Plant Physiol. (1987) 83, 483–489
0032-0889/87/83/0483/07/$01.00/0

Potential-Dependent Anion Transport in Tonoplast Vesicles from Oat Roots[1]

Received for publication May 2, 1986 and in revised form October 17, 1986

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ABSTRACT

Potential-dependent anion movement into tonoplast vesicles from oat roots (*Avena sativa* L. var Lang) was monitored as dissipation of membrane potentials ($\Delta\psi$) using the fluorescence probe Oxonol V. The potentials (positive inside) were generated with the H^+-pumping pyrophosphatase, which is K^+ stimulated and anion insensitive. The relative rate of $\Delta\psi$ dissipation by anions was used to estimate the relative permeabilities of the anions. In decreasing order they were: SCN^- (100) > NO_3^- (72) = Cl^- (70) > Br^- (62) > SO_4^{2-} (5) = $H_2PO_4^-$ (5) > malate (3) = acetate (3) > iminodiacetate (2). Kinetic studies showed that the rate of $\Delta\psi$ dissipation by Cl^- and NO_3^-, but not by SCN^-, was saturable. The K_m values for Cl^- and NO_3^- uptake were about 2.3 and 5 millimolar, respectively, suggesting these anions move into the vacuole through proteinaceous porters. In contrast to a H^+-coupled Cl^- transporter on the same vesicles, the potential-dependent Cl^- transport was insensitive to 4,4′-diisothiocyano-2,2′-stilbene disulfonate. These results suggest the existence of at least two different mechanisms for Cl^- transport in these vesicles. The potentials generated by the H^+-translocating ATPase and H^+-pyrophosphatase were nonadditive, giving support to the model that both pumps are on tonoplast vesicles. No evidence for a putative Cl^- conductance on the anion-sensitive H^+-ATPase was found.

The vacuoles in plant cells store metabolites, sustain turgor pressure, and maintain suitable cytoplasmic conditions. In cultivated plants, the osmolarity of the vacuole is around 400 to 600 mosmol/L (8) and inorganic salts provide a large part of it (19). The vacuolar concentrations range from 40 to 205 mM for K^+ and 38 to 170 mM for Cl^- for most higher plants (nonhalophytes; 17, 18). Compartmental analyses together with membrane potential measurements have indicated active transport into the vacuole for Na^+, K^+, and Cl^- (18, 24). However, the driving force and the mechanisms of transport of various ions across the tonoplast could not be tested directly in the intact cell. With the discovery of the electrogenic (potential-generating) H^+-pumping ATPase (1, 4, 6, 7, 14, 30) and PPase[2] (32 and references therein) on the tonoplast, anion uptake into the vacuole according to Mitchell's chemiosmotic theory (22) became plausible: the H^+ pumps generate a potential positive inside the vacuole, providing part of the driving force for anion movement into the vacuole.

In this paper, we generated membrane potentials in right-side out tonoplast vesicles with the anion-insensitive H^+-pyrophosphatase in order to exclude side effects of the anions on the enzyme activity, which might occur with the anion-sensitive H^+-ATPase. The membrane potential provides one driving force for anion uptake into the vesicles. We used the rate of dissipation of membrane potentials by anions to show their different permeabilities. Our evidence suggests that the potential-dependent Cl^- and NO_3^- transport into the vacuole is via proteinaceous porters. We also try to elucidate further a putative Cl^- conductance on the tonoplast H^+-pumping ATPase. A preliminary report of these results has been presented (11).

MATERIALS AND METHODS

Plant Material. Oat seeds (*Avena sativa* L. var Lang) were germinated in the dark over an aerated solution of 0.5 mM $CaSO_4$. After 3 or 4 d of growth, the roots were harvested.

Preparation of Low Density Microsomal Vesicles. The procedure of Churchill and Sze (6, 7) was used with some modifications. A microsomal preparation was obtained using differential centrifugation. This fraction was further purified by use of density gradient centrifugation. Low density vesicles, enriched in tonoplast membranes, were collected from the interface over a 6% (w/w) dextran cushion. For simplicity, we shall refer to this fraction as tonoplast vesicles (30, 33).

ATPase and PPase Activities. PPase activity was determined by measuring the release of Pi from PPi (32). Since two moles of Pi are released from 1 mol of PPi, we have expressed the activity as mol PPi consumed. The reaction mixture (0.5 ml) contained 30 mM BTP-Hepes (pH 8.0), 0.5 mM $MgSO_4$, 0.5 mM PPi-BTP, 4 μg/ml gramicidin, and 10 to 15 μg membrane protein. K^+-dependent PPase was determined by subtracting the activity measured in the absence of K^+. Salt additions were as indicated in figure legends.

ATPase activity was determined by a similar assay (33). The reaction mixture contained 30 mM Hepes-BTP (pH 6.75), 3 mM $MgSO_4$, 3 mM ATP-BTP, 17 μg membrane protein, 4 μg/ml gramicidin, plus 0.5 mM NaN_3, 0.1 mM Na orthovanadate, and 0.1 mM ammonium molybdate to inhibit the mitochondrial and plasma membrane ATPases and acid phosphatases, respectively (10).

Protein concentrations were estimated by the method of Lowry *et al.* (16) after precipitation with 10% TCA, using BSA as a standard.

