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Sodium and Sulfate Ion Transport in Yeast Vacuoles[1]

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The intra-luminal acidic pH of endomembrane organelles is established by a proton pump, vacuolar H^+-ATPase (V-ATPase), in combination with other ion transporter(s). The proton gradient (ΔpH) established in yeast vacuolar vesicles decreased and reached the lower value after the addition of alkaline cations including Na^+. As expected, the uptake of $^{22}Na^+$ was coupled with ΔpH generated by V-ATPase. Disruption of *NHX1* or *NHA1*, encoding known Na^+/H^+ antiporters, did not result in the loss of $^{22}Na^+$ uptake or the alkaline cation–dependent ΔpH decrease. Upon the addition of sulfate ions, the V-ATPase-dependent ΔpH in the vacuolar vesicles increased, but the membrane potential (ΔΨ) decreased. Consistent with this observation, radioactive sulfate was transported into the vesicles with a K_m value of 0.07 mM. The transport activity was unaffected upon disruption of the putative genes coding for homologues of plasma membrane sulfate transporters. These results indicate that the vacuoles exhibit unique Na^+/H^+ antiport and sulfate transport, which regulate the luminal pH and ion homeostasis in yeast.

Key words: acidic organelles, sodium/proton antiport, sulfate transport, V-ATPase, yeast vacuole.

Eukaryotic cells contain a wide variety of organelles with an acidic luminal pH. These organelles include lysosomes, vacuoles, endosomes, the Golgi apparatus, and synaptic vesicles (*1–4*). The acidic pH is required for organelle functions such as hydrolysis of macromolecules, release of ligands from receptors, processing of hormones, and protein sorting. The chemical gradient for protons (ΔpH) or membrane potential (ΔΨ) formed drives the transport of ions, transmitters, *etc.* (*5, 6*). The acidification is mediated by a proton pump, vacuolar H^+-ATPase (V-ATPase), and could be regulated by ion transport systems.

Yeast cells accumulate metabolically important compounds and ions in vacuoles, which are functionally similar organelles to mammalian lysosomes (*7*). The concentrations of ions and amino acids in the vacuoles vary with the growth conditions (*8–10*). We have expressed plant proton-translocating pyrophosphatase in vacuoles containing endogenous V-ATPase (*11*). The isolated chimeric vacuoles can form an electrochemical proton gradient with the addition of ATP or pyrophosphate. Interestingly, they form a similar ΔΨ with the addition of ATP or pyrophosphate, or the simultaneous addition of the two proton pump substrates. On the other hand, the addition of pyrophosphate increases ΔpH formed depending on ATP, and similarly ATP further increases the pyrophosphate-dependent ΔpH. These results showed that yeast vacuoles have a regulatory mechanism that maintains a constant ΔΨ, but that the proton transport by V-ATPase or pyrophosphatase is not strictly regulated by ΔpH (*11*).

In this study, we examined the effects of various ions on ΔpH formed in isolated yeast vacuolar vesicles: alkaline cations (Na^+, K^+, Li^+, or Rb^+) decreased ΔpH, whereas sulfate increased it. Consistent with these observations, radioactive Na^+ and SO_4^{2-} were taken up into the vacuolar vesicles. These activities were not affected by disruption of the genes coding for known Na^+/H^+ antiporters (*12–15*), or the homologues of plasma membrane sulfate transporters (*16, 17*), respectively. The antiport and sulfate transport systems may regulate vacuolar acidification and play important roles in yeast ion homeostasis.

EXPERIMENTAL PROCEDURES

The *Saccharomyces cerevisiae* strains used were: YPH499 (*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*) (*18*), and derivatives of it, TH1 (*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 Δnhx1::LEU2*), and TH2 (*MATα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 Δnha1::HIS3*). TH1 and TH2 are disruption mutants of *NHX1* and *NHA1*, respectively. TH3 (*MATα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 Δygr125::HIS3*) and TH4 (*MATα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 Δypr003::LEU2*), disruption mutants of the homologues of plasma membrane sulfate transporters, were constructed in this study.

