Planta (1998) 206: 272–277

Planta

Tonoplast inorganic pyrophosphatase in *Vicia faba* guard cells

Catherine P. Darley[1]*, Lorna, A. Skiera[1], Frederick D. Northrop[2], Dale Sanders[1], Julia M. Davies[2]

[1]Plant Laboratory, Department of Biology, University of York, P.O. Box 373, York YO1 5YW, UK
[2]Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA, UK

Received: 12 February 1998 / Accepted: 24 April 1998

Abstract. Lysed guard-cell protoplasts of *Vicia faba* L. exhibited hydrolytic activity characteristic of tonoplast inorganic pyrophosphatase (V-PPase; EC 3.6.1.1). Activity was inhibited by the specific V-PPase inhibitor aminomethylenediphosphonate, stimulated by K^+ (K_m = 51 mM) and inhibited by Ca^{2+} (80 nM free Ca^{2+} was required for 50% inhibition at 0.27 mM free Mg^{2+}). Patch-clamp measurements of electrogenic activity confirmed enzyme localisation at the tonoplast. This is the first report of V-PPase activity in guard cells; its possible involvement in stomatal opening is discussed.

Key words: Guard cell – Pyrophosphatase (V-PPase) – Stoma – Tonoplast – Vacuole – *Vicia* (guard cells)

Introduction

The plant tonoplast is accepted as being energised by two electrogenic H^+-pumps; an inorganic pyrophosphatase (V-PPase; EC 3.6.1.1) and an adenosine triphosphatase (V-ATPase; EC 3.6.1.3). Studies on non-stomatal vacuoles suggest the two pumps may have disparate physiological roles despite a common function of vacuolar acidification and establishment of a H^+ electrochemical potential difference (reviewed by Davies 1997). The V-PPase has been implicated in the maintenance of tonoplast energisation under stress conditions (low temperature and anoxia) which would restrict ATP supply and impair V-ATPase activity (Carystinos et al. 1995; Darley et al. 1995). Moreover, while the V-ATPase may be the prime determinant of the extent of vacuolar acidification (Davies et al. 1994), the V-PPase might provide a direct transport pathway for K^+ (Obermeyer et al. 1996).

Despite these advances in our understanding of vacuolar H^+-pumps their significance in stomatal physiology remains largely unexplored. Vacuolar ATPase activity has been reported in guard cells of *Commelina communis* (Fricker and Willmer 1990; Willmer et al. 1995) and *Vicia faba* (Hedrich et al. 1988) yet to date the existence of guard-cell tonoplast V-PPase activity has been merely assumed rather than tested. This lack of affirmation of guard-cell tonoplast V-PPase in turn renders incomplete not only our understanding of vacuolar energisation under stress but also the consequences of stomatal Ca^{2+} signalling. Of the two vacuolar H^+-pumps, the V-PPase of non-stomatal cells has been demonstrated to be extremely sensitive to inhibition by Ca^{2+}, with free cytosolic Ca^{2+} [$(Ca^{2+})_f$] as the most likely inhibitory species (Rea et al. 1992). Diffferential regulation of guard-cell vacuolar pumps by $(Ca^{2+})_f$ could afford subtle control of tonoplast energisation pertinent to stomatal function.

In this study, a pyrophosphatase hydrolytic activity (designated "PPase") has been partially characterised in lysed *V. faba* guard-cell protoplasts. This PPase activity has been tested for (i) sensitivity to the specific V-PPase inhibitor aminomethylenediphosphonate (AMDP; Zhen et al. 1994); (ii) stimulation by K^+ (Wang et al. 1986) and (iii) inhibition by Ca^{2+} (Rea et al. 1992) to assess its similarity to previously characterised V-PPases. The specific residency of PPase activity at the guard-cell tonoplast has been examined by measuring PPi-elicited currents from isolated, intact vacuoles using patch-clamp electrophysiology. This unequivocal localisation of an electrogenic PPase establishes for the first time that guard-cell vacuoles are indeed equipped with a V-PPase. Possible V-PPase involvement in stomatal physiology is discussed.

**Present address*: Department of Genetics, Faculty of Biology, Vrije Universiteit, DeBoelelaan 1087, NL-1081 HV Amsterdam, Netherlands

Abbreviations: AMDP = aminomethylenediphosphonate; BTP = bis-tris-propane; I/V = current/voltage; $K_{0.5}$ = constant for 50% maximal inhibition; V-ATPase = vacuolar-type ATPase; (V-)PPase = (Vacuolar-) inorganic pyrophosphatase; $(\ )_f$ = free ion concentration; $(\ )_{tot}$ = total species concentration

Correspondence to: J.M. Davies; E-mail: jmd32@cam.ac.uk; Fax: 44 (1223) 333 953; Tel.: 44 (1223) 333 939