Membrane Potential Generation. Relative $\Delta\psi$ formation at 20°C was measured as fluorescence quenching of Oxonol V (Excitation 580 nm, Emission 650 nm) with a Farrand System 3 spectrofluorometer according to Scherman and Henry (26). The reaction mixture contained 25 mM Hepes-KOH (pH 7.4), 200 mM sorbitol, 25 mM KIDA, 1 μM Oxonol V, 20 to 40 μg vesicle

[1] Supported in part by National Science Foundation grant PCM 83-04130 to H. S. and German Academic Exchange Service (DAAD) fellowship to K. H. K.

[2] Abbreviations: PPase, inorganic pyrophosphatase; BTP, bis-tris propane or 1,3-bis[tris(hydroxymethyl)-methylamine]propane; DIDS, 4,4′-diisothiocyanostilbene-2,2′disulfonate; $\Delta\psi$, membrane potential; ΔpH, pH gradient; IDA, iminodiacetate; Oxonol V, bis(3-phenyl-5-oxoisoazol-4-yl)pentamethine oxonol.

protein, and 100 μM PPi or 1.5 mM ATP. The contents in the cuvettes were continuously stirred. Potential generation caused by proton pumping was initiated by addition of 2 mM MgIDA. The fluorescence intensity decrease of Oxonol dyes induced by MgATP or MgPPi has been established to reflect $\Delta\psi$ generation (positive inside) by several laboratories (4, 23, 32). The ionophore gramicidin (2 μg/ml) was added at the end of each assay to show that fluorescence quenching was due to an ion gradient and not unspecific binding effects. The fluorescence quenching at steady state was quantified as

$$\Delta\psi = \Delta F/F \times 100$$

where F is the maximum fluorescence intensity before addition of Mg^{2+} and is usually set to 90 (arbitrary units), and ΔF is the maximal decrease in fluorescence intensity 4 to 5 min after addition of Mg^{2+} (Fig. 1). $\Delta\psi$ was usually 30 ± 5% with PPi, and 45 ± 5% with ATP as the substrate.

Relative $\Delta\psi$ Dissipation by Anions as a Measure of Relative Anion Permeabilities. To measure the relative permeabilities of anions, different anions at the concentrations indicated (in the respective legends) were added to vesicles with fully developed membrane potentials (steady state). The relative initial rates of electrical potential dissipation were estimated from the rate of fluorescence intensity increase after anion addition (Fig. 1). These relative rates are comparable since the driving force ($\Delta\psi$) is similar in all assays, independent of the anion and its concentration. The initial rates of fluorescence change was expressed as:

$$\Delta f/F \times \text{min}^{-1} \times 100$$

with Δf as fluorescence intensity increase after anion addition during the first 6 s. To account for the slight variation in steady state $\Delta\psi$ (= driving force for anion movement) from one experiment to another, we expressed the 'relative dissipation rate' as

$$\text{Relative dissipation rate} = \text{I.R.}/\Delta\psi \times 100$$

This normalization is only valid for small variation (<3%) in $\Delta\psi$ values. Gramicidin, an ion channel-former, dissipates $\Delta\psi$; and the rate of fluorescence change induced by gramicidin reflects the maximum rate of dissipation detectable with our method. Addition of 2 μg/ml gramicidin gave an average relative dissipation rate of 600. This value can also be obtained with SCN^- which is considered as a permeant anion and at low concentrations (50 μM) is a useful probe for measuring ATP-dependent electrical potential formation (positive inside) in microsomal vesicles (7, 31).

Dissipation rates could be limited by several factors, such as the rate of stirring, and the rate of dye response. These factors can, however, not account for the reproducible and large differences between dissipation rates of various anions (see "Results"). It is possible that $\Delta\psi$ dissipation by SCN^-, the most permeant anion (see "Results") is limited by these factors. Slower rates of dissipation by other anions must, however, be caused by other factors, *e.g.* the differential permeability of the membrane or the diffusion rate through the unstirred boundary layer surrounding the vesicles. Considering the similarity of the diffusion coefficients of the anions used, the large differences in dissipation rates must reflect the differential permeability of the membrane. Thus, the observed experimental differences among anions are useful for estimating the relative anion permeabilities.

Though indirect, measuring dissipation of $\Delta\psi$ as an indicator of anion permeabilities has several advantages. Fast responses (few seconds) can be detected and the fluxes can be monitored continuously with time. Inorganic anions (*e.g.* Cl^-) move across isolated vesicle membranes very fast, making direct measurements of $^{36}Cl^-$ flux quantitatively unreliable (unpublished observations). Measuring isotope fluxes across vesicles with a filtration technique is slow (>20 s) and less sensitive (30). Monitoring fast responses also reduces secondary effects resulting from direct anion effects on the primary pump (7, 33) or anion efflux via H^+-coupled anion transport pathways (27).

