20 ± 4
K-acetate	KCl	2 ± 0	8 ± 2 *

regulation of these enzymes. The effects of varying the intra- and/or extra-vesicular salt composition on ATP-dependent H^+-transport in the bone-derived membrane vesicles are summarized in Tables IV and V. The rates of hydrogen ion transport, both in the presence and absence of valinomycin, were the same with Cl^- or SO_4^{2-} present inside the vesicles, irrespective of the anion present in the outside medium. In contrast, the rates with acetate were lower, possibly indicating an inhibitory effect of acetate on intravesicular sites of the H^+-ATPase. An alternative explanation is that non-ionic diffusion of acetate could have detracted from the observed pH gradient. The relative effects of the anions in the extravesicular medium were almost the same, irrespective of the intravesicular salt; in the absence of valinomycin, NO_3^-, Cl^- or Br^- supported proton transport, whereas the rates in the presence of gluconate, SO_4^{2-} or acetate in the outside medium were lower. Under voltage-clamp conditions ($[K^+]_{out} = [K^+]_{in}$ in the presence of valinomycin) the rates with Cl^- or gluconate as the outside anions were not significantly different from each other, whereas the rates in the presence of acetate, SO_4^{2-}, Br^- or NO_3^- were lower. It should be noted that, although the initial rate of acidification was faster in the presence of NO_3^- compared to Cl^- in the absence of voltage clamp, the extent of acidification was lower with NO_3^- (Fig. 4).

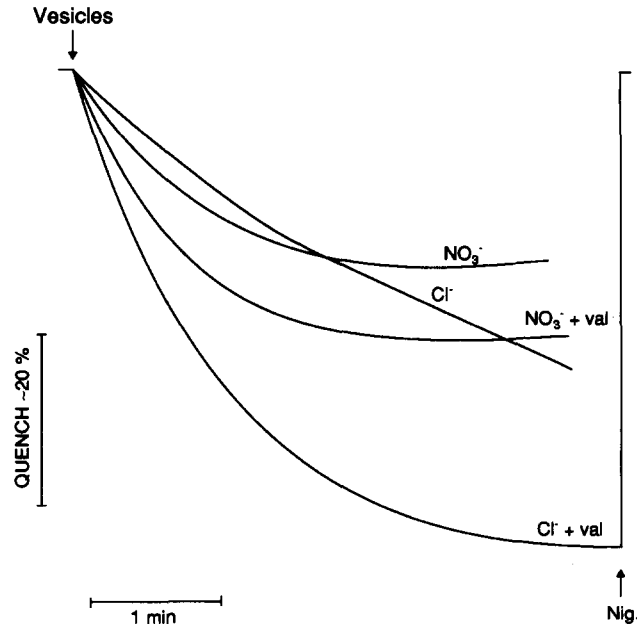

Fig. 4. Comparison of the effects of NO_3^- and Cl^- on ATP-dependent H^+-transport in medullary bone-derived membrane vesicles. Proton transport in K_2SO_4-loaded vesicles with Cl^- or NO_3^- as the external anions was measured as described in the legend to Table IV. At the time indicated nigericin, (1 μg/ml, final concentration) was added to dissipate the pH gradient. A representative experiment of three is shown.

TABLE V

Anion sensitivity of ATP-dependent H^+-transport in bone-derived membrane vesicles

The effects of the indicated external anions on ATP-dependent H^+-transport in membrane vesicles loaded with the indicated potassium salts were measured as described in the legend to Table III. The initial rate of H^+-transport is expressed as a percentage of the rate with Cl^- as the outside anion (control), measured in the same set of experiments. The mean rate of the controls is shown in Table IV. The values are the mean ± S.E. for 2–8 determinations on 2–4 separate preparations. The values were compared to 100% using a Student's *t*-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Salt inside	Salt outside	Initial rate of H^+-transport (% of control)			
		+ ethanol	(*n*)	+ valinomycin	(*n*)
KCl	KCl	100	(4)	100	(4)
	K-gluconate	48 ± 8	(2)	90 ± 0 *	(4)
	K_2SO_4	19 ± 2 ***	(3)	50 ± 6*	(2)
	K-acetate	25 ± 3 *	(2)	78 ± 6	(2)
K_2SO_4	KCl	100	(9)	100	(9)
	K-gluconate	40 ± 6 ***	(6)	97 ± 3	(7)
	K_2SO_4	20 ± 3 ***	(8)	63 ± 5 ***	(8)
	K-acetate	17 ± 3 ***	(3)	75 ± 22	(3)
	KBr	79 ± 12	(3)	73 ± 5 *	(3)
	KNO_3	137 ± 12	(3)	63 ± 7 *	(3)
K-acetate	KCl	100	(4)	100	(4)
	K-gluconate	50 ± 3 ***	(4)	85 ± 10	(4)
	K_2SO_4	16 ± 6 ***	(4)	44 ± 4 ***	(4)
	K-acetate	25 ± 7 **	(4)	76 ± 6 **	(4)

