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Comparative studies on the electrical properties of the H^+ translocating ATPase and pyrophosphatase of the vacuolar-lysosomal compartment

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The electrical properties of the vacuolar-lysosomal H^+ pumps were studied by direct measurement of the pump currents using the whole-cell configuration of the patch-clamp technique. Both pumps, the proton-translocating ATPase and pyrophosphatase, when activated by MgATP or inorganic Mg pyrophosphate ($MgPP_i$), transport protons into the vacuole and polarize the membrane potential (positive inside the vacuole). Accumulation of protons in the lumen of vacuole vesicles was monitored by absorbance changes of the pH probe, acridine orange. The electrochemical gradient provided by both the ATPase and pyrophosphatase stimulates effectively the uptake of various metabolites such as malate, citrate and sucrose. The maximal current density produced by the ATPase was ~ 2.5 $\mu A/cm^2$ and ~ 0.5 $\mu A/cm^2$ for the pyrophosphatase. K_m^{ATP} was 0.6 mM; K_m^{PPi} was 15-20 μM with progressive inhibition above 150 μM. At a cytoplasmic pH of 7.5 both enzymes were capable of pumping protons against a 10 000-fold concentration gradient (pH 3.5 inside the vacuole). Proton current produced by the ATPase was blocked reversibly by extravacuolar NO_3^- only.

Key words: Patch-clamp/tracer flux/H^+-ATPase/H^+-PP_iase/pump currents/pH dye

Introduction

The development of ion pumps enables cells to create and maintain ion or metabolite gradients across various cellular membranes. These membrane proteins consume energy from external sources (light) or cell metabolism (ATP, PP_i) to drive the ion transport process. Among the ATP-driven enzymes, electrogenic ion transport in animal cells is mediated by, for example, Na^+/K^+-, Ca^{2+}- or H^+-ATPases (Schuurmans Stekhoven and Bonting, 1981; Al-Awqati, 1986). In plants, proton pumps play a fundamental role in the balance of osmotic pressure and the generation and control of an electrical potential difference across the various membrane systems (for review see Serrano, 1985; Sze, 1985). In the vacuole-lysosome the proton-translocating ATPase and pyrophosphatase generate a pH gradient and potential difference across the vacuolar membrane (for review see Harikumar and Reeves, 1983; Rea and Sanders, 1987; Hedrich and Schroeder, 1989). These gradients in turn can drive the accumulation of other ions and metabolites inside the organelle. Regulation of the direction of metabolite transport across the vacuolar membrane, such as accumulation and release of solutes into and from the vacuole (Gerhardt and Heldt, 1987), implicates changes in the electrical potential difference and membrane permeability.

In our previous reports we described the electrical properties, selectivity, pharmacology and regulation of vacuolar ion channels using patch-clamp techniques (Hedrich *et al.*, 1986, 1988; Coyaud *et al.*, 1987; Hedrich and Neher, 1987). Patch-clamp studies also allow investigation of pump dynamics by direct measurement of the electrogenic current and electrical potential differences (Assmann *et al.*, 1985; Bentrup *et al.*, 1985, 1986; Gadsby *et al.*, 1985; Hedrich *et al.*, 1986, 1988; Lafaire and Schwarz, 1986; Schroeder, 1988; Serrano *et al.*, 1988).

It is now well established that the vacuolar ATPase and PP_iase are different enzymes both catalyzing electrogenic transport of the proton. Identification of protons as charge carriers was achieved by the use of protonophores, pH probes and H^+-pump inhibitors as well as by sequence homologies to other proton pumps (for review see Sze, 1985; Hedrich *et al.*, 1986; Rea and Sanders, 1987; Nelson and Taiz, 1989).

The present study elucidates the electrical and biochemical properties of the two vacuolar H^+ pumps from sugar-beet taproots. Since the proton-translocating phosphatases in the vacuolar membrane are two physically distinct enzymes (Rea and Poole, 1986) we will compare their pump currents, substrate affinities and specificities as well as the nature of nitrate inhibition. The physiological relevance of both enzymes will be demonstrated by MgATP- and $MgPP_i$-dependent accumulation of malate, citrate and sucrose into isolated sugar-beet vacuoles.

Results

A patch-clamp survey of the electrical properties of the vacuolar membrane from a large variety of plant material has demonstrated the presence of an electrogenic H^+-ATPase in higher plant vacuoles (Hedrich *et al.*, 1988). On the other hand, electrogenic proton currents produced by pyrophosphatase activity were described only recently in sugar-beet vacuoles (Hedrich and Kurkdjian, 1988). To elucidate the physiological relevance of the two electrogenic pumps, their biophysical properties were examined using the same experimental system, the sugar-beet vacuole.

