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Tris Is a Competitive Inhibitor of K^+ Activation of the Vacuolar H^+-Pumping Pyrophosphatase[1]

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The effects of a range of commonly used pH buffers on the hydrolytic activity of the plant vacuolar H^+-transporting inorganic pyrophosphatase (V-PPase) from mung bean (*Vigna radiata* L.) hypocotyls were tested. All of the buffers inhibited K^+ stimulation of the V-PPase, and the degree of inhibition was dependent on the concentrations of both the buffer and K^+. The effects were dependent on the organic cation used in the buffers, and those tested inhibited in the order: Tris > Bis-Tris-propane > Bicine = Tricine > imidazole. Detailed studies revealed that a model in which Tris affects both the K_m and V_{max} for K^+ stimulation provided an accurate description of the observed kinetics. The ability of different cations to stimulate the V-PPase was measured with a noncompeting buffer (5 mM imidazole-HCl) and the order of effectiveness was $K^+ = Rb^+ > NH_4^+ \gg Cs^+ > Na^+ > Li^+$, with the K_m for K^+ stimulation being about 1 to 2 mM. Published experiments performed in the presence of Tris were re-evaluated and all could be fitted to mixed inhibition kinetics, with kinetic parameters similar to those measured for the mung bean V-PPase. It is concluded that the variations in the published K_m for K^+ stimulation of the V-PPase are probably due to the effects of pH buffer cations and that the real value for this parameter is in the low millimolar range. The implications of this for regulation of the V-PPase by K^+ in vivo and for the role of the enzyme in K^+ transport into the vacuole are discussed.

The V-PPase is one of two H^+ pumps that are responsible for the acidification of vacuolar sap (Rea and Sanders 1987; Rea and Poole, 1993; Leigh et al., 1994), with the other being a V-type ATPase (Rea and Sanders, 1987; Sze et al., 1992). The V-PPase shows an absolute dependence on K^+ for maximum activity (Walker and Leigh, 1981; Rea and Poole, 1985; Wang et al., 1986; White et al., 1990; Davies et al., 1991; Obermeyer et al., 1996), and patch-clamp experiments have indicated that it may be involved in K^+ transport into the vacuole (Davies et al., 1992; Obermeyer et al., 1996), although attempts to measure PPi-dependent K^+ uptake into vacuolar membrane vesicles using radioisotopes (Sato et al., 1994) or fluorometric probes (Ros et al., 1995) have failed to confirm this.

A single polypeptide of approximately 80 kD is sufficient to catalyze all known properties of the V-PPase (Kim et al., 1994a), and analysis of the cDNAs encoding V-PPases from a number of species has indicated a high degree of sequence homology between them (Sarafian et al., 1992; Rea and Poole, 1993; Tanaka et al., 1993; Kim et al., 1994b; Sakakibara et al., 1996), suggesting that the biochemical properties are also likely to be highly conserved. Therefore, it is surprising that the reported values for the K_m for K^+ stimulation of the V-PPase differ significantly between species, ranging from less than 4 mM to more than 60 mM (Walker and Leigh, 1981; Rea and Poole, 1985; Wang et al., 1986; Marquardt and Lüttge, 1987; White et al., 1990; Baykov et al., 1993). Whereas it is possible that the effects may be due to real differences in the affinities for K^+, perhaps determined by large sequence differences in the K^+-binding sites, widely differing assay conditions have been used and offer a more likely explanation for the range of values. White et al. (1990) found that Tris partially stimulated the V-PPase of *Kalanchoë daigremontiana*, indicating that this buffer may be capable of interacting with the K^+-binding site of the V-PPase. Such an effect could explain the variation in the published results for K^+ stimulation, since Tris has been widely used as a pH buffer in studies of the V-PPase. In addition, Tris and other amine buffers affect the kinetics of substrate binding to the H^+-pumping pyrophosphatase of the photosynthetic bacterium *Rhodospirillum rubrum* (Baykov et al., 1996).

In this paper we analyze the effects of Tris and some other cationic buffers on the hydrolytic activity of the V-PPase and demonstrate that some are inhibitory to the enzyme, with Tris showing the greatest effect. Tris exerts its effect through mixed inhibition of K^+ stimulation. We use this information to re-evaluate published data for K^+ stimulation of the V-PPase in some studies in which Tris was used as the pH buffer.