In the absence of voltage-clamp conditions, proton transport could have been influenced both by a direct effect of anions on the H^+-ATPase and by the relative ability of these anions to cross the vesicle membrane and provide charge compensation for the electrogenic hydrogen ion transport. Under voltage-clamp conditions, however, the rate should not be limited by any anion conductance for charge compensation and should thus depend only on the direct effect of the anion. With the assumption that the initial rate of H^+-transport in the presence of Cl^- under voltage-clamp conditions represents the maximum rate of acidification, the results suggest the following influence of anions on the H^+-ATPase in the bone-derived membrane vesicles. (1) The H^+-ATPase has no absolute dependence on any specific anion for H^+-transport activity, as indicated by equal proton transport rates in the presence of Cl^- and gluconate under voltage-clamp conditions. (2) SO_4^{2-} is inhibitory to the H^+-ATPase at extravesicular sites, since SO_4^{2-} could substitute for Cl^- inside the vesicles but not, under voltage-clamp conditions, outside the vesicles. (3) Acetate is inhibitory to the H^+-ATPase when present on the inside of the membrane vesicles. (4) Br^- and NO_3^- are permeable anions in these membranes, as shown by good proton transport in the absence of valinomycin, but can not completely substitute for Cl^- under voltage-clamp conditions, as indicated by a small inhibitory effect of these anions on the ATPase.

Discussion

Vacuolar H^+-ATPases, although isolated and characterized from a variety of species and organelles, show many similarities in structure and functional properties [8]. The H^+-ATPase in this preparation of bone-derived membrane vesicles shows typical characteristics of a vacuolar H^+-ATPase in its inhibitor sensitivity, divalent cation sensitivity, its sensitivity to cold and its electrogenic mechanism. These results are in agreement with those obtained using membrane vesicles from purified osteoclasts [2,7]. The precise origin of these vesicles is unknown at present. Acidification processes might be expected to occur at both the ruffled border of the osteoclast and in a number of intracellular vesicles in osteoclasts and other cell types. However, Väänänen has shown that antibody staining of two subunits of the vacuolar H^+-ATPase was localized almost exclusively to osteoclasts and was not present to a significant extend in other cell types in medullary bone and bone marrow [4]. Furthermore, a similar microsomal fraction derived from an osteoblast cell line did not contain detectable H^+-transport activity [4]. This supports the idea that the H^+-transport activity studied here is derived from osteoclasts. It may be difficult, however, to define precisely the location for the H^+-ATPase within the osteoclast. Antibodies to several subunits of V-type H^+-ATPases have been reported to be polarized to the ruffled border of the osteoclast [2,4], however, in these same studies some osteoclasts were seen with diffuse staining throughout the cytoplasm, particularly in osteoclasts not attached to bone. Furthermore, stimulation by PTH was shown to increase the proportion of immunoreactivity in the ruffled border [4]. Taken together, these results suggest that the osteoclast pump cycles between the ruffled border and intracellular membranes as the cell goes from an active to a resting stage. Similar membrane cycling has been reported for the H^+-ATPase in the kidney [28], and for the gastric H^+/K^+-ATPase [29].

The estimate of vacuolar H^+-ATPase content, based on bafilomycin sensitivity, indicated that the H^+-ATPase constitutes at most only a small part of the total protein in this preparation. This explains the lack of correlation between the ATPase activity and the H^+-transport activity. However, all the H^+-transport activity in the preparation could be attributed to a vacuolar H^+-ATPase, since the transport signal could be totally abolished by low concentrations of bafilomycin A_1.

Acidification of membrane compartments by vacuolar H^+-ATPases, including osteoclast-derived membrane vesicles, has been shown to be supported by the presence of permeable anions, typically Cl^- [3,7,12–19]. Since vacuolar H^+-ATPases are electrogenic, these results have been interpreted as a requirement for a passive anion conductance to permit charge compensation during proton transport. In line with these results, proton transport in the bone-derived membrane vesicles was well supported in the presence of NO_3^-, Cl^- or Br^-, but not in the presence of SO_4^{2-}, gluconate and acetate. However, dissipation of the membrane potential by valinomycin increased proton transport significantly even in the presence of Cl^-. This result suggests that the Cl^- permeability in these membranes is insufficient to support maximal rates of acidification. Modulation of the Cl^- conductance of membranes has been suggested to be one way for the cell to regulate the pH within membrane compartments acidified by vacuolar H^+-ATPases [18]. It was recently suggested that a distinct Cl^- conductance channel is present in the ruffled border membrane of the osteoclast and that it is coupled to the vacuolar H^+-ATPase [25].