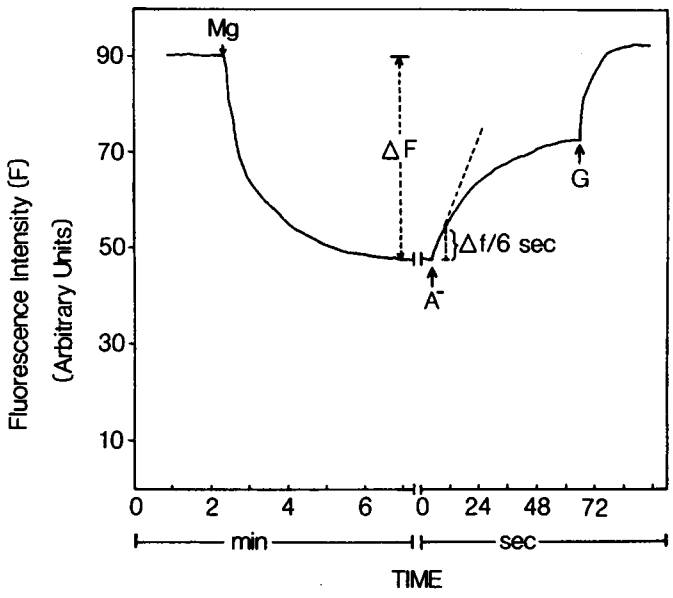

FIG. 1. Quantitative expression of the steady state $\Delta\psi$ formed by H^+-PPase or H^+-ATPase and the rate of $\Delta\psi$ dissipation by anions. The fluorescence intensity of a reaction mixture (containing 25 mM Hepes-KOH (pH 7.4), 200 mM sorbitol, 25 mM KIDA, 1 μM Oxonol V, 100 μM PPi, or 1.5 mM ATP and 20 to 40 μg membrane protein) is set to 90. Addition of 2 mM MgIDA induced fluorescence quenching until a steady state is reached (in 4–5 min). The chart speed is then increased 4-fold, and an aliquot (10 μl) of anion (A^-) stock solutions was added to give the final concentration. The rate of fluorescence increase is determined from the change observed in 6 s. The steady state $\Delta\psi = \Delta F/F \times 100$. The initial rate (I.R.) of fluorescence increase induced by anions = $\Delta f/F \times \text{min}^{-1} \times 100$. The relative dissipation rate = I.R./$\Delta\psi \times 100$.

RESULTS AND DISCUSSION

Anion-Insensitivity of the Electrogenic H^+-Pumping PPase. Dissipation of membrane potentials generated by an anion-sensitive H^+-translocating ATPase has been used to get qualitative estimates of anion permeabilities (4, 6, 14). To achieve a more quantitative comparison we took advantage of the K^+-dependent H^+-pumping PPase associated with tonoplast vesicles from oat roots, which is insensitive to anions (32; Table I). Table I shows the PPi hydrolysis activity of the K^+-dependent PPase in the presence of anions under conditions closely resembling those used for the Oxonol V studies (see below). None of the anions has a substantial effect on the enzyme. The initial rate of dissipation of membrane potentials by anions is therefore a valid measure of their relative permeabilities and not biased by side effects on the H^+-PPase itself.

Differential $\Delta\psi$ Dissipation by Anions: An Indicator of Relative Anion Permeabilites. The H^+-PPase was used to generate membrane potentials (positive inside) in tonoplast vesicles; these potentials were monitored with the fluorescence probe Oxonol V. Though it is difficult to calibrate the $\Delta\psi$ response of Oxonol V because of unspecific interactions with K^+ and valinomycin, we estimate the $\Delta\psi$ is at least +45 mV (31) and perhaps as high as +100 mV (1). After establishing a steady state potential (no further decrease in fluorescence intensity), anions were added. Addition of K^+-salts to the outside of the vesicles, in theory, should generate a diffusion potential (in ms) according to the Goldman equation (17). However, experimentally, little or no change in fluorescence intensity is detectable with any K-salt in

Table I. *Effect of Anions on K⁺-Dependent PPase Activity in Tonoplast Vesicles from Oat Roots*

Reaction mixtures contained 30 mM BTP-Hepes at pH 8.0, 0.5 mM $MgSO_4$, 0.5 mM PPi, 4 μg/ml gramicidin, 10 μg membrane protein, 25 mM KIDA, and additional salts (20 mM). Nonspecific PPase activity ($-K^+$) has been subtracted. The data represent the average (±SE) of four determinations. Activity in the presence of 45 mM KIDA was set to 100%.

Additional Salts (20 mM)	K⁺-Dependent PPase Activity	
	μmol PPi/mg protein·h	%
KCl	4.88 ± 0.34	105
KBr	4.28 ± 0.49	92
KSCN	4.32 ± 0.61	93
KNO_3	4.39 ± 0.09	95
K_2SO_4	4.69 ± 0.81	101
KIDA	4.64 ± 0.27	100
Acetate/K	4.61 ± 0.44	99
Malate/K	3.71 ± 0.39	80

