Sensitivity to Nitrate and Other Oxyanions Further Distinguishes the Vanadate-Sensitive Osteoclast Proton Pump from Other Vacuolar H^+-ATPases†

Diptendu Chatterjee, Lynn Neff, Munmun Chakraborty, Christopher Fabricant, and Roland Baron*

Departments of Orthopedics and Cell Biology, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06510

Received December 9, 1992

ABSTRACT: The osteoclast proton pump (OC H^+-ATPase) differs from other vacuolar H^+-ATPases (V-ATPases) in its sensitivity to vanadate and in the subunit composition of its catalytic domain, where isoforms of subunits A and B are expressed [Chatterjee et al. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89,* 6257–6261]. In the present study, the sensitivity of the osteoclast H^+-ATPase to various oxyanions was tested. The results indicate that H^+ transport by microsomal preparations isolated from chicken osteoclasts is 20–100-fold more sensitive to nitrate that any other animal and fungal V-ATPases and 10–20-fold more sensitive than plant V-ATPases, as is the ATPase activity of the affinity-purified enzyme. This inhibition by nitrate is not due to a chaotropic effect of the oxyanion and is complete at 1 mM concentrations with an IC_{50} of 100 μM. In contrast, proton transport by the OC H^+-ATPase was insensitive to other oxyanions (phosphate, sulfate, and acetate) which inhibit other V-ATPases. These results further demonstrate that the proton pump present in osteoclast membranes differs from other vacuolar ATPases. It is speculated that, since cells of the macrophage lineage can generate high intracellular concentrations of nitrate, it may be possible to physiologically or therapeutically regulate the activity of the OC H^+-ATPase in the osteoclast without affecting the other V-ATPases in the same or in other cells.

Vacuolar ATPases (V-ATPases) are multisubunit enzymes expressed in all cells, where they transport protons across the limiting membranes of various organelles in order to acidify their lumen and/or energize the membrane, allowing the transport of small molecules (Mellman et al., 1986; Forgac, 1989; Nelson, 1991). Examples of such acidified organelles are plant and fungal vacuoles, Golgi cisternae, endosomes, and lysosomes (Forgac, 1989; Nelson & Taiz, 1989). Coated vesicles (Arai et al., 1987), synaptic vesicles (Moriyama & Futai, 1990; Sudhof et al., 1989), and secretory granules of chromaffin cells (Cidon & Nelson, 1983) also contain V-ATPases in their membranes and the proton gradient generated by the proton pump is utilized to energize the import of neurotransmitters (Forgac, 1989; Nelson, 1991). Vacuolar H^+-ATPases are also present at the plasma membrane of several cell types, including the kidney tubule intercalated cell (Gluck et al., 1982; Gluck & Caldwell, 1987, 1988) and the osteoclast (Baron et al., 1985; Blair et al., 1989; Bekker & Gay, 1990; Chatterjee et al., 1992), where they are involved in the acidification of the extracellular environment and may participate, in cells such as macrophages (Swallow et al., 1990), in intracellular pH regulation via the extrusion of protons from the cytoplasm.

Despite this ubiquitous distribution, there is a need for the various organelles, cells, and organs to differentially regulate H^+ transport. This requirement implies the existence of structural or regulatory differences between the vacuolar H^+-ATPases found within each cell and/or in the plasma membrane of more specialized cell types such as the cells of the kidney tubule and osteoclasts in bone. Although the sequences of most of the subunits composing the vacuolar proton pumps are highly conserved between species and closely related to corresponding subunits of the mitochondrial F_o–F_1-ATPase (Bowman et al., 1989; Nelson et al., 1989; Sudhof et al., 1989), recent evidence suggests the existence of isoforms for some of these subunits, possibly explaining the subtle differences observed in their apparent molecular weight and/or in the properties of the enzymes purified from various sources. For instance, Puopolo et al. (1992) have isolated cDNA clones encoding at least two isoforms of subunit B, one predominantly expressed in brain and the other in kidney.