Beside its role as a charge compensator, Cl^- has also been suggested to activate directly vacuolar H^+-ATPases [12]. However, the degree of Cl^- activation has been reported to vary widely among vacuolar H^+-ATPases [12–19]. For example, absolute Cl^- dependence has been reported for proton transport in renal cortical endosomes [15], whereas proton transport in clathrin-coated vesicles was observed in the complete absence of Cl^- [16]. By studying proton transport in the presence and absence of voltage-clamp conditions and systematically varying the intravesicular and extravesicular anion, we have extended the scope of previous studies [3,7] and discriminate the effects of anions as charge compensators from their direct effects on the H^+-ATPase. In the present study, maximal proton transport rates were observed in the complete absence of Cl^- under voltage-clamp conditions, suggesting neither any absolute requirement of the H^+-ATPase for Cl^- nor any direct stimulatory effect by Cl^- on the ATPase. However, on the basis of a nonlinear dependence of proton transport on Cl^- in the absence of voltage clamp, Blair et al. [3] suggested some direct influence of Cl^- on the osteoclast H^+-ATPase. Reconciliation of these results would require that the interaction of chloride with the H^+-ATPase was potential sensitive.

Some anions, in particular SO_4^{2-} and acetate, were found to be directly inhibitory to the H^+-ATPase. Interestingly, a sidedness of the inhibition was observed; SO_4^{2-} inhibited the H^+-transport only when present outside the vesicles, whereas the inhibition by acetate was more pronounced when the anion was present inside rather than outside the vesicles. The results are in line with the view that vacuolar H^+-ATPases exhibit anion-sensitive inhibitory sites [12]. The anion sensitivity of the proton transport in the medullary bone-derived membrane vesicles is somewhat similar to that recently reported for the chromaffin granule proton pump [19]. However, in contrast to

our result indicating that SO_4^{2-} has an extravesicular site of action, an intravesicular inhibitory effect by SO_4^{2-} on the chromaffin granule H^+-ATPase was suggested.

The results obtained with NO_3^- are somewhat different from those reported for other vacuolar H^+-ATPases [3,7,12,15–17]. Under voltage-clamp conditions the inhibitory effect of NO_3^- is in line with what has generally been found. However, in the absence of voltage clamp, no inhibitory effect of NO_3^- could be found on the initial transport rate. In contrast, a stimulation relative to Cl^- of the initial rate of AO quench was seen. The initial rate of proton transport in membrane vesicles from chromaffin granule and multivesicular bodies has been shown to be stimulated by the addition of NO_3^- [12,17]. However, since pre-incubation in the presence of KNO_3 or intravesicular KNO_3-loading resulted in inhibition of proton transport in these preparations, an intravesicular location of a NO_3^- inhibitory site was suggested. In contrast, we did not observe any inhibition by NO_3^- after 10 min preincubation in the presence of this anion (result not shown). Our results suggest that, due to good membrane permeability of NO_3^- in the bone-derived membrane vesicles, NO_3^- can act as an effective charge compensator, resulting in high initial transport rates despite the counteracting inhibitory effect. The explanation for the decreased steady-state acidification in the presence of NO_3^-, as compared to Cl^-, is at the present not known. At first sight, it could be interpreted as being in line with an intravesicular inhibitory site of NO_3^-, but the lack of inhibition found after preincubation with NO_3^- contradicts such an explanation. The effect of NO_3^- on proton transport has to be further investigated before any definite conclusions can be made on the effect of this anion on the H^+-ATPase, particularly in the light of claims that anions, in especially NO_3^-, might interfere directly with the Acridine orange technique for measuring proton transport [26,27]. The significance of such possible interference in relation to the results presented here, and elsewhere by others, is presently not known. However, NO_3^- has been shown to inhibit ATP hydrolysis by vacuolar H^+-ATPases, supporting a direct effect of this anion on the H^+-ATPase [16].

In summary, we conclude that the H^+-ATPase found in this bone-derived membrane vesicle preparation shows the characteristics of a vacuolar H^+-ATPase in its inhibitor-, divalent cation-, and cold-sensitivity and its electrogenic mechanism. The results suggest an electrogenic hydrogen ion transport mechanism with a parallel anion conductance to permit charge compensation across the membrane, but without an absolute requirement for any specific anion. These results are in agreement with the recently proposed mechanism of osteoclast acidification [3]; a vacuolar H^+-ATPase working in parallel with a Cl^- channel resulting in electroneutral HCl secretion. The recent reports of tissue specific isozymes of the H^+-ATPase [30–32] suggest further studies to define the exact nature of this ion pump in the osteoclast.

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