MATERIALS AND METHODS

All chemicals were from Sigma or BDH (Poole, Dorset, UK). Seeds of mung bean (*Vigna radiata* L.) were germinated and grown on water-saturated vermiculite in the dark at 25°C, and hypocotyls were harvested after 5 d. Tonoplast vesicles were isolated from the hypocotyls by the method of Rea et al. (1992). Vesicles were frozen in

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Abbreviations: Tricine, *N*-[Tris-(hydroxymethyl)methyl]glycine; V-PPase, vacuolar H^+-transporting inorganic pyrophosphatase.

liquid N_2 and stored at −80°C. V-PPase activity was measured as the hydrolysis of PPi at pH 8.0. Earlier studies (Wang et al., 1986; Leigh et al., 1992) showed that the kinetics and inhibitor sensitivities of PPi hydrolysis match those of H^+-pumping, and, thus, hydrolysis is a good measure of V-PPase activity. Since large concentrations of Tris were used in some experiments, H^+-pumping was not used as a measure of activity, because changes in the internal pH-buffering capacity of the tonoplast vesicles would have compromised an accurate comparison of the H^+-transport rates between different treatments. PPi hydrolysis was measured as described by Leigh et al. (1992), except that the medium contained 10 μg/mL L-α-lysophosphatidylcholine and 300 μg/mL Triton X-100. The concentrations of $MgSO_4$ and PPi-Bis-Tris-propane were 1.5 and 0.3 mM, respectively, and the pH buffer was as indicated in the figures and tables. Protein was assayed by a modification of the method of Appleroth and Angsten (1987) with BSA as a standard. V-PPase activities are reported as the amount of PPi hydrolyzed (Pi release ÷ 2), except in Figure 4 where they are reported in the units that were used in the original papers. Curve fitting was done with Sigmaplot 2.1 (Jandel Scientific, Ekrath, Germany).

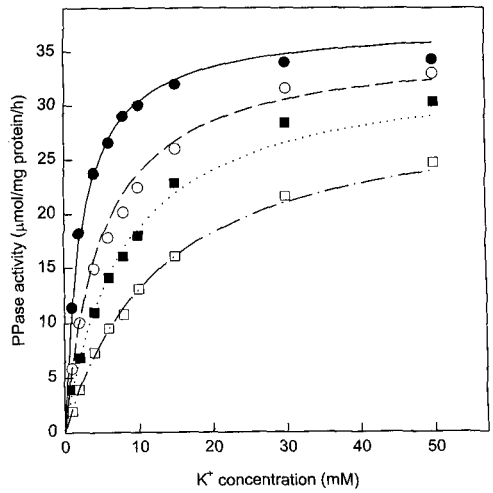

Figure 2. K^+ stimulation of the mung bean V-PPase activity in the presence of 5 (●), 25 (○), 50 (■), or 100 (□) mM Tris. The symbols indicate the experimental data and the lines are the activities calculated using Equation 1 with values for the kinetic parameters of V_{max} = 38.0 μmol mg^{-1} protein h^{-1}, K_m = 1.6 mM, K_{ic} = 11.0 mM, and K_{iu} = 395 mM.

RESULTS

The hydrolytic activity of the V-PPase was measured in the presence of 1 and 50 mM K^+ with different buffers present at concentrations of 25 to 100 mM (Fig. 1). With 50 mM KCl in the assay medium, activity was greatest with all of the buffers when they were at 25 mM. With the exception of imidazole-HCl, increasing concentrations of all of the buffers progressively decreased V-PPase activity (Fig. 1A). Bicine and Tris were the most inhibitory and, when present at 100 mM, these buffers supported only 47 and 57%, respectively, of the activity measured at 25 mM imidazole-HCl. When the analysis was repeated at a K^+ concentration of 1 mM, the results showed that the activity was maximal with imidazole. Activity was intermediate with Tricine and Bicine and least with Tris (Fig. 1B). Since Cl^- was the counteranion for most of the buffers (Cl^- concentration was assumed to be the same as that of the buffer cation), these inhibitory effects must be due to the nature of the organic cation used in the buffer.