Furthermore, studying proton transport by inside-out vesicles isolated from highly purified preparations of chicken osteoclasts, we have recently reported that the osteoclast H^+-ATPase, although vacuolar in nature, differs in its structure and properties from other H^+-ATPases (Chatterjee et al., 1992). First, the OC H^+-ATPase was found to be sensitive not only to the classical inhibitors of V-ATPases (NEM and Bafilomycin A1) but also to vanadate, which blocked H^+ transport at a concentration of 1 mM, with an IC_{50} of 100 μM. Second, immunochemical and/or apparent M_r differences were found between the osteoclast V-ATPase and V-ATPases from bone marrow cells, from macrophages, and from kidney in the two subunits of the catalytic portion of the enzyme. Subunit A of the osteoclast V-ATPase had an M_r of 63 kDa and was not recognized by antibodies to the 70-kDa subunit of bovine coated vesicles or chromaffin granules, and subunit B differed from the kidney 60-kDa subunit in its cross-reactivity with antibodies to the *Neurospora crassa* or bovine chromaffin granule B subunits (Chatterjee et al., 1992).

Taken together, these results further supported the concept that variations in isoforms of some of the subunits that assemble to form vacuolar H^+-ATPases not only occur but also can confer specific properties to proton pumps expressed in different organelles or in different cell types.

Sensitivity to oxyanions being one of the characteristic properties that varies among V-ATPases, with a 2–7-fold difference between kidney and plant tonoplast sensitivities for instance, the present study was designed to determine how oxyanions affected the osteoclast H^+-ATPase. We report that

† This work has been supported by Grants DE-04724 and AR-41339 from the National Institutes of Health (R.B.).

* Corresponding author.