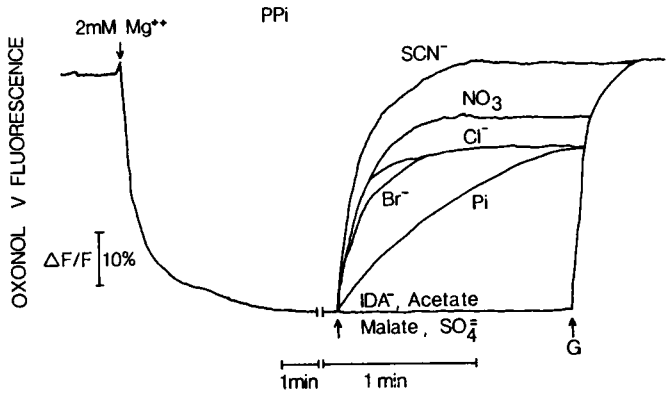

FIG. 2. Permeant anions dissipate Δψ generated by H⁺-PPase. Reaction mixtures contained 25 mM Hepes-KOH at pH 7.4, 200 mM sorbitol, 1 μM Oxonol V, 100 μM PP_i, 25 mM KIDA, and 30 μg membrane protein. MgIDA (2 mM) was added to start the reaction. K-salts (20 mM) were added at the first arrow. G = 2 μg/ml gramicidin.

the absence of PPi unless valinomycin is added (not shown). In the presence of PPi, addition of KIDA or K_2SO_4 did not cause any change (Fig. 2). One explanation is that the fluorescence quenching (Δψ) in Figure 2 reflects mostly activity from the electrogenic H⁺ pump and that diffusion potentials from other ions (*e.g.* K⁺) are relatively small.

If anions are relatively permeant, they enter the vesicles and would decrease the electrical potential, thereby increasing the fluorescence intensity. The order of rate of dissipation of the potential by the anions tested was $SCN^- > NO_3^- = Cl^- > Br^- > H_2PO_4^-$ > acetate = malate = sulfate = IDA if 20 mM anions were added (Fig. 2). Organic anions, such as acetate, sulfate, malate, and IDA, did not dissipte PP_i-generated Δψ, possibly because the rate of proton pumping into the vesicles exceeded anion influx. However, small decreases in Δψ could be detected when the tonoplast H⁺-ATPase was used to generate membrane potentials. The order of rate of potential dissipation by these anions is $SO_4^{2-} = H_2PO_4^-$ > malate = acetate > IDA (Table II). Sulfate, malate, and acetate had little or no effect on the ATPase activity in these vesicles (Table III), excluding the possibility that potential dissipation was caused by inhibition of the H⁺-ATPase.

The permeability of phosphate is certainly less than that of Br^- and the other more permeant anions, although it could not be ranked precisely, as ΔpH formation by both PPase or ATPase was decreased in the presence of phosphate (data not shown).

Table II. *Anion Dissipation of Δψ Generated by the H⁺-ATPase*

Reaction mixtures and assay conditions as in Figure 1, except that PPI was replaced by 1.5 mM ATP-BTP. Relative dissipation rates were quantified as described in "Materials and Methods."

K⁺-Salt (20 mM)	Relative Dissipation Rate
IDA	17
Malate	28
Acetate	32
$H_2PO_4^-$	43
SO_4^{2-}	43
Cl^-	320

Table III. *Effect of Anions on ATPase Activity in Tonoplast Vesicles from Oat Roots*

Reaction mixtures contained 30 mM Hepes-BTP at pH 6.75, 3 mM $MgSO_4$, 3 mM ATP-BTP, 4 μg/ml gramicidin, 0.1 mM Na orthovanadate, 0.1 mM ammonium molybdate, 0.5 mM NaN_3, and 17 μg membrane protein. Unspecific ATPase activity ($-MgSo_4$) has been subtracted. The activity in absence of salt was set to 100%. The data represent the average (± SE) of four determinations.

Additional Salts (20 mM)	ATPase Activity	
	μmol Pi/mg protein·h	%
None	9.94 ± 0.18	100
Malate/K	10.55 ± 0.21	106
Acetate/K	11.78 ± 0.19	118
K_2SO_4	8.70 ± 0.17	88
KCl	17.68 ± 0.24	178

This is probably due to feedback inhibition of PPase or ATPase activity, as Pi is an end product for both enzyme reactions.

The ability of anions to dissipate electrical potential was correlated generally with their ability to stimulate ΔpH formation (4, 6, 7, 23, 32). Relatively permeant anions, like Cl^- or Br^-, stimulated ΔpH formation more than SO_4^{2-} or HPO_4^{2-}. These results are consistent with the idea that the H⁺ electrochemical gradient consists of two interconvertible components: Δψ and ΔpH. Anion uptake into the vesicles neutralized the potential difference (Δψ), stimulating H⁺ pumping. The increase in H⁺ concentration within the vesicles results in a ΔpH generation (acid inside) as shown previously by our laboratory (6, 7, 32) as well as by other laboratories (1, 4, 23). When anions have no other effects on the primary pumps, the relative rate of potential dissipation by anions or the stimulation of ΔpH formation in tonoplast-enriched vesicles directly reflects the relative permeability of the membrane to anions.

Since electrical potential changes can be induced by only a small amount of anions, we have used the relative rates of potential dissipation as a sensitive indicator of relative anion permeabilities. Estimation of anion permeabilities using the stimulation of ΔpH formation as an assay might be biased by the effects of H⁺-coupled transport systems (27). Using initial rates of potential dissipation instead of net dissipation we also minimized possible stimulatory effects on the primary pump that might be caused by decreasing Δψ after anion addition. Furthermore, initial rates reflect activity when the driving force (Δψ) for anion uptake is similar for each anion and each concentration used.