Two electrogenic pumps in the vacuolar membrane

After access to the lumen of the vacuoles was gained by patch pipettes the pipette solution equilibrated with the vacuolar sap. Using solutions with symmetric ion composition on both sides of the membrane, steady-state conditions were indicated

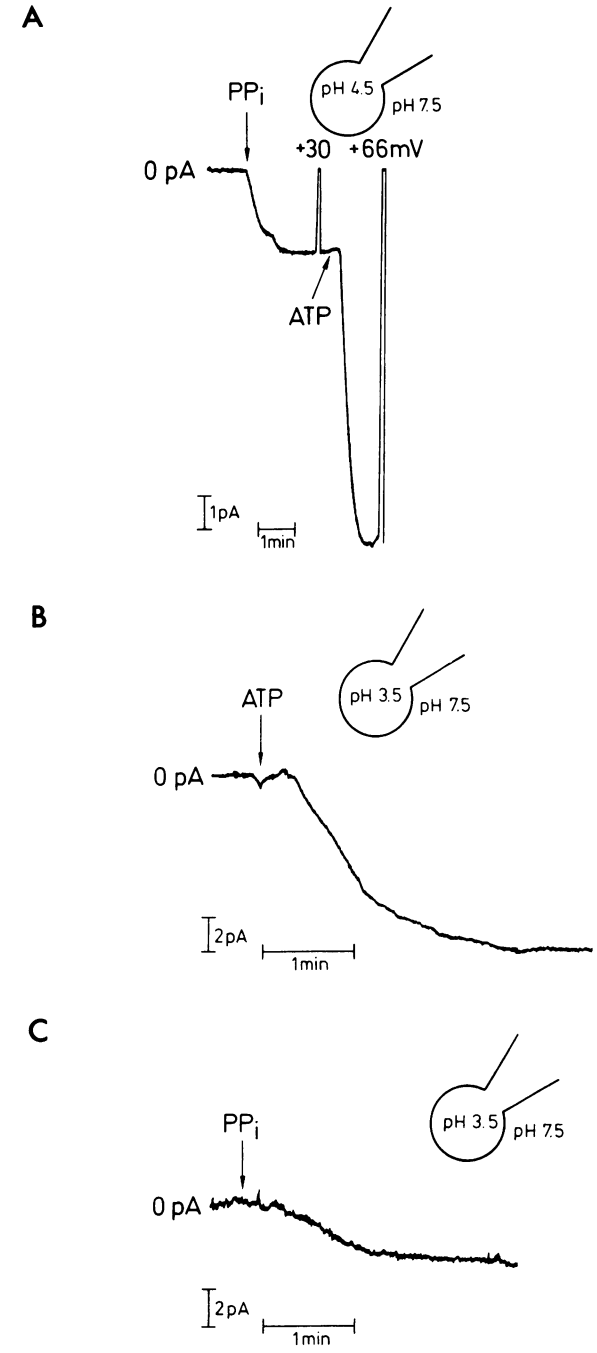

Fig. 1. Proton-translocating phosphorylases on the vacuolar membrane of sugar-beet taproots. (**A**) Voltage-clamp recording of pump currents from a whole vacuole. The membrane potential was clamped to 0 mV. Application of 100 μM pyrophosphate ($MgPP_i$) to the extravacuolar solution generated inward currents of ~2.5 pA which increased to 11.5 pA when 5 mM MgATP was present in addition. Polarization of the transmembrane potential by +30 and +66 mV resulting from pump activities as recorded in the current-clamp mode (measuring the zero-current potential). (**B,C**) $MgPP_i$ and MgATP-driven pumps catalyze uphill transport of the proton. pH gradients were pH 7.5 in the extravacuolar space and 4.5 (A) or 3.5 (B,C) inside the vacuole.

by a resting potential of 0 mV (which was reached within 1–5 min for a 20 pF vacuole). Under voltage-clamp conditions the vacuolar potential was clamped to its resting potential and pulses of MgATP and $MgPP_i$ were applied to the cytoplasmic side of the vacuoles by bath perfusion. The