Yeast cells were grown aerobically at 30°C in YPDA medium (2% glucose, 1% yeast extract, 2% polypeptone, and 20 μg/ml adenine hemisulfate). For Na^+ transport experiments, cells were harvested at the early logarithmic

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MES, 2-morpholinoethanesulfonic acid; V-ATPase, vacuolar H^+-ATPase; ΔpH, chemical gradient for protons; ΔΨ, membrane potential.

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phase (2×10^7 cells/ml), washed with distilled water, and then transferred to a carbon source-free medium, containing 0.67% yeast nitrogen base with appropriate supplements. Cells were incubated for five hours aerobically at 30°C, and then used for vacuolar vesicle preparation. When indicated, they were grown up to 2×10^7 cells/ml in YPDA medium containing 1 M NaCl or KCl, and used directly for vacuolar vesicle preparation without incubation in the carbon source–free medium. Cells grown in YPDA medium were used directly for sulfate transport studies.

Vacuoles were prepared by the previously described method (*19*) as modified by Takeshige *et al.* (*20*), and their purity was confirmed by the ~30-fold increase of α-mannosidase activity (*21*). They were converted to vesicles by dilution with three volumes of buffer C (10 mM MES-Tris, pH 6.9, containing 5 mM $MgCl_2$ and 5 mM magnesium gluconate for cation and anion transport, respectively), the suspension was centrifuged at 169,000 ×*g* for 60 min, and the precipitated vesicles were suspended in buffer C for storage at –80°C. The formation of ΔpH and ΔΨ was observed by measuring the quinacrine and oxonol V (a membrane potential sensitive dye) fluorescence, respectively (*11*).

For radioactive sodium uptake, vacuolar vesicles (0.2 mg/ml) were suspended in 20 mM MES-Tris, pH 7.0, containing 20 mM ^{22}NaCl (3.7 MBq/m mol), 150 mM choline chloride and 5 mM $MgCl_2$. After 10 min incubation at 25°C, 0.5 mM ATP (Tris-salt) was added. An aliquot (100 μl) was applied to a membrane filter (Millipore HATF, 0.45 μm pore size), which was washed with 5 ml of ice-cold 20 mM MES-Tris, pH 7.0, containing 500 mM sucrose, and then dried for radioactivity measurement with an Aloka ARC-361 γ counter. For the uptake of radioactive sulfate, vacuolar vesicles (0.1 mg/ml) were incubated in 20 mM MES-Tris, pH 7.2, containing 0.5 mM ATP, 50 mM sucrose, and 5 mM magnesium gluconate at 25°C for 1 min. Then, $Na_2{}^{35}SO_4$ (185 MBq/m mol) was added. Samples were filtered and washed with 20 mM MES-Tris, pH 7.2, 50 mM sucrose, and 5 mM magnesium gluconate. Radioactivity was measured with a Beckman LS6500 Liquid Scintillation Counter. Protein concentrations were determined as previously described (*22*).

Reagents were obtained from commercial sources: ^{22}NaCl (37 MBq/ml, 13.2 GBq/mg), NEN Life Science Products; $Na_2{}^{35}SO_4$ (3.7 GBq/m mol), Amersham; bafilomycin A_1, Wako Chemical; and ATP (Tris-salt), FCCP, and CCCP, Sigma. All other chemicals used were of the highest grade commercially available.

RESULTS AND DISCUSSION

Decrease of ΔpH in Vacuolar Vesicles with Alkaline Cations—We showed previously that the vacuolar membrane has a regulatory mechanism that maintains a constant ΔΨ (*11*). This finding prompted us to study vacuolar ion transport systems using purified vesicles.