0006-2960/93/0432-2808$04.00/0 © 1993 American Chemical Society

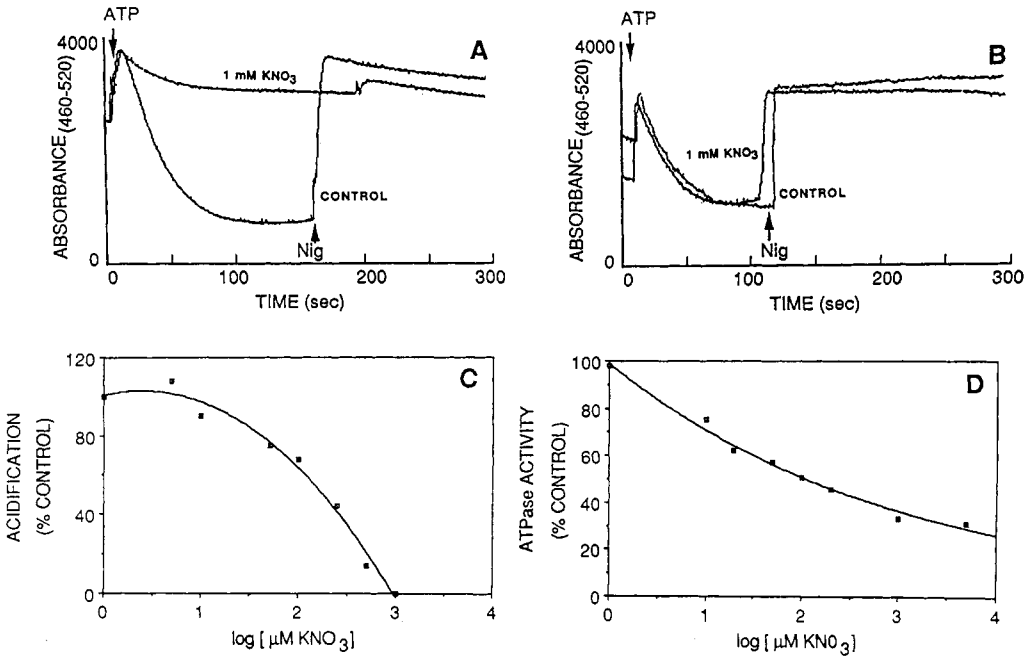

FIGURE 1: Low concentrations of KNO_3 inhibit H^+ transport by osteoclast membrane vesicles and NEM-and-vanadate-sensitive H^+-ATPase activity of the purified enzyme. (A and B) Actual tracings showing the effects of 1 mM KNO_3 on H^+ transport by osteoclast membrane vesicles and kidney microsomes, respectively. Purified membrane vesicles (40 and 120 μg of protein/2 mL of acidification buffer for osteoclast and kidney, respectively) were loaded with acridine orange (1.4 μM) in the presence of 0.5 μM valinomycin and incubated with or without (control) 1 mM KNO_3 for 10 min before addition of Mg^{2+}-ATP. The drop in the fluorescence intensity (λ_{ex} = 460 nm and λ_{em} = 520 nm) was monitored by a Hitachi 2000 spectrofluorometer. The proton gradient was disrupted after 2 min by addition of 1 μM nigericine. (C) Dose-dependent inhibition of the acidification of osteoclast membrane vesicles by KNO_3. Acidifications were carried out as described above after preincubation of the membrane vesicles in the presence of indicated doses of KNO_3. (D) Dose-dependent inhibition of NEM-and-vanadate-sensitive H^+-ATPase activity of the affinity-purified enzyme from osteoclast. Enzymes were preincubated in the presence or absence of different concentrations of KNO_3 and enzyme activity was measured as described in Materials and Methods.

this proton pump is 20–100-fold more sensitive to nitrate than any previously reported animal and fungal V-ATPases and 10–20 times more sensitive than plant V-ATPases. As reported for the kidney enzyme (Wang & Gluck, 1990), and in contrast to the *N. crassa* (Bowman et al., 1988), chromaffin granule (Moriyama & Nelson, 1989), Golgi, and coated vesicle V-ATPases (Forgac, 1989), this inhibition is not due to a chaotropic dissociation of the catalytic domain of the enzyme (V_1) from the transmembrane elements (V_0).

MATERIALS AND METHODS

Osteoclast-Derived, Kidney, and Chromaffin Granule Membrane Vesicle Preparation. Osteoclasts were isolated from laying hens which were kept for 14 days under a calcium-deficient diet as described before (Chatterjee et al., 1992). Purified membrane vesicles were isolated by differential centrifugation followed by a Percoll gradient and characterized by assaying organelle marker enzymes (Chatterjee et al., 1992). Briefly, osteoclasts are routinely purified approximately 400-fold over the starting cell population (>90% purity in terms of membranes), and the microsomal fraction is further enriched approximately 10-fold in plasma membrane markers, with little contamination from ER, Golgi, mitochondrial, and lysosomal markers (Chatterjee et al., 1992). For comparison, kidney vesicles and chromaffin granule membrane vesicles were prepared according to Wang and Gluck (1990) and Cidon and Nelson (1986), respectively. Electron microscopy of the membrane vesicles was performed according to Bowman et al. (1988) and as described (Chatterjee et al., 1992).

Acidification Assay. The acidification assays were carried out with 40 μg of membrane proteins in 2 mL of acidification buffer (150 mM KCl, 5 mM $MgSO_4$, and 20 mM HEPES, pH 7.4, adjusted with tetramethylammonium hydroxide) according to Fuchs et al. (1989).

Immunoblot Analysis. To see the effects of NO_3 on subunit dissociation, 500 μg of membrane vesicle proteins from osteoclast and chromaffin granules was incubated in the presence of various concentrations of KNO_3 in the presence of 5 mM ATP and 5 mM $MgSO_4$ for 1 h in a final volume of 500 μL of acidification buffer at 0 °C. The supernatants were collected by centrifugation at 100000*g* for 30 min, and the proteins in the supernatant were analyzed by SDS–PAGE (Laemmli, 1970) and used for immunoblot analysis with antibodies against the 70-kDa subunits of the *N. crassa* vacuolar proton pump (Bowman et al., 1989) (for osteoclast membranes) and chromaffin granule vacuolar pump (Wang et al., 1988) (for chromaffin granule membranes).

Affinity Purification of the OC H^+-ATPase. The IgG fraction from the immune serum against the 70-kDa subunit of *N. crassa* vacuolar pump (kindly provided to us by B. Bowman and W. Dschida) was purified and coupled with CNBr-activated Affi-Gel 10 (according to Bio-Rad). The microsomal fraction was solubilized in $C_{12}E_9$ (1% w/v) and passed through the column. The column was washed thoroughly with binding buffer (Bio-Rad) and eluted with elution buffer (Bio-Rad). The protein so obtained was neutralized with 1 M HEPES buffer, pH 9.0.