In spite of possible limitations in our assay (*e.g.* see "Materials and Methods"), the observed differences in membrane potential dissipation by anions were reproducible and thus provided estimates of the relative anion permeabilities. The anion permeabilities in decreasing order and the relative rates were:

$$\begin{array}{c} SCN^- > NO_3^- = Cl^- > Br^- > H_2PO_4^{2-} > SO_4^{2-} > \\ 100 > 72 = 70 > 62 > 13 > 5 > \end{array}$$

$$\begin{array}{c} \text{acetate} = \text{malate} > IDA^-; \\ 3 = 3 > 2 \end{array}$$

The relative anion permeabilities of the vacuolar membrane shown here are similar to those reported for tonoplast vesicles where the $\Delta\psi$ was generated by ATP (4, 6, 14). In most cases, the decreasing order of anion permeabilities was $SCN^- > NO_3^- > Cl^- \gg SO_4^{2-}$ as in the plasma membrane vesicles of *Neurospora* (23). As mentioned before, our estimates of anion permeabilities using the PPase to generate membrane potentials are not influenced by direct effects of the anions on the pump and, thus, may be more reliable than those using the tonoplast ATPase. Interestingly, the relative anion permeabilities shown in Figure 2 are analogous to those found for the anion transport protein of red blood cells. Band 3 catalyzes the exchange of many anions, with Cl^- and HCO_3^- being transported most rapidly; while the rate of transport of SO_4^{2-} and HPO_4^{2-} are slower by as much as 10^4-fold (13). It is yet to be established whether $\Delta\psi$-driven anion transport across the tonoplast of plant cells is mediated by one or more protein pathway(s).

These results suggest that under physiological conditions Cl^- and NO_3^- are the major anions moving into the vacuole. The driving force is provided in part by the electrical gradient (positive inside) generated by the electrogenic H^+-ATPase and H^+-PPase. Movement of Cl^- and NO_3^- into the vacuole partially neutralizes positive charges inside, and could account for the low potential difference (+10 to +20 mV [2, 18, 19]) in the vacuole relative to the cytoplasm. These results are consistent with high concentrations of Cl^- found in the vacuole (17, 21) and its role as a possible storage compartment for NO_3^- (19–21).

Less information is available about transport across the tonoplast for the other anions tested. However, compartmental analysis of $^{35}SO_4^{2-}$ tracer kinetics in intact cells (9) revealed that sulfate accumulation is regulated principally at the tonoplast. For phosphate it was reported that its uptake into the vacuole is a very slow process, although about 90% of the cellular phosphate is stored in the vacuole (nonmetabolic or storage pool) (3, 21). Both observations are in good agreement with the relatively low permeability of the tonoplast for these anions described above.

Potential-Dependent NO_3^- and Cl^- Transport is Saturable. As an attempt to differentiate between potential-dependent anion movement through proteinaceous porters or the lipid bilayer, we did a kinetic analysis of Cl^-, NO_3^-, and SCN^- concentration on $\Delta\psi$ dissipation. The working hypothesis is that protein-mediated anion uptake would depend on the anion concentration and the maximum rate is reached when all the transport sites are saturated. Transport via a lipid pathway would be linearly related to the substrate concentration until the driving force becomes limiting.

Figure 3 shows the dissipation of membrane potentials by different concentrations of NO_3^-, Cl^-, and SCN^-. The dissipation by high concentrations (20 mM) of SCN^- was as fast or even faster than that of gramicidin (Fig. 3). Gramicidin gives the maximum rate of $\Delta\psi$ dissipation detectable with this assay and also indicates the magnitude of the driving force ($\Delta\psi$). Complete decrease of $\Delta\psi$ by SCN^- at rates comparable to gramicidin, suggest SCN^- uptake was extremely fast and was limited at high concentrations (20 mM) by the driving force and the detection method. Therefore, we suggest that SCN^- transport might have a high K_m and V_{max} or it might be transported mainly by the lipid pathway.

In contrast, the initial rate of dissipation by Cl^- and NO_3^- is saturable (Fig. 3, A and B). This could not be due to limitations of the assay (driving force, detection limit) as the maximum rates