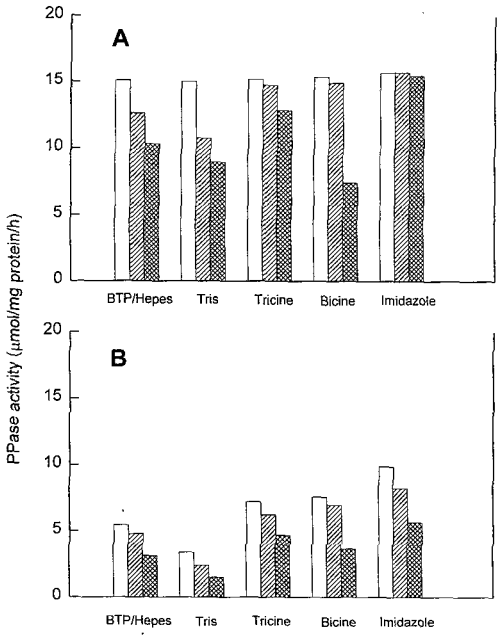

Figure 1. Effect of different pH buffers on the activity of the mung bean V-PPase. A, Activity measured in the presence of 50 mM KCl. B, Activity measured in the presence of 1 mM KCl. The buffer concentrations were 25 (open bars), 50 (hatched bars), or 100 mM (cross-hatched bars). BTP, Bis-Tris-propane.

To study the effect of Tris further, its influence on K^+ stimulation of the V-PPase was investigated over a wide range of K^+ concentrations. Increasing concentrations of Tris progressively inhibited the V-PPase at all K^+ concentrations (Fig. 2). Although results with small concentrations of Tris (up to 25 mM) could apparently be described by competitive inhibition between Tris and K^+ (data not shown), this model gave progressively poorer fits at larger Tris concentrations. However, a mixed inhibition model in which Tris affected both the K_m and the V_{max} for K^+ stimulation gave good fits at all Tris concentrations. This model is described by the following equation (Cornish-Bowden, 1976):

$$v = \frac{V_{max}s}{K_m\left(1 + \frac{i}{K_{ic}}\right) + s\left(1 + \frac{i}{K_{iu}}\right)} \quad (1)$$

where v is the rate of PPi hydrolysis, s is the K^+ concentration, V_{max} and K_m are the kinetic constants for K^+ stimulation, and K_{ic} and K_{iu}, respectively, are the inhibitor constants for competitive and uncompetitive inhibition of K^+ stimulation by Tris. Fitting this equation to results from four independent experiments yielded mean ± SE values

for the kinetic parameters of: K_m = 1.85 ± 0.15 mM, K_{ic} = 11.75 ± 0.75 mM, and K_{iu} = 392.5 ± 56.8 mM.

In view of this effect of Tris on the V-PPase, the stimulation of the enzyme by K^+ and other monovalent cations was re-evaluated using 5 mM imidazole-HCl as the buffer, i.e. conditions that promoted maximal activity at low K^+ concentrations (Fig. 1). Based on the V_{max} values, the tested cations stimulated the V-PPase in the order: $K^+ = Rb^+ > NH_4^+ \gg Cs^+ > Na^+ > Li^+$ (Fig. 3). This order of effectiveness is similar to that reported by others (Walker and Leigh, 1981; Wang et al., 1986; Marquardt and Lüttge, 1987; White et al., 1990; Obermeyer et al., 1996). The K_m values show the same ordering (Table I; Fig. 3). K^+, Rb^+, NH_4^+, and Cs^+ had K_m values in the 1 to 3 mM range, whereas the affinity of the V-PPase for Na^+ and Li^+ was about 10-fold lower.

To determine whether these effects of Tris can explain the variation in the published K_m values for K^+ stimulation of the V-PPase, the data from some earlier papers in which Tris was used as a pH buffer were re-examined to determine whether they could be fitted by the mixed inhibition model (Eq. 1). Baykov et al. (1993) determined the kinetics of the K^+ stimulation for the mung bean hypocotyl V-PPase with the Tris concentration either fixed at 100 mM or varied inversely with the KCl concentration to maintain a fixed ionic strength of 100 mM. They reported a K_m for K^+ of 40 mM in the presence of 83 μM Mg_2PPi (the substrate of the V-PPase; Leigh et al., 1992; Baykov et al., 1993; Gordon-Weeks et al., 1996) and 61 mM with 3.3 μM Mg_2PPi. However, as shown in Figure 4A, the results can be simulated very well by Equation 1 with values for the K_m for K^+ and K_{ic} and K_{iu} for Tris very similar to those derived from the experiments described above (Fig. 2). Walker and Leigh (1981) measured the K^+ stimulation of the red beet (*Beta vulgaris* L.) V-PPase with 49.5 mM Tris present in the medium and found that the results did not conform to Michaelis-Menten kinetics; therefore, they were unable to derive an accurate K_m for K^+ stimulation. Again, however, the results can be fitted with a mixed inhibition model, although the predicted K_m value for K^+ is higher than that for the mung bean enzyme (Fig. 4B). Finally, Rea and Poole (1985) assayed the red beet V-PPase in the presence of 52 mM Tris and, like Walker and Leigh (1981), were unable to derive a K_m for K^+ because of non-Michaelian kinetics. Their data can also be fitted to a mixed inhibition model and, in this case, the kinetic parameters are similar to those for the mung bean enzyme (Fig. 4C).