Assay of NEM-Sensitive ATPase Activity of the Purified H^+-ATPase. The purified osteoclast H^+-ATPase (10 μL, 1 μg of protein/assay) was incubated at room temperature for 10 min with 4 μL of phosphatidylserine (0.5 μg/mL in 0.1% $C_{12}E_9$). Samples were diluted with H_2O up to 50 μL in the presence or absence of 10 mM NEM or KNO_3 at different concentrations. The reaction was started with 50 μL of substrate solution [10 mM $MgCl_2$, 80 mM histidine, 1 mM ouabain, 3 mM sodium azide, 5 mM EDTA, and 10 mM [^{32}P]ATP (0.5 mCi/10 mL)]. After a 30-min incubation at 37 °C, the reaction was stopped with 0.75 mL of perchloric

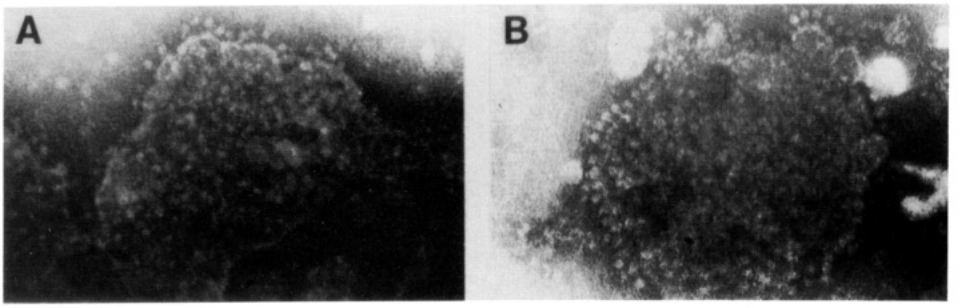

FIGURE 2: Electron micrographs of negatively stained osteoclast membrane vesicles. Osteoclast membrane vesicles (0.8 mg of protein/mL in acidification buffer containing 5 mM ATP and 5 mM $MgSO_4$) were incubated for 1 h in the absence (A) or in the presence (B) of 100 mM KNO_3. The membranes were then applied to Formvar-coated grids. After 1 min, the solution was drawn off; the membranes remaining attached to the Formvar film were negatively stained for 5–15 s with 1% phosphotungstate, pH 6.5. Specimens were examined on a JEOL CX-100 electron microscope at a magnification of 58000×. There were no differences in the results whether the incubation was carried out at 0 °C or at room temperature.

acid, reacted with 0.25 mL of a 5% ammonium molybdate solution and extracted with 2 mL of 2-isobutanol + toluene (1:1) by vortexing for 15 s. The phases were allowed to separate and 100 μL of the organic phase was counted.

RESULTS

As reported previously (Chatterjee et al., 1992), acidification of the osteoclast microsomes was sensitive to NEM and Bafilomycin A1 but also to vanadate at concentrations of 1 mM or lower, confirming the unique pharmacological properties of the OC H^+-ATPase (data not shown). When the acidification assay was performed in the presence of nitrate, proton transport in osteoclast-derived microsomes was found to be highly sensitive to this oxyanion (Figure 1A). In contrast, the same concentrations of NO_3 did not affect acidification of microsomes prepared from chicken kidney (Figure 1B). Although concentrations higher than 70 mM are required for inhibiting kidney or organelle V-ATPases (Bowman et al., 1988; Wang & Gluck, 1990; Ried et al., 1992), 1 mM NO_3 was sufficient to block 100% of acidification in osteoclast membranes, with an IC_{50} of 100 μM (Figure 1C).

In order to determine whether NO_3 inhibited H^+ transport by directly affecting the H^+-ATPase or by indirectly affecting other components of the membrane preparations, the osteoclast H^+-ATPase was affinity purified and the NEM-sensitive ATPase activity was measured in the presence of various concentrations of nitrate. As shown in Figure 1D, 1 mM NO_3 inhibited not only H^+-transport but also up to 70% of the H^+ ATPase activity, thereby demonstrating that the enzyme is directly inhibited by these low concentrations of the oxyanion.