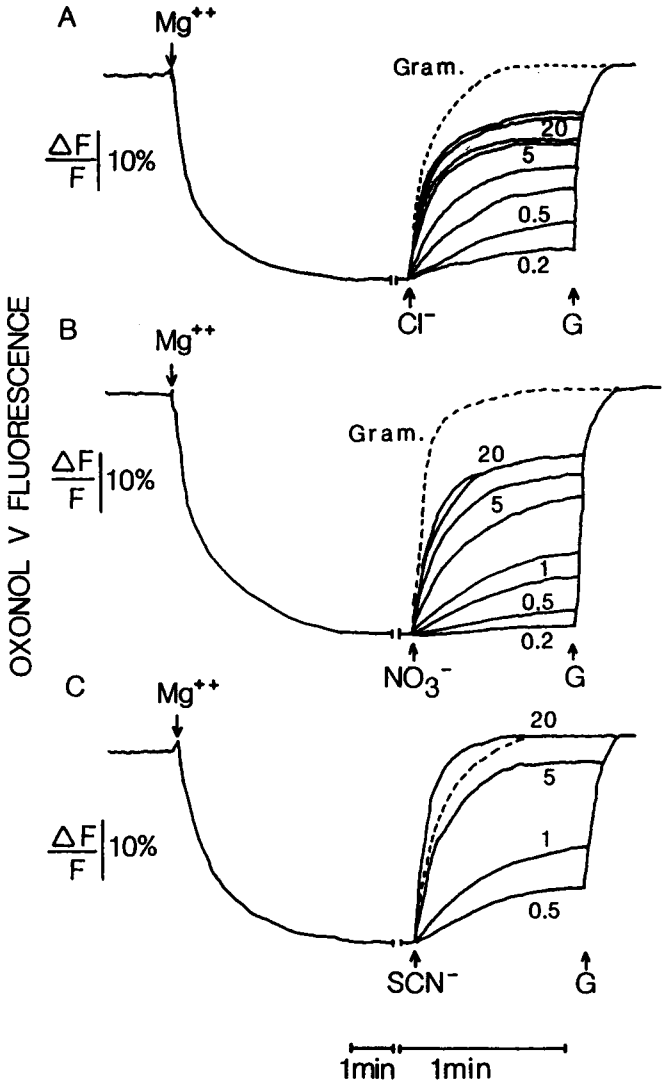

FIG. 3. Kinetics of $\Delta\psi$ dissipation by (A) Cl^-, (B) NO_3^-, and (C) SCN^-. Reaction mixtures as in Figure 2 (membrane protein: 28 or 35 μg). BTP-anions (at 0.2, 0.5, 1, 2, 5, 10, 20, or 40 mM) were added as indicated. The dotted traces show complete dissipation of $\Delta\psi$ by 2 μg/ml gramicidin in the absence of anions. G or gramicidin was added at the end of each assay with anions to give a final concentration of 2 μg/ml.

seen with SCN^- or gramicidin were higher than the V_{max} for Cl^- and NO_3^- (Fig. 3C). Michaelis-Menten plots of the effect of Cl^- and NO_3^- concentration on $\Delta\psi$ dissipation are shown in Figure 4, A and B. Hanes-Woolf plots (not shown) gave apparent K_m values for Cl^- and NO_3^- of 2.3 and 5 mM, respectively. These results suggest that Cl^- and NO_3^- move into the vacuole via proteinaceous porters. Further characterization of these transport activities, especially inhibitor sensitivities and substrate specificities, is necessary to be able to define whether these saturation kinetics are the result of one or several porter(s). Surprisingly, an independent study with isolated barley vacuoles showed the apparent K_m for $^{36}Cl^-$ uptake as 2.3 mM (21). Although the authors suggested ATP is involved in the uphill transport of Cl^- and NO_3^- into vacuoles, they did not show how ATP energized the uptake.

Potential-Dependent Cl^- Transport is DIDS-Insensitive. DIDS, an amino-reactive disulfonic stilbene derivative, inhibits

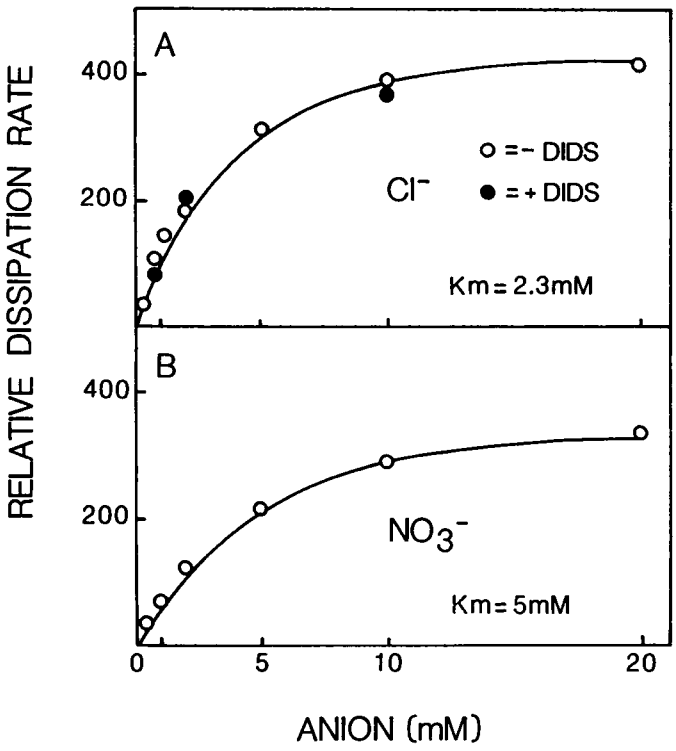

FIG. 4. Effects of (A) Cl^- and (B) NO_3^- concentrations on dissipation of $\Delta\psi$ generated by H^+-PPase. Initial rates of dissipation from Figure 3 were estimated from the first 6 s after anion addition and converted to relative dissipation rate (see "Materials and Methods"). In (A), the rate of dissipation was examined in the absence (O) and presence (●) of 3 μM DIDS. The data represent the average of two or three experiments. Apparent K_m values were obtained from Hanes-Woolf plots (not shown).

anion transport in animal and plant cells (5, 12, 13, 15). Recently, a H^+-coupled Cl^- transport on the oat root tonoplast was also shown to be sensitive to DIDS at low micromolar concentrations (27). Unlike the H^+-ATPase (7, 33), H^+-pumping by the K^+-dependent PPase is DIDS insensitive (32). This enabled us to test whether the potential-dependent Cl^- transport is inhibited by DIDS. As is shown in Figure 4A, there is no significant decrease in the initial rates of potential dissipation by Cl^- in the presence of 3 μM DIDS, a concentration which reduced the activity of the H^+-coupled Cl^- transport by about 50% (27). We therefore suggest that these two Cl^- transport systems, the potential-driven Cl^- porter(s) and the H^+-coupled Cl^- porter(s), are different entities.