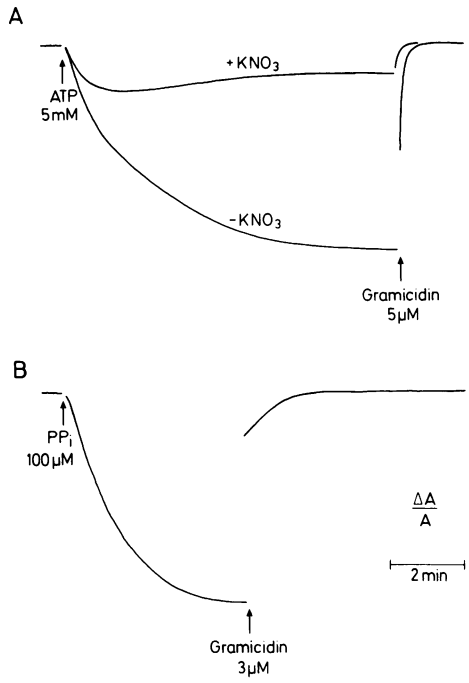

Fig. 2. Time course of MgATP-dependent (**A**) and $MgPP_i$-dependent (**B**) acridine orange absorbance in the presence of vacuole vesicles. At the times indicated by arrows, 5 mM ATP, 150 μM $MgPP_i$ or 3–5 μM Gramicidin was added. Traces shown are representative absorbance changes obtained in at least five different experiments each. Instantaneous change of AO absorbance due to the addition of chemicals has been corrected in the traces.

presence of MgATP as well as $MgPP_i$ elicited inwardly directed pump currents in the entire vacuole (Figure 1). Pump currents generated by an ATPase and by a PP_iase could be induced separately and subsequently on the same vacuole, indicating that both enzymes are located on the same membrane (Figure 1).

In the absence of a pH gradient across the vacuolar membrane the maximal current produced by the PP_iase was ~0.5 $\mu A/cm^2$, which accounts for ~15–25% of that caused by ATPase activity (2.5 $\mu A/cm^2$). This ratio was not significantly altered by the application of pH gradients between the vacuolar space and the extravacuolar medium. Similar observations could be made for pump-induced membrane polarization. Upon the application of saturating concentrations of MgATP to whole vacuoles, rapid polarizations of +30 to +70 mV could be observed, 50–100 μM $MgPP_i$, on the other hand, polarized the membrane potential to +10 to +30 mV.

Electrogenic pumps catalyze uphill transport of protons

The nature of the ion transported into the vacuolar space was determined by absorbance change in the pH dye, acridine orange (AO), using purified vacuole vesicles. Starting from isolated vacuoles (Willenbrink and Doll, 1979), vesicles were further purified by differential and density gradient centrifugation (Rea and Poole, 1986; Pope and Leigh, 1988). A decrease in AO absorbance after the application of MgATP and $MgPP_i$ indicates proton accumulation inside the vacuole vesicles (Figure 2). Proton release from the vesicles could be induced by addition of

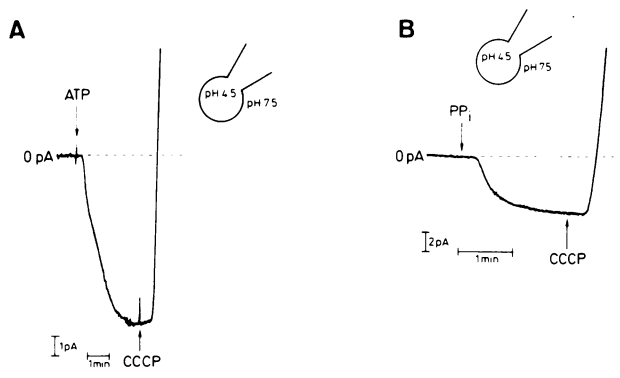

Fig. 3. Effect of the protonophore, CCCP, on MgATP- and $MgPP_i$-induced pump currents and proton conductivity of the vacuolar membrane. (**A**,**B**) In the presence of a pH gradient (pH 7.5/4.5 acidic inside the vacuole) 1 μM CCCP elicited an outward current.

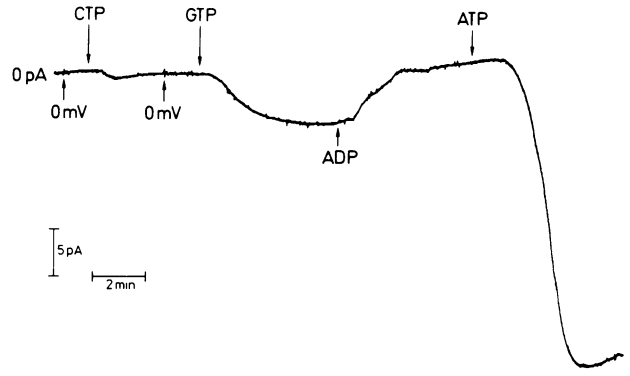

Fig. 5. Nucleotide specificities of the ATP-induced pump currents associated with the vacuolar membrane. Nucleotides were applied successively by bath perfusion as Mg complexes at a final concentration of 1 mM.