Upon the addition of Na^+, the ΔpH formed by V-ATPase decreased significantly, as detected as the reversal of quinacrine fluorescence quenching (Fig. 1A). We could obtain vesicles showing a slightly greater response to Na^+ when cells were incubated in the medium lacking a carbon source (Fig. 1B). Thus, the transport system was further characterized using these vesicles. This observation is consistent with the presence of a Na^+-dependent proton efflux system in the vesicles. We found that vacuolar vesicles obtained from cells grown with high salt showed increased reversal of quinacrine fluorescence quenching with Na^+ or K^+ (Fig. 1, C and D). The K_m for Na^+ was difficult to determine because it is relatively high (> 50 mM) (data not shown).

The quenching was also reversed with K^+, Li^+ and Rb^+, whereas choline$^+$ had no effect. However, it was difficult to conclude that the proton efflux caused by these cations was carried out by the same system as that for Na^+. As shown in Fig. 1, the ratio of the extent and initial rate of the reversal by K^+ and Na^+ varied with the growth conditions.

$^{22}Na^+$ Uptake into Vacuolar Vesicles through a Na^+/H^+ Antiport System—If the decrease in ΔpH with Na^+ is due to proton efflux coupled with Na^+ influx through Na^+/H^+ antiport, vacuolar vesicles should show Na^+ uptake that is sensitive to dissipation of ΔpH. As expected, ATP-dependent $^{22}Na^+$ uptake was observed with such vesicles (Fig. 2). The $^{22}Na^+$ concentration inside was estimated to be 170 mM, assuming that the vesicle volume was 5.2 μl/mg (*23*), indicating that Na^+ is concentrated against an electrochemical gradient for Na^+. The $^{22}Na^+$ uptake was inhibited by bafilomycin A_1, a specific V-ATPase inhibitor. Furthermore, the uptake was inhibited by CCCP, and $^{22}Na^+$ that accumulated in the vesicles was rapidly released on the addition of the same uncoupler. These results indicate that $^{22}Na^+$ uptake is coupled with ΔpH generated by V-ATPase, and that vacuole membranes possess a Na^+/H^+ antiport system.

Effects of Antiporter Mutations on Na^+/H^+ Antiport—It was of interest to determine whether or not the antiporters identified previously were responsible for vacuolar antiport activity. Nhx1p was identified as a Na^+/H^+ antiporter in the

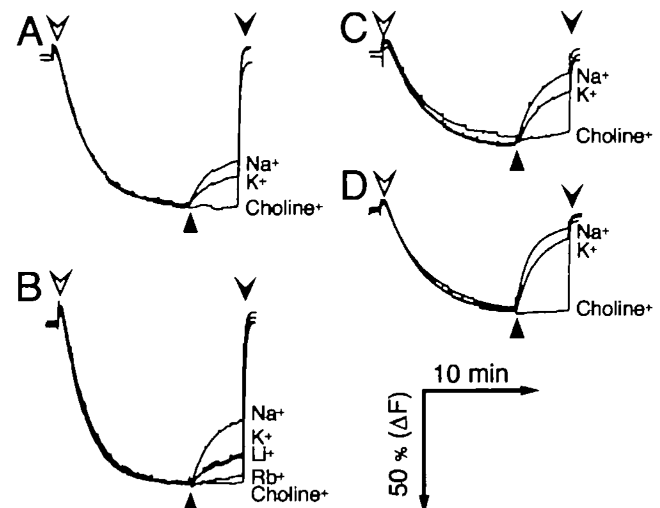

Fig. 1. **Effects of cations on ΔpH formed with V-ATPase.** Various vacuolar vesicles (15 μg protein) isolated from strain YPH499 were examined for ΔpH formation by quinacrine fluorescence quenching. A, vesicles isolated from the yeast growing in YPDA medium; B, vesicles from the same strain after incubation in the carbon source-free medium; C and D, the same strain grown with 1 M NaCl (C) or 1 M KCl (D). The quenching was assayed at 25°C in 1 ml of 20 mM MES-Tris, pH 6.8, containing 150 mM choline chloride, 5 mM $MgCl_2$, and 5 μM quinacrine dihydrochloride. ATP (0.5 mM Tris-salt, open arrowheads), chloride salts of various cations (40 mM, closed triangles), and 5 mM NH_4Cl (closed arrowheads) were added at the times indicated.