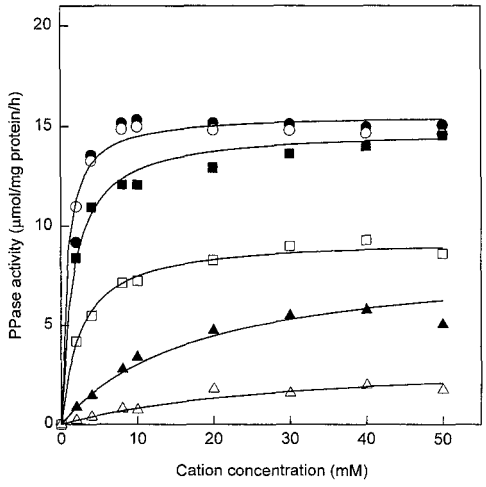

Figure 3. Cation stimulation of the mung bean V-PPase measured with 5 mM imidazole-HCl as the pH buffer. Symbols are experimental data for activities in the presence of K^+ (●), Rb^+ (○), NH_4^+ (■), Cs^+ (□), Na^+ (▲), or Li^+ (△). Lines are fitted using the Michaelis-Menten equation with the following parameters: K^+ and Rb^+, K_m = 0.75 mM, V_{max} = 15.59; NH_4^+, K_m = 1.57 mM, V_{max} = 14.84; Cs^+, K_m = 2.53 mM, V_{max} = 9.37; Na^+, K_m = 17.89 mM, V_{max} = 8.47; and Li^+, K_m = 32.57 mM, V_{max} = 3.43.

Table I. *K_m values for monovalent cation stimulation of the mung bean V-PPase*

Activity was measured with 5 mM imidazole-HCl as the pH buffer. Values are means ± SE of three separate independent experiments.

Cation	K_m
	mM
K^+	1.27 ± 0.23
Rb^+	1.20 ± 0.35
NH_4^+	1.83 ± 0.25
Cs^+	2.37 ± 0.44
Na^+	12.14 ± 2.48
Li^+	17.01 ± 6.45

Although the above results clearly show that Tris affects the V-PPase by decreasing K^+ stimulation, its effect on Mg^{2+} activation was also investigated because Baykov et al. (1993) reported that the kinetics of Mg^{2+} activation of the mung bean V-PPase were dependent on ionic strength when Tris concentrations were varied to alter this parameter. Thus, it is important to establish whether the effects they observed could be due to this buffer. In addition, Baykov et al. (1996) found that Tris can affect the binding of Mg_2PPi to the Mg^{2+}-loaded form of the PPase from *R. rubrum*. Therefore, the Mg^{2+} dependency of the mung bean V-PPase was determined at two PPi concentrations (0.1 or 1.5 mM) with the ionic strength allowed to vary with the $MgSO_4$ concentration or with it fixed at 100 mM by altering the concentration of imidazole-HCl to compensate for the different $MgSO_4$ additions. The results clearly show that the kinetics for Mg^{2+} activation of the enzyme are not affected by ionic strength (Fig. 5). Similar results were obtained when ionic strength was kept fixed at 100 mM by changing the concentration of either Tris-HCl or KCl (not shown). The kinetics are similar to those reported by Leigh et al. (1992) for the oat root V-PPase, and, in particular, the decline in activity at large Mg^{2+} concentrations in the presence of 1.5 mM PPi was observed under both ionic strength regimes (Fig. 5). This decline is thought to be due to noncompetitive inhibition by Mg_2PPi (Leigh et al., 1992).