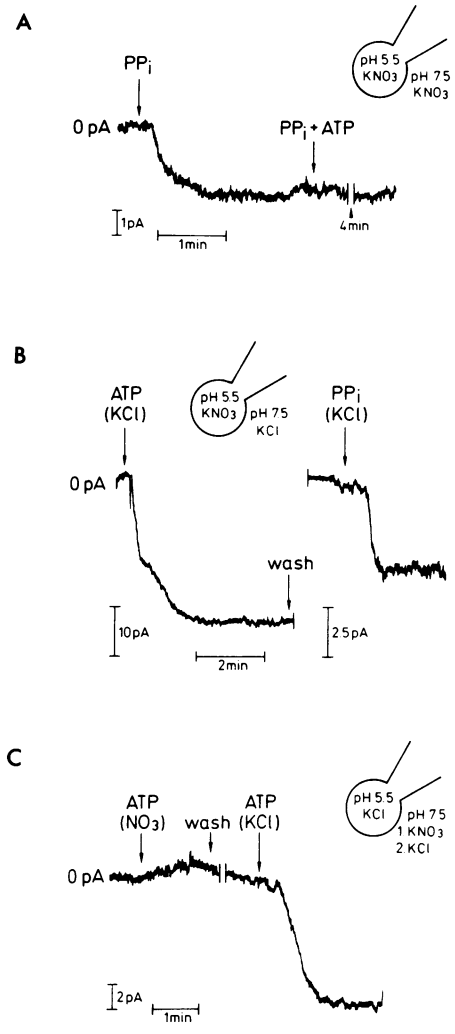

Fig. 4. Effect of nitrate on the ATP- and PP_i-driven proton pumps of the vacuolar membrane. (**A**) PP_i induced currents in the presence of 200 mM KNO_3 on either membrane side. A concentration of 200 mM nitrate totally suppressed ATPase activity (no pump current after addition of MgATP). (**B**) Vacuolar nitrate is neither affecting H^+-translocation by the ATPase nor by the PP_iase, reflecting the location of the nitrate inhibition side on the cytoplasmic face of the H^+-ATPase. (**C**) Recovery of ATPase activity after replacement of nitrate (1) by chloride (2) on the cytoplasmic surface of the enzyme.

micromolar concentrations of the proton-channel forming peptide, Gramicidin (Figure 2) or protonophore, carbonyl-cyanide-dichlorophenylhydrazone (CCCP, not shown here). In the presence of 50 mM KNO_3, MgATP-induced acidification was reduced by ~85% with respect to the KCl control curve (Figure 2A). Further analysis of the nitrate effect on the H^+-ATPase will be presented below.

In patch-clamp records (Figure 1B and C), proton pumping from the cytoplasmic side, pH 7.5, to the vacuolar side, pH 3.5, demonstrates the capability of the H^+-ATPase and H^+-PP_iase of the sugar-beet taproot to pump protons against a 10 000-fold proton gradient.

When pH gradients were imposed across the membrane in the absence of MgATP or $MgPP_i$, the resting potential of the vacuoles stayed at 0 mV, indicating the absence of a measurable proton conductance (Figure 3A,B). However, a proton conductance was induced by extravacuolar MgATP or $MgPP_i$ (pumping H^+ inside the vacuole) or the protonophore, CCCP, mediating H^+ release (outward currents) through a CCCP-induced conductance (at pH 7.5 in the cytoplasm and 4.5 in the vacuole, Figure 3A,B).

Location and nature of the nitrate inhibition on the ATPase

The specific properties of the vacuolar H^+-ATPase such as stimulation by chloride and malate and inhibition by nitrate (Figure 2A) caused this protein to be used widely as a vacuolar marker (Sze, 1985). However, the localization of the nitrate-sensitive side on this enzyme was not cleared up until now.

In pump current records of Figure 4 we demonstrate that nitrate obviously blocks the H^+-ATPase only when present at the cytoplasmic face of the vacuolar membrane. Up to 200 mM NO_3^- inside the vacuole did not block this enzyme (Figure 4B). The H^+-pump activity of the PP_iase, on the other hand, was affected neither by vacuolar nor by cytoplasmic nitrate (Figure 4A,B). During the exchange of NO_3^- for Cl^- at the cytoplasmic face of the vacuole the H^+-pump activity of the ATPase was, at least partially, restored (Figure 4C).