We also tested duramycin, a polypeptide antibiotic, found to inhibit Cl^- transport in clathrin-coated vesicles from bovine brain (29). We found no specific inhibition of Cl^- transport (measured as dissipation of PPi dependent $\Delta\psi$) with concentrations up to 50 μg duramycin/100 μg vesicle protein (data not shown). This concentration is 10-fold higher than that found to be effective in clathrin-coated vesicles (29). Thus, the Cl^- porter of the oat root tonoplast might be structurally different from the one found in clathrin-coated vesicles.

Is Cl^--Dependent $\Delta\psi$ Dissipation Associated Partly with the Anion-Sensitive H^+-ATPase? The tonoplast H^+-pumping ATPase from various higher plants is anion-sensitive (1, 4, 6, 7, 14, 30); especially provocative is the strong stimulation by Cl^-. One interpretation for this characteristic is that a Cl^- channel is physically associated with the H^+-ATPase (1). Two observations appeared to support this idea: (a) the membrane-bound (1, 7, 33) and partially purified (25) H^+-ATPase was inhibited by the anion channel blocker, DIDS; and (b) the similarity in kinetic constants for Cl^- stimulation of the ATPase and Cl^- stimulation of ATP-driven H^+ transport (1).

Our results suggest that the $\Delta\psi$-dependent Cl^- uptake in tonoplast vesicles described here is not via part of the H^+-ATPase as it is not inhibited by DIDS (Fig. 4A). This conclusion assumes that a putative Cl^- conductance associated with the H^+-ATPase would be inhibited by DIDS as suggested above. We were able to test the effect of DIDS directly on $\Delta\psi$-dependent anion transport by using the H^+-PPase as the primary pump. Unlike the H^+-ATPase, the H^+-PPase is resistant to low concentrations of DIDS (32). The inhibition by DIDS of the Cl^--stimulated ΔpH generated with ATP (1, 7) can now be explained by the direct inhibition of the H^+-ATPase alone, independent of any Cl^- conductance (see below).

We tested whether an additional Cl^- conductance was associated with the H^+-ATPase as follows. Assuming that H^+-ATPase and H^+-PPase are on the same vesicles, Cl^- should dissipate the $\Delta\psi$ generated by both pumps via the same DIDS-insensitive porter(s). An additional Cl^- conductance associated with the H^+-ATPase might become available only in the presence of ATP. To test for this possibility, we compared Cl^- dissipation of $\Delta\psi$ generated with ATP or PP_i. Since the rate of membrane potential dissipation by Cl^- was identical whether PPi or ATP was used as the substrate (Table IV), we tentatively conclude there is no evidence for an additional Cl^- conductance associated with the anion-sensitive ATPase. It is possible, however, that the stimulatory effect of Cl^- on the tonoplast H^+-ATPase offsets an additional Cl^- conductance induced by ATP addition. Reconstitution of the purified tonoplast ATPase might settle this question.

One assumption we have made is that H^+-PPase and H^+-ATPase are on the same vesicle population, so Cl^- would dissipate the $\Delta\psi$ via the same porter(s). Several observations support this model: (a) Both H^+-PPase and H^+-ATPase are associated with the vacuolar membrane (30, 32 and references therein) and it is unlikely the two pumps are located in separate domains of the membrane or on separate vacuoles; and (b) the Oxonol V signals generated by H^+-ATPase and H^+-PPase in tonoplast vesicles were nonadditive (Fig. 5). If the two enzymes were on different vesicle populations, one might expect the signals to be additive. However, if the two H^+ pumps were on the same vesicles, there might be a maximum $\Delta\psi$ that can be reached before H^+ efflux increases to a level equal to the rate of H^+ uptake (*i.e.* steady state). The steady state $\Delta\psi$ generated by ATP plus PP_i was close to that generated by ATP alone, suggesting that the two pumps are on the same vesicle population (Fig. 5). Maximal fluorescence quenching in Figure 5 is not limited by the dye concentration as up to 75% quenching of fluorescence could be achieved with more active vesicle preparations (not shown). Recently, a direct study using permeabilized *Nitella* cell has shown that both H^+-ATPase and H^+-PPase are found on the same vacuolar membrane (28).

Table IV. *Cl^- Dissipation of Membrane Potentials Generated with H^+-ATPase or H^+-PPase*

Reaction mixtures as in Figure 1 for the H^+-PPase, and as in Table II for the H^+-ATPase. Assays were conducted at 12°C to obtain comparable signals. Membrane protein was 35 μg per assay. Relative dissipation rates were quantified as described in "Materials and Methods."