DISCUSSION

The observation that Tris directly affects the interaction of K^+ with the V-PPase offers an explanation for discrepancies in published values for the K_m for K^+ stimulation of this enzyme and for the non-Michaelis-Menten kinetics

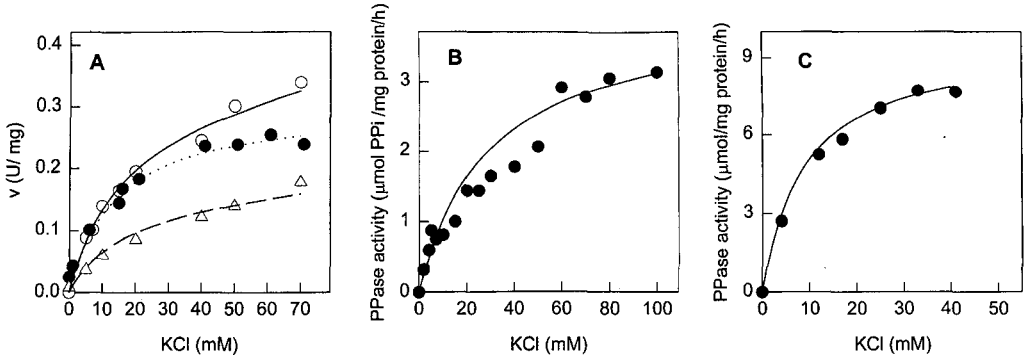

Figure 4. Re-evaluation of published experiments in which the K^+ stimulation of the V-PPase was measured in the presence of Tris. Symbols are the original experimental data and lines are activities calculated using Equation 1. A, Experiment from Baykov et al. (1993) with activities of the mung bean V-PPase measured in the presence of 83 (○, ●) or 3.3 (△) µM Mg_2PPi with the Tris concentration fixed at 100 mM (●) or varied to maintain an ionic strength of 100 mM (○, △). The kinetic parameters for the modeled lines are K_m = 1.90 mM, K_{ic} = 12.0 mM, K_{iu} = 395 mM, and V_{max} = 0.38 (solid and dotted lines) or 0.19 (dashed line) units mg^{-1} protein. B, Experiment from Walker and Leigh (1981) in which K^+ stimulation of the red beet V-PPase was measured with a constant Tris concentration of 49.5 mM. The parameters for the fitted line are K_m = 5.0 mM, K_{ic} = 9.0 mM, K_{iu} = 275 mM, and V_{max} = 4.75 µmol mg^{-1} protein h^{-1}. C, Experiment from Rea and Poole (1985) in which the K^+ stimulation of the red beet V-PPase was measured with a constant Tris concentration of 52 mM. The parameters for the fitted line are K_m = 2.0 mM, K_{ic} = 12.0 mM, K_{iu} = 350 mM, and V_{max} = 11.0 µmol mg^{-1} protein h^{-1}.

measured by some authors (Walker and Leigh, 1981; Rea and Poole, 1985). It appears from the analysis presented above that Tris (and probably some other buffer cations; Fig. 1) interferes with the K^+ stimulation of the V-PPase and that both the K_m and the V_{max} are changed. A reanalysis of published data (Fig. 4) shows that nonstandard kinetics or high K_m values for K^+ stimulation can be explained by mixed inhibition and that the K_m for K^+ is 1 to 2 mM (Table I; Fig. 2). The similarity of the K_m values for K^+ for V-PPases from different species is consistent with the high degree of sequence homology of the enzyme from different sources (Sarafian et al., 1992; Rea and Poole, 1993; Tanaka et al., 1993; Kim et al., 1994b; Sakakibara et al., 1996).

The low K_m for K^+ makes it unlikely that this cation has a regulatory role in modulating the activity of the enzyme in vivo. The cytoplasmic activity of K^+ has been measured at 70 to 80 mM except under conditions of extreme K^+ deficiency, when it can decline to about 40 mM in some cells (Walker et al., 1996; see also Maathuis and Sanders, 1993). Thus, under all conditions of K^+ supply the cytosolic K^+ concentration will be sufficient to fully stimulate the V-PPase, and so the ion will never limit the enzyme's activity.