Specificity and substrate affinity

To prove the substrate specificity of the vacuolar ATPase, inwardly directed pump currents were recorded during the

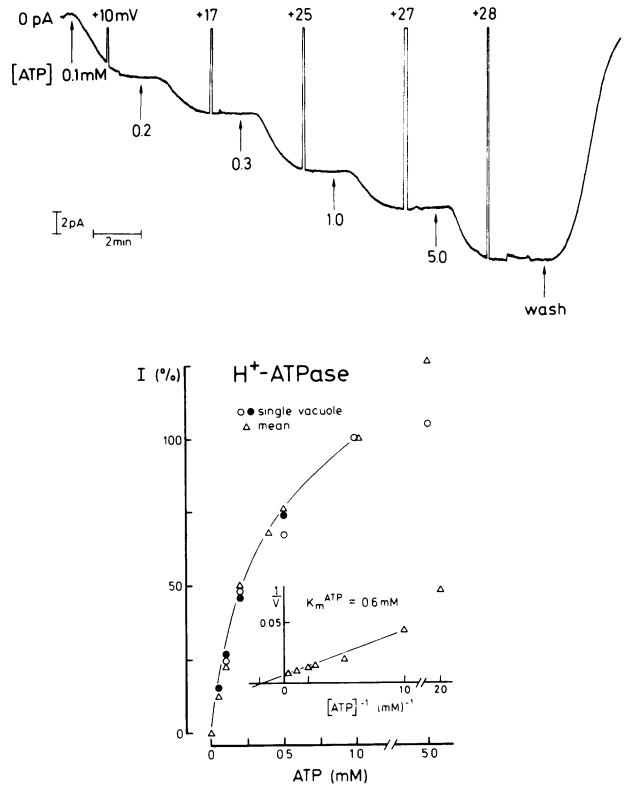

Fig. 6. Substrate affinity of the H^+-translocating ATPase. (**Upper panel**) Stepwise increase of inward pump current upon stepwise increase of millimolar MgATP. Increase in extravacuolar MgATP polarizes the transmembrane potential up to +28 mV (positive inside the vacuole). (**Lower panel**) Michaelis–Menten and Lineweaver–Burk plot of MgATP-induced pump currents. Open and filled circles, results from two experiments where all MgATP concentrations were successively tested on the same vacuole; triangles, results from 36 individual experiments, curves were normalized by setting steady-state currents in the presence of 1 mM ATP to 100%.

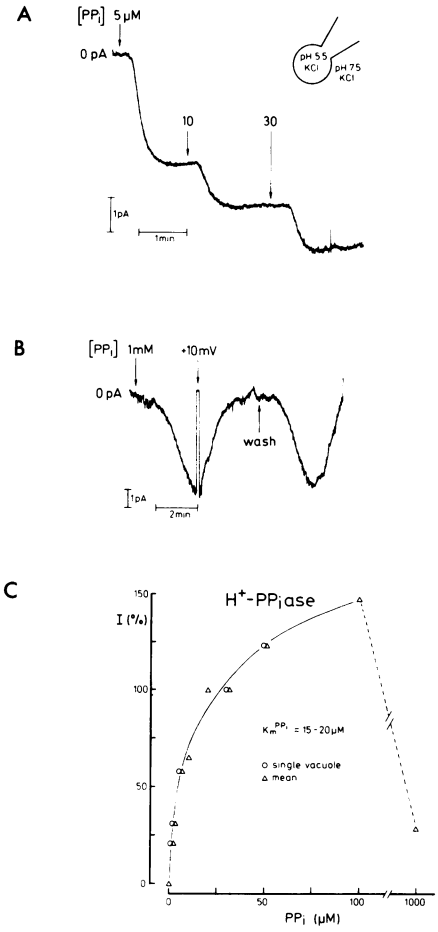

Fig. 7. Substrate affinity of the H^+-translocating PP_iase. (**A**) Stepwise increase of inward pump current upon stepwise increase in micromolar $MgPP_i$ concentrations in the extravacuolar space. (**B**) Transient activation of inward current upon the perfusion as well as removal of 1 mM $MgPP_i$ at the cytoplasmic surface of the vacuolar membrane. Initial current minimum and minimum after removal of 1 mM MgPPi demonstrate PP_i-free solutions. Current minimum in the presence of PP_i reflects the equilibration of the bath solution with 1 mM $MgPP_i$. Peak currents reflect the presence of $MgPP_i$ concentrations in the range of 50–150 μM. (**C**) Michaelis–Menten plot of PP_i-induced currents. Circles, mean of three experiments where all $MgPP_i$ concentrations were successively tested on the same vacuole; triangles, results from 24 individual experiments, currents were normalized by setting steady-state currents in the presence of 30 μM $MgPP_i$ to 100%.

subsequent application of equimolar concentrations of purine and pyrimidine nucleotides (Figure 5). Among CTP, GTP, ADP and ATP, only GTP and ATP produced pronounced H^+ currents. As the current elicited by GTP is only 25–30% compared to the current elicited by ATP, the physiological function of this protein is presumably that of a H^+-pumping ATPase.