KCl	Relative Dissipation Rate	
	ATPase	PPase
mM		
0.5	81	99
2	162	190
10	284	287
40	351	350

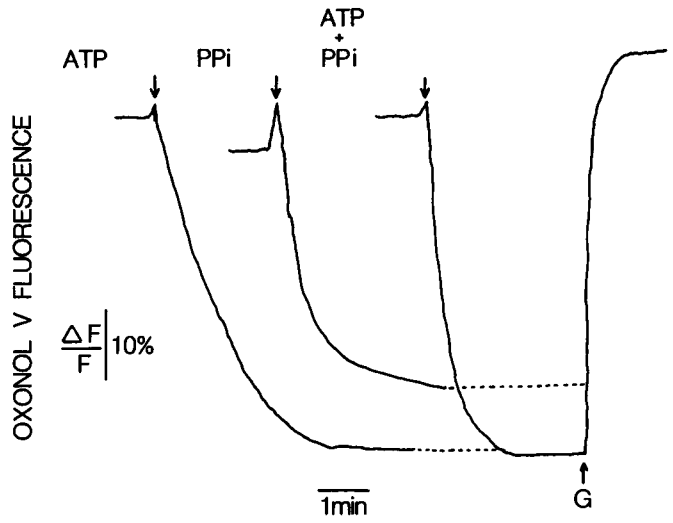

FIG. 5. Membrane potential generation by H^+-ATPase and H^+-PPase is nonadditive. Reaction mixtures were as in Figure 2, except the substrates were 3 mM ATP-BTP or 100 μM PPi, or both, as shown. MgIDA (4 mM) was added at the arrows. G = 2 μg/ml gramicidin.

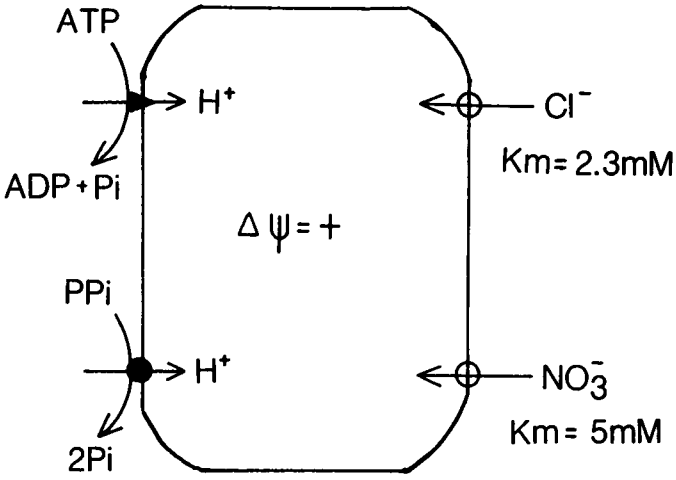

FIG. 6. Model for Δψ-dependent anion transport into the vacuole. The electrogenic tonoplast ATPase or PPase pumps H^+ into the vacuolar lumen, generating an electrical potential (Δψ), positive inside. The Δψ component of the total proton motive force provides one driving force for anion uptake into the vacuole. Although the Δψ-dependent Cl^- and NO_3^- transport are shown as mediated by individual porters, there is no evidence yet whether one protein catalyzes transport of both anions or whether each anion is transported by one or more pathways.

Model of Potential-Dependent Anion Transport Systems on the Tonoplast. A model incorporating the putative Δψ-dependent anion porters described above is given in Figure 6. One driving force for Cl^- and NO_3^- uptake into the vacuole is provided by the proton motive force generated by the H^+-ATPase and the H^+-PPase. This transport via putative proteinaceous porters utilizes only the electrical gradient (membrane potential) part of the total $\Delta\bar{\mu}_{H^+}$. Movement of Cl^- and NO_3^- down their electrochemical gradient would be consistent with the compartmental analyses and flux studies done in oat coleoptiles by Pierce and Higinbotham (24). In that study, the question of whether Cl^- uptake into the vacuole was passive or active depended heavily on the assumed potential difference between the cytoplasm and vacuole (see discussion in Ref. 24). If one assumes a membrane potential of only +10 mV (positive inside the vacuole), movement of Cl^- into the vacuole would be down its electrochemical gradient when Cl^- concentration in the cytoplasm and vacuole are estimated to be 85 and 65 mM, respectively. This transport would be considered "passive" as defined by MacRobbie (18) and by Pierce and Higinbotham (24). In some cells, such as *Chara*, the concentration of Cl^- in the vacuole (100 mM) is 10-fold higher than in the cytoplasm. If the membrane potential across the tonoplast is about +18 mV (positive inside), then Cl^- is not at equilibrium but must be pumped from the cytoplasm to the vacuole (18). The mechanism for this active transport is not known. One possibility is a H^+/Cl^- exchange. The experiments reported here were not designed to test this model, though we have evidence for H^+-coupled Cl^- transport in another study (27).

In summary, we suggest the low steady state Δψ across the tonoplast (+10 to +20 mV) *in vivo* is due to the high permeability of the tonoplast to certain anions. The Cl^- found inside the vacuole is taken up in part by the membrane potential generated by the H^+-ATPase or the H^+-PPase. The potential-dependent Cl^- and NO_3^- uptake are mediated by putative proteinaceous porter(s).

Acknowledgment—Duramycin was a generous gift from Dr. E. Racker.

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