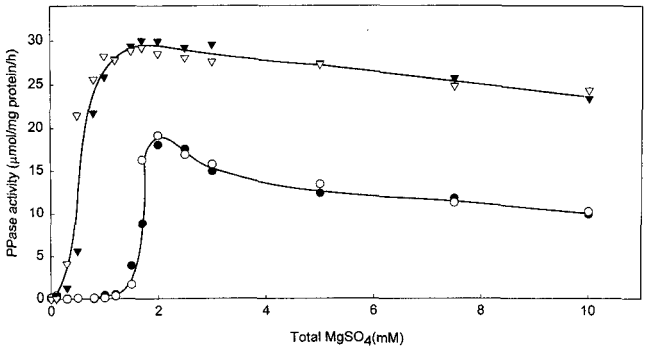

Figure 5. Effect of varying the total Mg^{2+} concentration on activity of the mung bean V-PPase in the presence of 50 mM KCl at fixed total PPi concentrations of 0.1 mM (▽, ▼) or 1.5 mM (●, ○). Ionic strength was kept constant at 100 mM by varying the concentration of imidazole-HCl (●, ▼) or allowed to vary at a constant imidazole-HCl concentration of 25 mM (○, ▽).

There is currently some conflict over the possible role of the V-PPase in K^+ transport into the vacuole. Davies et al. (1992) and Obermeyer et al. (1996), using patch clamping, obtained evidence for such a role, but radiotracer experiments with reconstituted V-PPase (Sato et al., 1994) and fluorescent probe measurements with tonoplast vesicles (Ros et al., 1995) have failed to confirm this. It is interesting that the patch-clamp experiments of Davies et al. (1992) were done with up to 37 mM Tris in the extravacuolar medium, and K^+ concentrations on each side of the membrane varied between 30 and 100 mM. The vacuoles used in this study were from sugar beet, and calculations using the kinetic parameters for red beet in Figure 4, B and C, suggest that the increase in K^+ concentration would stimulate the V-PPase by 25 to 66%, depending on which set of values are used. Thus, at least part of the increased current flow measured by Davies et al. (1992) could have resulted from this relief of Tris inhibition. However, Obermeyer et al. (1996) also found that PPi-dependent currents into vacuoles of *Chenopodium rubrum* were larger with K^+ than with other monovalent cations. These authors used 5 mM Hepes-Bis-Tris-propane as the pH buffer in the presence of 50 mM KCl, and under these conditions it is unlikely that the buffer was interfering with interactions between K^+ and the V-PPase (Fig. 1). In addition, a key observation of Davies et al. (1992) was that the reversal potential for the PPi-dependent current was dependent on the concentrations of both H^+ and K^+, as expected if both ions are transported. Even if Tris was interfering with K^+ stimula-

tion in their experiments, this would not have affected the reversal potential. Overall, the results are still consistent with a role for the V-PPase in K^+ transport. Nonetheless, there would seem to be value in a re-examination of these experiments using a pH buffer that does not affect K^+ stimulation.

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LITERATURE CITED

Appleroth KJ, Angsten H (1987) An improvement of the protein determination in plant tissues with the dye binding method according to Bradford. Biochem Physiol Pflanz **182:** 85–89

Baykov A, Bakaleva NP, Rea PA (1993) Steady-state kinetics of substrate hydrolysis by vacuolar H^+-pyrophosphatase. A simple three state model. Eur J Biochem **217:** 755–762

Baykov AA, Sergina NV, Evtushenko OA, Dubnova EB (1996) Kinetic characterization of the hydrolytic activity of the H^+-pyrophosphatase of *Rhodospirillum rubrum* in membrane-bound and isolated states. Eur J Biochem **236:** 121–127

Cornish-Bowden A (1976) Principles of Enzyme Kinetics. Butterworths, London

Davies JM, Poole RJ, Rea PA, Sanders D (1992) Potassium transport into plant vacuoles energized directly by a proton-pumping inorganic pyrophosphatase. Proc Natl Acad Sci USA **89:** 11701–11705

Davies JM, Rea PA, Sanders D (1991) Vacuolar proton pumping pyrophosphatase in *Beta vulgaris* shows vectorial activation by potassium. FEBS Lett **278:** 66–68

Gordon-Weeks R., Steele SH, Leigh RA (1996) The role of magnesium, pyrophosphate and their complexes as substrates and activators of the vacuolar H^+-pumping inorganic pyrophosphatase. Studies using ligand protection from covalent inhibitors. Plant Physiol **111:** 195–202

Kim EJ, Zhen R-G, Rea PA (1994a) Heterologous expression of plant vacuolar pyrophosphatase in yeast demonstrates sufficiency of the substrate-binding subunit for proton transport. Proc Natl Acad Sci USA **91:** 6128–6132

Kim Y, Kim EJ, Rea PA (1994b) Isolation and characterization of cDNAs encoding the vacuolar H^+-pyrophosphatase of *Beta vulgaris*. Plant Physiol **106:** 375–382