Since it has been reported that high concentrations (140 mM) of nitrate can dissociate the catalytic portion of the V-ATPase (V_1) from the proton channel (V_0) in *N. crassa*, we then performed electron microscopy and immunoblots on osteoclast microsomes incubated in the presence of various concentrations of nitrate. When observed in EM after negative staining, numerous ball-and-stalk structures were observed (Figure 2A), as previously reported in these preparations (Chatterjee et al., 1992), in kidney membrane vesicles (Brown et al., 1987) or in *N. crassa* vacuolar membranes (Bowman et al., 1988). However, and in contrast with *N. crassa* and chromaffin granule V-ATPases, no dissociation was observed between the V_1 domain and the V_0 domain in the membranes of the microsomal vesicles after treatment with up to 150 mM KNO_3 (Figure 2B), a concentration of nitrate that is 150-fold higher than what is required for complete inhibition of H^+ transport of H^+-ATPase activity in these preparations.

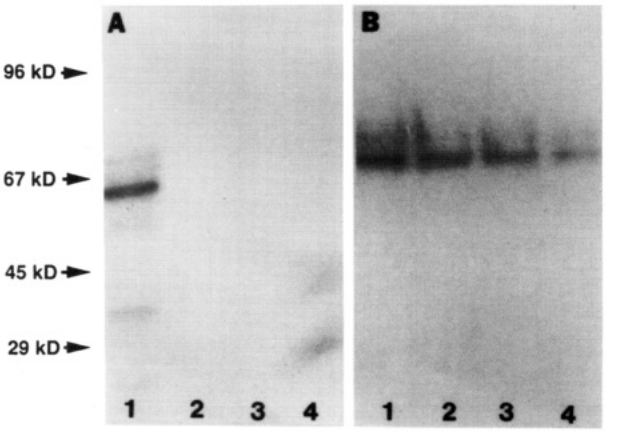

FIGURE 3: Analysis of the release of 70-kDa and 63-kDa subunits of the osteoclast H^+-ATPase by KNO_3. The membrane fractions (500 μg of protein) from osteoclast (A) and chromaffin granules (B) were incubated in the presence of 50, 100, and 150 mM KNO_3 for 1 h at 0 °C in a final volume of 500 μL of acidification buffer in the presence of 5 mM ATP and 5 mM $MgSO_4$. After centrifugation at 100000*g* for 30 min, the supernatants were subjected to SDS–PAGE (100 μL/lane) (Laemmli, 1970), transferred to nitrocellulose, and immunoblotted with the antibody against the 70-kDa subunit of *N. crassa* vacuolar pump for osteoclast membranes (A) and anti-72-kDa subunit of the chromaffin granule vacuolar pump for chromaffin granule membranes (B). Lane 1 represents the microsomal fraction (80 μg of protein): the antibody recognizes a 63-kDa band in osteoclast membranes and a 72-kDa band in the case of chromaffin granule membrane. Lanes 2–4 were the supernatants from the membrane vesicles incubated in the presence of 150, 100, and 50 mM KNO_3, respectively. No release of the 63-kDa catalytic subunit from osteoclast membranes was detected even in presence of 150 mM KNO_3; in contrast, a concentration-dependent release of the 72-kDa subunit was observed in chromaffin granule membranes in the presence of nitrate.

Similarly, no detectable release of the A subunit(s) was observed by western blot of the supernatants from osteoclast-derived vesicles (Figure 3A) after incubation in the presence of 5 mM ATP with increasing concentrations of NO_3 up to 50 mM, further confirming the results obtained by electron microscopy. In contrast, A subunits were released from chromaffin granule-derived vesicles under the same experimental conditions (Figure 3B). Taken together, these results demonstrate that, although the OC H^+-ATPase is highly sensitive to nitrate, the inhibition of proton transport is not due to a chaotropic effect of the oxyanion.

Since other oxyanions have been found to be inhibitory for H^+ transport in V-ATPases, we then determined the sensitivity of the OC H^+-ATPase to various anions and cations. As shown in Table I, the OC H^+-ATPase sensitivity to anions and cations was overall similar to that of other V-ATPases.