When the MgATP concentration was increased stepwise, the pump current also increased stepwise, reaching saturation at 5–10 mM MgATP (Figure 6, upper panel). The K_m value for MgATP was 0.6 mM in sugar-beet taproot vacuoles (Figure 6, lower panel).

In contrast to the ATPase, saturation of $MgPP_i$-induced current occurred at ~100 μM $MgPP_i$, revealing a $K_m^{MgPP_i}$ of 15–20 μM (Figure 7A,C). Increasing the $MgPP_i$ concentration above 150 μM progressively inhibited the enzyme (Figure 7B,C). When a vacuole was incubated in $MgPP_i$-free solution and 1 mM $MgPP_i$ was allowed to equilibrate with the extravacuolar solution (using bath perfusion), a transient activation of pump currents could be observed (Figure 7B). Identical behaviour of the H^+-pump resulted during the subsequent removal of $MgPP_i$ from the extravacuolar space. These activation transients demonstrate directly the non-linear concentration dependence of the PP_iase.

ATP and PP_i stimulate solute uptake

Sucrose, citrate and malate together with potassium constitute the main solutes in beet vacuoles (Marin, 1987). Thus uptake of these metabolites from the extravacuolar solution in the presence of ATP and PP_i was used to indicate the involvement of both electroenzymes in vacuolar solute transport. Vacuole suspensions from sugar-beet taproots prepared according to Willenbrink and Doll (1979) were incubated with ^{14}C-labeled metabolites in the presence and absence of MgATP and $MgPP_i$. The results shown in Table I demonstrate the enhancement of malate, citrate and sucrose uptake into isolated sugar-beet taproot vacuoles by both MgATP and $MgPP_i$. MgATP-stimulated metabolite uptake always exceeded $MgPP_i$-stimulated accumulation. ATP and PP_i in the presence of the Mg^{2+}-chelator, EDTA (2 mM), did not stimulate metabolite uptake, identifying the Mg^{2+} complex as the substrate for energizing the transport process.

Table I. MgATP and $MgPP_i$ stimulate the uptake of metabolites into sugar-beet vacuoles

	Metabolite (μmol/mg protein/min)		
	Malate	Citrate	Sucrose
–	29 ± 5.0	26 ± 2.8	70 ± 5.0
MgATP (5 mM)	76 ± 10.0	65 ± 6.5	189 ± 25.0
$MgPP_i$ (50 μM)	61 ± 12.0	40 ± 6.3	161 ± 7.0

Uptake of ^{14}C-labeled malate, citrate and sucrose into isolated sugar-beet vacuoles was performed as described in Materials and methods. The rates of metabolite uptake into the vacuoles were related to the number of vacuoles as evaluated from the sorbitol-impermeable ^3H_2O space assuming that 2×10^8 sugar-beet vacuoles (equivalent to 0.3 mg vacuolar membrane protein, H.P.Getz, personal communication) cover a volume of 100 μl. The linearity of the transport process was recorded to confirm that initial rates were measured. The values represent averages of at least five different experiments.

Similarly, ATP in the absence of Mg^{2+} failed to activate pump currents or acidify the vacuolar compartment (not shown here).

Whereas our previous studies on vacuoles from photosynthetic tissue have already demonstrated that the ATP hydrolysis generates enough current to provide a driving force for malate accumulation (Martinoia *et al.*, 1985; Hedrich *et al.*, 1986), the uptake studies presented here unequivocally demonstrate the H^+-pyrophosphatase as an additional mechanism for the energization of metabolite transport into isolated vacuoles.

Discussion

Using chromatographic separation techniques it was shown by Rea and Poole (1986) that ATP and PP_i hydrolysis belong to two different molecular entities. The present work on single sugar-beet vacuoles further demonstrates that both enzymes are located on the same membrane.

Both proton pumps are active under physiological conditions

The K_m^{ATP} of 0.6 mM is in the same range of values found for vacuoles from photosynthetic tissues (Hedrich *et al.*, 1986, 1988) and thus the ATPase is capable of operating at under cytoplasmic ATP levels (1–2 mM, Stitt *et al.*, 1982).

The H^+-ATPase and H^+-PP_iase were shown to mediate an uphill transport of protons even if the gradient across the vacuolar membrane was up to 4 pH units, proton gradients that can be observed *in vivo*, e.g. in vacuoles of plants performing crassulacean acid metabolism (pH 3.5, Lüttge, 1987), in lemon fruit vacuoles (lemon juice, pH 2–2.2) or the brown alga *Deresmeratia* (pH 0.8–1.2, McClintock *et al.*, 1982). These findings may indicate that the proton binding site at the cytoplasmic mouth of the 'proton channel' within the pump complex may possess an apparent pK above 7.5 and a pK of proton release below 3.5 at its vacuolar face.