Leigh RA, Gordon-Weeks R, Steele SH, Koren'kov VD (1994) The H^+-pumping pyrophosphatase of the vacuolar membrane of higher plants. *In* MR Blatt, RA Leigh, D Sanders, eds, Membrane Transport in Plants and Fungi. Company of Biologists, Cambridge, UK, pp 61–75

Leigh RA, Pope AJ, Jennings IR, Sanders D (1992) Kinetics of the vacuolar H^+-pyrophosphatase from higher plants. The role of magnesium, pyrophosphate and their complexes as substrates, activators and inhibitors. Plant Physiol **100:** 1698–1705

Maathuis FJM, Sanders D (1993) Energization of potassium uptake in *Arabidopsis thaliana*. Planta **191:** 302–307

Marquardt G, Lüttge U (1987) Proton transporting enzymes at the tonoplast of leaf cells of the CAM plant *Kalanchoë daigremontiana*. II. The pyrophosphatase. J Plant Physiol **129:** 269–286

Obermeyer G, Sommer A, Bentrup FW (1996) Potassium and voltage dependence of the inorganic pyrophosphatase of intact vacuoles from *Chenopodium rubrum*. Biochim Biophys Acta **1284:** 203–212

Rea PA, Britten CJ, Sarafian V (1992) Common identity of substrate-binding subunit of vacuolar H^+-translocating inorganic pyrophosphatase of higher plant cells. Plant Physiol **100:** 723–732

Rea PA, Poole RJ (1985) Proton-translocating inorganic pyrophosphatase of red beet (*Beta vulgaris* L.) tonoplast vesicles. Plant Physiol **77:** 46–52

Rea PA, Poole RJ (1993) Vacuolar H^+-translocating pyrophosphatase. Annu Rev Plant Physiol Plant Mol Biol **44:** 157–180

Rea PA, Sanders D (1987) Tonoplast energisation: two H^+ pumps, one membrane. Physiol Plant **71:** 131–141

Ros R, Romieu C, Gibrat R, Grignon C (1995) The plant inorganic pyrophosphatase does not transport K^+ in vacuole membrane vesicles multilabeled with fluorescent probes for H^+, K^+, and membrane potential. J Biol Chem **270:** 1–7

Sakakibara Y, Kobayashi H, Kasamo K (1996) Isolation and characterisation of cDNAs encoding vacuolar H^+-pyrophosphatase isoforms from rice (*Oryza sativa* L.). Plant Mol Biol **31:** 1029–1038

Sarafian V, Kim Y, Poole RJ, Rea PA (1992) Molecular cloning and sequence of cDNA encoding the pyrophosphate-energized vacuolar membrane proton pump of *Arabidopsis thaliana*. Proc Natl Acad Sci USA **89:** 1775–1779

Sato MH, Kasahara M, Ishii N, Homareda H, Matsui H, Yoshida M (1994) Purified vacuolar inorganic pyrophosphatase consisting of a 75-kDa polypeptide can pump H^+ into reconstituted proteoliposomes. J Biol Chem **269:** 6725–6728

Sze H, Ward JM, Lai S (1992) Vacuolar H^+-translocating ATPases from plants: structure, function and isoforms. J Bioenerg Biomembr **24:** 371–381

Tanaka Y, Chiba K, Maeda M, Maeshima M (1993) Molecular cloning of cDNA for vacuolar membrane proton translocating inorganic pyrophosphatase in *Hordeum vulgare*. Biochem Biophys Res Commun **190:** 1110–1114

Walker DJ, Leigh RA, Miller AJ (1996) Potassium homeostasis in vacuolate plant cells. Proc Natl Acad Sci USA **93:** 10510–10514

Walker RR, Leigh RA (1981) Mg^{2+}-dependent, cation-stimulated inorganic pyrophosphatase associated with vacuoles isolated from storage roots of red beet (*Beta vulgaris* L.). Planta **153:** 150–155

Wang Y, Leigh RA, Kaestner KH, Sze H (1986) Electrogenic H^+-pumping pyrophosphatase in tonoplast vesicles of oat roots. Plant Physiol **81:** 497–502

White PJ, Marshall J, Smith JAC (1990) Substrate kinetics of the tonoplast H^+-translocating inorganic pyrophosphatase and its activation by free Mg^{2+}. Plant Physiol **93:** 1063–1070