Table I: Effects of Different Ions on Acidification of Osteoclast Microsomes[a]

salt (150 mM)	acidification (% of control) with valinomycin	without valinomycin
A. Monovalent, Divalent, and Trivalent Anions		
K^+ salts		
Cl	100	83 ± 4
Br	79 ± 3	70 ± 6
NO_3	0	0
PO_4	82 ± 2	20 ± 2
acetate	77 ± 2	28 ± 6.0
SO_4	75 ± 3	21 ± 6.0
Na^+ salts		
Cl	90 ± 6	70 ± 4
Br	74 ± 5	68 ± 6
NO_3	0	0
PO_4	30 ± 5	31 ± 4
acetate	23 ± 4	22 ± 6
SO_4	26 ± 2	20 ± 4
F	0	0
Cs^+ salts		
Cl	80 ± 3	78 ± 6
choline salt		
Cl	60 ± 4	62 ± 6
Br	46 ± 3	42 ± 8
B. Mixtures of Cl (75 mM) and Nonsupporting Anions (75 mM)		
acetate + Cl	76 ± 4	70 ± 6
F + Cl	75 ± 6	70 ± 4
SO_4 + Cl	80 ± 5	77 ± 5
PO_4 + Cl	80 ± 3	76 ± 5
C. Nonionic Solute		
sucrose	16 ± 4	14 ± 6

[a] The acidification assays were performed as described in Figure 1, but KCl was substituted in the acidification buffer by various anions and cations in the presence and absence of valinomycin and at a constant concentration of 150 mM. Maximum acidification was obtained with KCl in the presence of valinomycin, and all other values are calculated as a percentage of maximum acidification. In the absence of valinomycin, Cl^- could give maximum support of acidification in the form of K^+ salt. Br^-, acetate, and sulfate in the form of K^+ salt could significantly support the acidification in the presence of valinomycin. Na^+ salts of Cl^- or Br^- could also support the acidification whereas the Na^+ salts of acetate or sulfate were found to be ineffective. CsCl or choline chloride could also support the acidification. Nitrates, in the form of either K^+ or Na^+ salt, were completely ineffective in supporting the acidification.

The proton pump in osteoclast membranes was electrogenic, requiring either valinomycin and K^+ or Cl^- outside; bromide could effectively substitute for Cl^- to support H^+ transport; monovalent cations (K^+, Na^+, Cs^+, or choline) were not inhibitory. However, and in contrast to other V-ATPases, the oxyanions SO_4^{2-}, PO_4^{2-}, and acetate were not found to inhibit H^+ transport. All three oxyanions could support H^+ transport in the presence of valinomycin or Cl^-.

DISCUSSION

We have previously reported that the proton pump present in osteoclast membranes can be distinguished from other vacuolar H^+-ATPases by its sensitivity to vanadate and by the presence of different isoforms of subunits A and B which, together, form the catalytic portion of the enzyme (Chatterjee et al., 1992). We report here that the OC H^+-ATPase differs also very dramatically from other vacuolar H^+-ATPases in its sensitivity to various oxyanions. First, concentrations of nitrate approximately 20–100-fold lower than those required to inhibit other pumps, including the kidney H^+-ATPase, block both H^+ transport and, for the most part, the ATPase activity in osteoclast-derived microsomes. Unlike the chromaffin granule, coated vesicle, Golgi, and fungal V-ATPases (Bowman et al., 1988; Forgac, 1989; Moriyama & Nelson, 1989; Arai et al., 1988; Ried et al., 1992), but similar in this respect to the kidney ATPase (Wang & Gluck, 1990), inhibition by nitrate did not induce a dissociation between the catalytic and transmembrane domains of the pump. Furthermore, and differing again from other V-ATPases, the OC H^+-ATPase could function quite efficiently in the presence of other oxyanions (SO_4^{2-}, PO_4^{2-}, acetate) in the acidification buffer (in the presence of Cl^- or valinomycin/K^+), while others cannot (Moriyama & Nelson, 1987a; Wang & Gluck, 1990; Arai et al., 1988). These results further demonstrate that, at least in chicken, the proton pump that is present in osteoclast membranes, although of an electrogenic vacuolar type, is different from the other known V-ATPases, whether from kidney or from other cells or organelles.