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prevacuolar compartment (*12–14*). This antiporter was suggested to play important roles in cation detoxification and osomoregulation in yeast cells. We can not exclude the possibility that Nhx1p is localized to discrete patches on the vacuoles, as suggested by Nass and Rao (*13*). However, the disruption of *NHX1* did not result in the loss of vacuolar Na^+-dependent quinacrine fluorescence reversal or $^{22}Na^+$ uptake (Table I). Previous workers found the presence of *NHX1*-dependent Na^+/H^+ antiport in isolated vacuoles (*24*). The Na^+ uptake rate estimated from the atomic absorption was at least 1,000-fold faster than the rate obtained in our study, and 300-fold higher than the V_{max} rate of V-ATPase in vacuoles (*23*). These results are inconsistent with their suggestion that Nhx1p mediates electro-neutral Na^+/H^+ antiport. Furthermore, they did not show that the uptake was coupled with ΔpH generated by V-ATPase. These findings indicating that Na^+ uptake shown by previous worker is not dependent on ΔpH generated by V-ATPase on vacuolar membrane. The apparent discrepancy between our and previous workers' observations may be due to the presence of other organelles in the vacuolar preparation.

NHA1 encodes the plasma membrane antiporter that mediates Na^+ and K^+ efflux from cells (*15*). Consistent with the role of *NHA1*, disruption of this gene also did not significantly affect the vacuolar Na^+/H^+ antiport activity (Table I). These results suggest that vacuoles have a unique Na^+/H^+ antiport system(s) not coded for by *NHX1* and *NHA1*. It should be noted that no homologues of Nhx1p and Nha1p have been found in the yeast genome (*16*), indicating that the vacuolar antiporter has a previously unknown primary structure.

Effects of Sulfate on ΔpH and ΔΨ in Vacuolar Vesicles—The addition of chloride increased the formation of ΔpH in vacuolar vesicles and decreased ΔΨ (Fig. 3A), confirming previous results that suggested the presence of a chloride channel (or transporter) in vacuoles (*25*). It was of interest to examine other anions for a similar effect. Of those tested, sulfate increased the ATP-dependent formation of ΔpH ~five-fold, and significantly decreased ΔΨ (Fig. 3B). These results suggest that sulfate was taken up into the lumen of the vesicles, where it decreased ΔΨ and thus increased ΔpH. Although sulfate was added as a sodium salt (0.5 mM Na_2SO_4), the sodium ion concentration was lower than the value for significant Na^+/H^+ antiporter activity (Fig. 1). Other anions (5–10 mM) including phosphate and bicarbonate had essentially no effects on ΔpH or ΔΨ formed in vacuolar vesicles (data not shown). Nitrate (5 mM) increased ΔpH similar to sulfate, confirming previous results (*25*).

Properties of the Sulfate Uptake System—Consistent with its effects on ΔpH and ΔΨ, radioactive sulfate was transported into vesicles (Fig. 4A). The uptake was sensitive to bafilomycin A_1, CCCP, and FCCP, and the sulfate that accumulated inside the vesicles was released upon the addition of the uncouplers. However, NH_4^+ or nigericin had no effect on the uptake, indicating that sulfate transport was driven by ΔΨ, but not by ΔpH (Fig. 4B).