The $K_m^{PP_i}$ is 15–20 μM in the presence of 0–150 μM $MgPP_i$ on the cytoplasmic surface of the membrane. Higher $MgPP_i$ concentrations cause a 'substrate inhibition' of the enzyme. $MgPP_i$ saturation of the pump currents as recorded from intact sugar-beet vacuoles (Figure 7) is remarkably similar to values previously obtained by Leigh and Pope (1987) on PP_i hydrolysis and H^+ accumulation in vacuolar vesicles from oat roots. The observed PP_i inhibition in H^+ pumping may thus reflect alterations in the hydrolytic activity of the enzyme (as proposed by Leigh and Pope, 1987) rather than on its 'proton channel'.

Similarly, the nitrate inhibition of ATP-driven proton pumping (Figure 4) seems to affect mainly the hydrolase, as the activity of the isolated enzyme is reduced by nitrate in the same way (Rea *et al.*, 1987). In addition we could identify the nitrate inhibition side as being located on the cytoplasmic face of the pump complex. The finding that vacuolar nitrate is ineffective in pump inhibition is in agreement with the vacuolar function of a nitrate store. Rea *et al.* (1987) have shown that the ATPase activity of vacuolar vesicles obtained from red beet was irreversibly inhibited by nitrate. In contrast to studies on vacuolar vesicles, our patch-clamp investigations on whole vacuoles could demonstrate that the nitrate inhibition of H^+ currents produced by the ATPase was at least partially reversible (Figure 4C). This discrepancy between the two findings may be due to alterations of the enzyme during isolation and purification of vacuolar vesicles or incomplete removal of nitrate from the extravacuolar space.

Energization of metabolite uptake

Besides evidence for the physiological relevance of both enzymes provided by correlation between the K_m^{ATP,PP_i} value and cytoplasmic substrate pools, further evidence was provided by the finding that MgATP and $MgPP_i$ stimulated metabolite uptake into isolated vacuoles (Table I). Whereas the MgATP-induced currents exceeded the currents induced by $MgPP_i$ by ~400–600% (under substrate saturation and the membrane potential clamped to 0 mV) the uptake rates were only 20–60% higher in the presence of MgATP than with $MgPP_i$. This controversy might reflect that in the presence of ion gradients and a 'free running' membrane potential both enzymes may possess different efficiencies.

Whereas in the case of the organic anions, malate and citrate, membrane polarization may have provided the driving force, sucrose uptake may be driven by the H^+ gradient (Sze, 1985).

Regulation of proton-translocating enzymes in the vacuolar membrane

Measurements on photosynthetic cells did show that during day–night cycles the cytoplasmic adenylate ratio does not change significantly (Stitt *et al.*, 1982; Heineke, 1987). Similar observations on the cytoplasmic proton concentration revealed the pH to be stable, too (for review see Kurkdjian and Guern, 1989). Regulation of the H^+-ATPase may thus involve cytoplasmic factors other than the substrates of the pump reaction. Activation cycles of nitrate reductase activity (Campbell and Smarrelli, 1986) may produce changes in the cytoplasmic nitrate concentration. However, evidence for these changes being sufficient to modulate H^+-ATPase activity is still lacking.

As described for the adenylate ratio, the cytoplasmic PP_i level (0.2–0.3 mM) does not remarkably change during the day–night cycle of metabolism (Weiner *et al.*, 1987), indicating a strong regulation in PP_i production and utilization. The vacuolar H^+-PP_iase, exhibiting an optimum-type PP_i dependence (Figure 7B,C), is likely to be involved in the balance of the cytoplasmic pyrophosphate concentration.

The free energy of PP_i hydrolysis can be transformed into an electrochemical gradient of the proton (see Figures 1 and 6 and Sze, 1985) which in turn could be used for the accumulation of metabolites (e.g. see Table I). The H^+-translocating pyrophosphatase could thus serve as an interface between metabolism and energization of the vacuolar membrane.

Materials and methods

Patch-clamp measurements

The application of the whole-vacuole configuration (in analogy to 'whole-cell', Marty and Neher, 1983) of the patch-clamp technique (Hamill *et al.*, 1981) to isolated vacuoles was used to control buffer capacities, ionic gradients and electrical potentials across the vacuolar membrane (cf. Hedrich *et al.*, 1988). Vacuoles were directly isolated from intact tissue by a procedure modified by Coyaud *et al.* (1987) from that of Klercker (1892). In short, the surface of a freshly cut tissue slice is rinsed with buffer solution to wash the liberated vacuoles directly into the recording chamber. Fresh vacuoles were isolated for each experiment. Vacuole isolation and sealing of patch pipettes on the vacuolar membrane was performed within 2–5 min. Membrane capacitance of the vacuoles used were in the range of 15–30 pF. Membrane surface area calculated from the whole-vacuole capacitance (assuming a specific capacitance of 1 $\mu F/cm^2$ for biological membranes) were in agreement with those obtained from vacuole diameters.