As hypothesized by Wang and Gluck (1990) for the kidney H^+-ATPase and by Moriyama and Nelson for chromaffin granules (1987b) and as suggested by our electron microscopy and western blot analysis of nitrate-treated microsomes, inhibition by NO_3 could involve mechanisms other than a purely physical chaotropic effect. Although the structures observed by electron microscopy of osteoclast-derived membrane vesicles were similar to that of the F_o–F_1 mitochondrial ATPase, several observations rule out the possibility that these were contaminating mitochondrial membranes. First, our membrane preparations have negligible amounts of mitochondrial markers (Chatterjee et al., 1992), whereas 30–40% of the vesicles in our membrane preparation contained ball-and-stalk structures. Second, the conditions used for cell disruption should not be sufficiently stringent to generate vesicles from mitochondria. Third, the characteristics of H^+ transport measured in our acidification assay in the same preparations are not of the F_o–F_1 mitochondrial type. Finally, our western blot studies should have independently identified the subunits of the V_1 component of the ATPase if they had been chaotropically released in the supernatants. It can, therefore, safely be concluded that although the OC H^+-ATPase is highly sensitive to nitrate, the mechanisms by which nitrate inhibits this pump are not via the dissociation of the catalytic domain of the enzyme (V_1) from the transmembrane proton channel (V_0).

In the absence of a chaotropic effect, and as suggested for the kidney and chromaffin granules V-ATPases, it is tempting to speculate that direct binding of the oxyanion to specific sites on one or more subunits of the osteoclast H^+-ATPase is responsible for its inhibition (Wang & Gluck, 1990; Moriyama & Nelson, 1987b). These sites would however most likely differ between the osteoclast and the kidney pumps, at least in their affinity for nitrate, since 1 mM nitrate is sufficient to block the osteoclast proton pump when a concentration of 70 mM is required to stop the kidney ATPase (Wang & Gluck, 1990) and 20–30 mM for chromaffin granule proton pump (Moriyama & Nelson, 1987a). Wang and Sze (1985) reported that the acidification of oat root tonoplast was also highly sensitive to NO_3. The IC_{50} for KNO_3 on vesicle acidification by oat root tonoplast proton pump was however about 1–2 mM, and 10 mM KNO_3 was required to block 90% of the acidification as compared with 100 μM and 1 mM, respectively, for osteoclast membranes. Both the IC_{50} value and concentration of KNO_3 required for complete inhibition of the oat root tonoplast acidification is therefore about 10–20-fold higher than that required for the osteoclast membrane vesicles. Despite this difference in the concentration of NO_3 required to inhibit the OC and the other V-ATPases, the OC H^+-ATPase showed the same discrepancy between the effects of

nitrate on H^+ transport and on the ATPase activity, as reported in other systems (Moriyama & Nelson, 1987a). It is possible that nitrate interferes both with the catalytic activity of the enzyme, which can be inhibited only by 70%, and with ion transport itself, which can be entirely blocked, albeit at different concentrations.

In addition to further emphasizing the differences between the osteoclast proton pump and the other V-ATPases, this marked difference in sensitivity between V-ATPases could have important functional implications. Macrophages, a cell type developmentally closely related to the osteoclast (Udagawa et al., 1990; Suda et al., 1992), can generate concentrations of intracellular nitrate up to 17 mM (Marletta et al., 1988) that, as shown here, would be sufficient to block the osteoclast proton pump. Thus, if osteoclasts can generate nitrate, and even in much lower amounts (the IC_{50} being 100 μM), this would provide a means for regulating the activity of the H^+-ATPase within a range where other V-ATPases in the same cell, in the endocytic or secretory pathways for instance, would remain unaffected. Hence, sensitivity to nitrate further distinguishes the vanadate-sensitive osteoclast H^+-ATPase from other vacuolar proton pumps. This might be a process by which proton pumps of the OC-type may be specifically regulated under physiological conditions.

In conclusion, these observations further establish the fact that the vacuolar H^+-ATPases that are present in different cells or organelles differ in their properties and sensitivities to various ions and inhibitors. The OC H^+-ATPase, already unique in its sensitivity to vanadate and in the subunit composition of its catalytic domain (Chatterjee et al., 1992), is also distinguishable by its sensitivity to oxyanions and, in particular, to nitrate, an anion that can be generated inside cells under physiological conditions. These differences between the osteoclast proton pump and that of other cells may allow their differential regulation in the cell itself, potentially permitting the design of new methods to specifically inhibit bone resorption in disease situations where it is increased.

ACKNOWLEDGMENT

D.C. was the recipient of a Swebilius Cancer Research Award from the Yale Comprehensive Cancer Center. We are very grateful to Drs. N. Nelson and B. Bowman for providing us with antibodies to subunit A and for their very helpful advice.