The uptake showed K_m and V_{max} values of 70 μM and 50 n mol/mg protein·min, respectively. The sulfate concentration in vacuolar vesicles under the V_{max} conditions was estimated to be 33 mM (outside SO_4^{2-}, 0.5 mM), indicating that a ~70-fold concentration gradient was established. These results indicate that the transport system is different from those found in the plasma membrane coded by *SUL1* and

TABLE I. **Sodium transport into wild-type and mutant vacuolar vesicles.**

Source of vacuolar vesicles	Na^+ uptake (nmol/mg·min)	ΔpH change with Na^+ (% ΔF/min)
YPH499 (wild-type)	61	15
TH1 (Δ*nhx1*)	56	13
TH2 (Δ*nha1*)	48	10

Sodium uptake by wild-type or disruption mutant vesicles (0.2 mg protein/ml) was assayed with radioactive Na^+, the initial rates being shown. See Fig. 2 and text for the assay conditions. ATP-dependent formation of ΔpH was assayed as quinacrine fluorescence quenching, and its decrease on Na^+ addition was also followed. The initial rate of the ΔpH change (initial rate of fluorescence quenching reversal) with Na^+ was expressed as relative fluorescence. See Fig. 1 and text for more details.

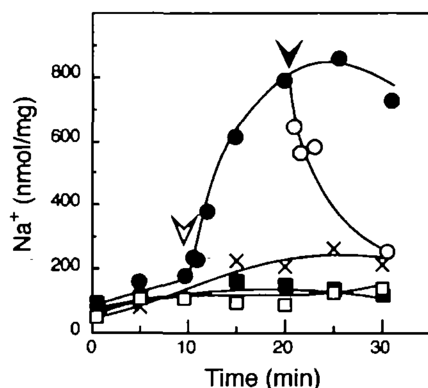

Fig. 2. **Na^+ uptake into vacuolar vesicles coupled with ΔpH generated by V-ATPase.** Vacuolar vesicles (0.2 mg protein/ml) from strain YPH499 were incubated with 20 mM $^{22}NaCl$ for 10 min at 25°C. Assays were started by the addition of 0.5 mM ATP-Tris (open arrowhead) (closed circles, crosses, or closed squares). A control without ATP is also shown (open squares). Na^+ uptake was also assayed in the presence of 25 μM CCCP (closed squares) or 100 nM bafilomycin A_1 (crosses). At the indicated time (closed arrowhead), 25 μM CCCP was added, and the uptake was followed (open circles).

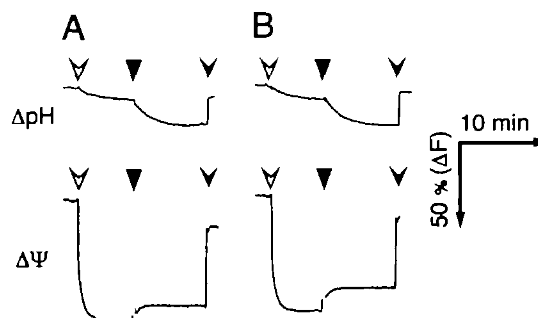

Fig. 3. **Effects of anions on ΔpH and ΔΨ formed in vacuolar vesicles.** Cl^- (A) or SO_4^{2-} (B) was examined as to the steady state ΔpH and ΔΨ formed by V-ATPase. Yeast vacuolar vesicles (15 μg protein) isolated from strain YPH499 were incubated with 5 μM quinacrine dihydrochloride or 0.5 μM oxonol V in 1 ml of 20 mM MES-Tris, pH 7.2, containing 50 mM sucrose and 5 mM magnesium gluconate. 0.5 mM ATP-Tris (open arrowheads), 5 mM NaCl (A, closed triangles), 0.5 mM Na_2SO_4 (B, closed triangles), 5 mM NH_4Cl (ΔpH, closed arrowheads), or 25 μM CCCP (ΔΨ, closed arrowheads) was added at the times indicated.