Inwardly directed pump currents were recorded after addition of 0.05–10 mM MgATP or 2–1000 μM $MgPP_i$ by bath perfusion. Since ions in the pipette and the bath solution were always maintained at equimolar concentrations, holding the voltage clamp at the resting potential of the vacuole ensured isolation of pump currents. Membrane potentials were measured in the current-clamp mode (measuring the zero current potential).

Control of membrane potential and measurement of current and membrane capacitance were performed with an EPC-7 patch-clamp amplifier (List Electronic, Darmstadt, FRG). Data were low-pass-filtered at 10 Hz, digitized (VR 10, Instrutec Inc., List Electronic) and processed on a PDP-11/73 computer.

Patch-clamp solutions. Vacuoles were exposed to solutions containing either 200 mM KCl or KNO_3. Both bathing media included 5 mM $MgCl_2$, 0 or 0.1 mM $CaCl_2$ and 5 mM Tris–MES or citrate–KOH buffered to pH 7.5. The vacuole was equilibrated with either 200 mM KCl or KNO_3 including 5 mM $MgCl_2$, 1 mM $CaCl_2$ and 5 mM MES–Tris, pH 7.5 and 5.5, or citrate–KOH, pH 3.5 and 4.5. The osmotic pressure of the cell sap was measured osmometrically and patch-clamp solutions were adjusted accordingly with sorbitol. The protonophore CCCP was applied at a final concentration of 0.1–10 μM.

H^+ flux measurements on vacuole vesicles using a pH probe

Vacuoles from sugar-beet taproots were isolated according to Willenbrink and Doll (1979). Vacuole suspensions were osmotically shocked and freeze-thawed to form vacuole vesicles. Adhering mitochondria and cell debris were removed by precentrifugation at 10 000 *g* for 15 min. Vesicles were sedimented from the supernatant at 100 000 g for 1 h, resuspended and layered onto a continuous sucrose density gradient. Membrane fractions corresponding to 18–21% sucrose exhibiting highest activity in PP_iase and nitrate-inhibited ATPase were used for pH dye experiments.

Acidification of the vesicle lumen caused by MgATP or $MgPP_i$ application was monitored by decrease in acridine orange (AO) absorbance at 492/550 nm with an AMINCO DW-2 dual-wavelength spectrophotometer (Rasi-Caldogno *et al.*, 1985).

Shocking-washing solution contained 250 mM sucrose, 3 mM $MgCl_2$, 1 mM EDTA, 5 mM Tris–MES, pH 7.5. Standard pH assay medium contained in 1 ml: 250 mM sorbitol, 25 mM Bis-Tris propane–MES, 50 mM KCl, 5 mM $MgSO_4$, 20–50 μg membrane protein and 5 μM AO.

Tracer flux experiments on vacuole suspensions

Uptake of ^{14}C-labeled metabolites into vacuoles was measured at 20°C by silicon layer filtering flotation (Martinoia *et al.*, 1985). The standard incubation mixtures for metabolite uptake contained 850 mM sorbitol, 30 mM Hepes–KOH, pH 8.0, 0.08% polyvinylpyrrolidone (PVP-40), 0.08% bovine serum albumin, 5% White's medium (v/v; Nickell and Maretzki, 1969), 1.5 mM dithiothreitol, ^{14}C-labeled substrates (sp. act: 1 Ci/mol; Amersham-Buchler, Braunschweig, FRG) at a final concentration of 1.1 mM, ^3H_2O (6 μCi/ml) and 0.2–2.0 × 10^6 vacuoles/ml. The concentrations of MgATP and $MgPP_i$ were 5 mM and 50 μM respectively. Metabolite uptake was allowed to last 10 min and was terminated by rapid centrifugation of 100 μl aliquots through a layer of 100 μl silicon oil (AP/100 Wacker Chemie, München, FRG) into 20 μl 2 M sorbitol containing 10% perchloric acid. Simultaneously [^{14}C]sorbitol (2 μCi/ml) was added instead of labeled metabolites in order to calculate the amount of medium adhering to the surface of the vacuole. The pellets were resuspended in 250 μl H_2O and centrifuged for 1 min at 10 000 *g*. An aliquot of 180 μl from the supernatant was measured for radioactivity by scintillation counting. The internal volume of the vacuole suspension was defined by evaluation of sorbitol-impermeable space and ^3H_2O space.

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