REFERENCES

Arai, H., Berne, M., Terres, G., Terres, H., Puopolo, K., & Forgac, M. (1987) *Biochemistry 26*, 6632.
Arai, H., Terres, G., Pink, S., & Forgac, M. (1988) *J. Biol. Chem. 263*, 8796.
Baron, R., Neff, L., Louvard, D., & Courtoy, P. J. (1985) *J. Cell Biol. 101*, 2210.
Bekker, P. J., & Gay, C. V. (1990) *J. Bone Min. Res. 5*, 569.
Blair, H. C., Teitelbaum, S. L., Ghiselli, R., & Gluck, S. (1989) *Science 245*, 855.
Bowman, B. J., Allen, R., Wechser, M. A., & Bowman, E. J. (1988) *J. Biol. Chem. 263*, 14002.
Bowman, B. J., Dschida, W. J., Harris, T., & Bowman, E. J. (1989) *J. Biol. Chem. 264*, 15606.
Brown, D., Gluck, S., & Hartwig, J. (1987) *J. Cell Biol. 105*, 1637.
Chatterjee, D., Chakraborty, M., Leit, M., Jamsa-Kellokumpu, S., Fuchs, R., & Baron, R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6257.
Cidon, S., & Nelson, N. (1983) *J. Biol. Chem. 258*, 2892.
Cidon, S., & Nelson, N. (1986) *J. Biol. Chem. 261*, 9222.
Forgac, M. (1989) *Physiol Rev. 69*, 765.
Fuchs, R., Male, P., & Mellman, I. (1989) *J. Biol. Chem. 264*, 2212.
Gluck, S., & Caldwell, J. (1987) *J. Biol. Chem. 262*, 15780.
Gluck, S., & Caldwell, J. (1988) *Am. J. Physiol. 254*, F71.
Gluck, S., Kelly, S., & Al-Awqati, Q. (1982) *J. Biol. Chem. 257*, 9230.
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Marletta, M. A., Yoon, P. S., Iyengar, R., Leaf, C. D., & Wishnok, J. S. (1988) *Biochemistry 27*, 8706.
Mellman, I., Fuchs, R., & Helenius, A. (1986) *Annu. Rev. Biochem. 55*, 663.
Moriyama, Y., & Nelson, N. (1987a) *J. Biol. Chem. 262*, 9175.
Moriyama, Y., & Nelson, (1987b) *Biochem. Biophys. Res. Commun. 149*, 140.
Moriyama, Y., & Nelson, N. (1989) *J. Biol. Chem. 264*, 3577.
Moriyama, Y., & Futai, M. (1990) *J. Biol. Chem. 265*, 9165.
Nelson, H., Mandiyan, S., & Nelson, N. (1989) *J. Biol. Chem. 264*, 1775.
Nelson, N. (1991) *Trends Pharmacol. Sci. 12*, 71.
Nelson, N., & Taiz, L. (1989) *Trends Biochem. Sci.*, 113.
Puopolo, K., Kumamoto, C., Adachi, I., Magner, R., & Forgac, M. (1992) *J. Biol. Chem. 267*, 3696.
Ried, T., Baldini, A., Rand, T. C., & Ward, D. C. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 1388.
Suda, T., Takahashi, N., & Martin, T. J. (1992) *Endocr. Rev. 13*, 66.
Sudhof, T. C., Fried, V. A., Stone, D. K., Johnston, P. A., & Xie, X.-S. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6067.
Swallow, C. J., Grinstein, S., & Rotstein, O. D. (1990) *J. Biol. Chem. 265*, 7645.
Udagawa, N., Takahashi, N., Akatsu, T., Tanaka, H., Sasaki, T., Nishihara, T., Koga, T., Martin, T. J., & Suda, T. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7260.
Wang, S. Y., Moriyama, Y., Mandel, M., Hulmes, J. D., Pan, Y. C. E., Danho, W., Nelson, H., & Nelson, N. (1988) *J. Biol. Chem. 263*, 17638.
Wang, Y., & Sze, H. (1985) *J. Biol. Chem. 260*, 10434.
Wang, Z.-Q., & Gluck, S. (1990) *J. Biol. Chem. 265*, 21957.