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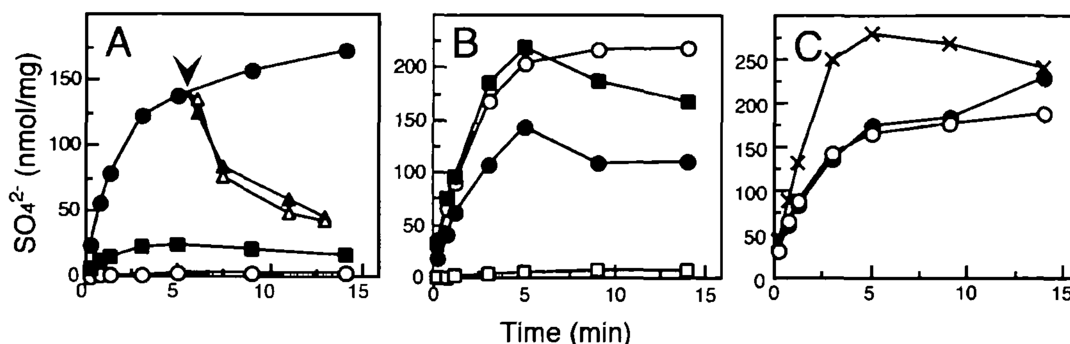

Fig. 4. **Sulfate uptake into vesicles coupled with the electrochemical proton gradient generated by V-ATPase.** A, sulfate uptake dependent on the electrochemical proton gradient generated by V-ATPase. Vacuolar vesicles (0.1 mg protein/ml) from strain YPH499 were incubated with or without 0.5 mM ATP. Uptake was started by the addition of 0.5 mM $Na_2{}^{35}SO_4$ (closed circles). Sulfate uptake was also assayed in the absence of ATP (open circles), or in the presence of 0.5 mM ATP and 200 nM bafilomycin A_1 (closed squares). At the indicated time (arrowhead), 25 μM CCCP (closed triangles) or 2 μM FCCP (open triangles) was added and the uptake was followed. No uptake was observed when CCCP or FCCP was added before the assay (not shown). B, effects of NH_4^+ and ionophores on sulfate uptake into vacuolar vesicles. Sulfate uptake was assayed in the presence of 5 mM NH_4Cl (closed circles), 50 nM nigericin (closed squares), and 25 μM CCCP (open squares). Other conditions were as given in A except that 25 mM potassium gluconate was included instead of sucrose. C, disruption of putative sulfate transporters. Vacuolar vesicles were prepared from TH3 (*Δygr125::HIS3*) (crosses) and TH4 (*Δypr003::LEU2*) (open circles) lacking the genes for putative sulfate transporters, homologous to plasma membrane sulfate transporters, and assayed for uptake. The uptake into control vesicles (YPH499) is also shown (closed circles). Other conditions were as given in A.

SUL2, because the K_m values were 4–10 μM (*26, 27*). The initial rate of vacuolar sulfate uptake was not affected by disruption of *YGR125* and *YPR003*, which code for homologues of the yeast plasma membrane sulfate transporters (Fig. 4C) (*16, 17*), indicating that the uptake is not due to previously identified genes.

Regulation of ΔΨ and Acidic Luminal pH in Yeast Vacuoles—It is obvious that the luminal pH of vacuoles is established by V-ATPase in combination with ion transporters. We found in this study that the yeast vacuoles have Na^+/H^+ antiport and sulfate transport systems. The Na^+/H^+ antiport could lower ΔpH, thus, it could be responsible for the regulatory mechanism. In addition, the sulfate transporter found in this study decreased ΔΨ and increased ΔpH established by V-ATPase, similar to the Cl^- transporter (or channel) (*25*). Thus, the combination of the Na^+/H^+ antiporter and sulfate transporter together with other system(s) established the electrochemical proton gradient and acidic luminal pH of vacuoles. Similar systems may be present in mammalian endomembrane organelles including lysosomes, endosomes and synaptic vesicles. In this regard, the presence of a sulfate transporter in rat liver lysosomes has been suggested (*28*), although transport driven by V-ATPase was not studied.

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