Materials and methods

Isolation of guard-cell protoplasts and vacuoles. *Vicia faba* L. (cv. Long Pod) plants were grown and guard-cell protoplasts prepared as described by Allen and Sanders (1994). For spectrophotometric studies, protoplasts were suspended in 500 µl Buffer A (in mM: 10 ascorbic acid, 1 $CaCl_2$, 500 D-sorbitol, pH 6.4 with NaOH). Only preparations containing over 90% guard-cell protoplasts (identified by chloroplast morphology) were used. Lysis was by 4-fold dilution (to a total 2 ml volume) with Buffer B [in mM; 2 EGTA, 10 Hepes-KOH (pH 8.0), 100 D-sorbitol (>99% purity; Fluka Chemicals, Gillingham, UK)]. For patch clamping, protoplasts were suspended in 2 ml Buffer A; a 200-µl sample was placed in a recording chamber and lysed by superfusion with Buffer C (as Buffer B but with 100 mM KCl replacing D-sorbitol).

Hydrolysis assay solutions and protocol. The standard basal reaction mixture for PPase and total ATPase hydrolytic activity determinations contained (in mM): 50 KCl, 1 Na_2MoO_4, 0.005 gramicidin-D, 30 BTP-Mes (Bistrispropane and 2-(N-morpholino) ethanesulfonic acid, respectively), pH 8.0. For standard PPase assays, the medium contained 1.6 mM $MgSO_4$ and 0.6 mM BTP-PPi; for ATPase assays, 3 mM $MgSO_4$ and 3 mM BTP-ATP. Preparation of BTP-PPi and BTP-ATP from sodium salts (Sigma, Poole, UK) was by cation-exchange chromatography and titration with BTP. Aminomethylenediphosphonate (AMDP) was added to a final concentration of 100 µM for specific inhibition of V-PPase activity (Zhen et al. 1994). The specific V-ATPase inhibitor bafilomycin A_1 (Bowman et al. 1988) was added to a final concentration of 100 nM from a stock solution in dimethylsulfhoxide (DMSO). The final concentration of DMSO did not exceed 0.1% (v/v) and control experiments showed no DMSO effect on ATPase activity. In PPase K^+-dependency experiments, the KCl concentration of the standard assay was varied from 0 to 200 mM reciprocally with choline-Cl to maintain constant ionic strength and Cl^- concentration. For PPase Ca^{2+}-sensitivity assays at fixed free Mg^{2+} [$(Mg^{2+})_f$], the basal reaction mixture contained (in mM): 100 KCl, 5 EGTA, 0.005 gramicidin-D, 1 Na_2MoO_4, 50 mM BTP-Mes, 0.25 mM BTP-PPi, pH 7.5. Free Mg^{2+} and Ca^{2+} [$(Ca^{2+})_f$] were generated by the addition of $MgSO_4$ (0–3.3 mM total) and $CaCl_2$ (0–4.81 mM total) respectively. Concentrations of free ions and PPi-metal complexes were estimated with either the "SOLCON" program (D.C.S. White, University of York, UK and Y.E. Goldman, University of Pennsylvania, USA) or "calcium.exe" (W. Warchol, M. Gratzl and K.J. Foehr, University of Ulm, Germany) using the dissociation constants given by Leigh et al. (1992).

Contamination of the assay solutions by vacuolar calcium was considered to be insignificant. It was estimated that even if all the vacuoles contained and then released 5 mM calcium, that addition would be in the sub-micromolar range in the final assay and (with the buffering employed) would make a negligible impact on final free calcium levels. Reagent-derived total calcium contamination of the hydrolysis assay was estimated to be approximately 25 µM at maximum, which would not have had a significant effect on final free calcium. This was supported by previous Ca^{2+}-selective microelectrode measurements on assay media (data not shown).

Hydrolysis assay protocol and protein determination. Guard-cell protein (12–14 µg; approximately 7500–8400 protoplasts respectively) was added to the assay reaction mixture (final volume 300 µl) and incubated for 20 min at 37 °C (Darley et al. 1995). The reaction was stopped by the addition of 0.9 ml Ames reagent [6 parts 0.4% (w/v) ammonium molybdate in 0.5 M H_2SO_4 to 1 part 10% (w/v) ascorbic acid] and the mixture allowed to stand at room temperature for 30 min. Absorbance at 820 nm was measured in cuvettes with a 1-cm light path (OPI-2 spectrophotometer; Shimadzu, Tokyo, Japan). Absorbance readings were corrected using controls containing membrane protein but no pump substrate in the assay solution. To allow PPase and ATPase activities to be compared on a mole/mole basis, PPase activity was calculated as one-half the rate of Pi liberation (equivalent to nmol PPi consumed per unit time; Darley et al. 1995). Three separate guard-cell preparations were tested and triplicate samples used for each Pi determination. Protein was determined using the BioRad assay (BioRad, Munich, Germany) using bovine γ-globulin as a standard.

Patch-clamp procedures and analysis. Vacuoles originating from guard-cell protoplasts were identified by the adherence of grey-green chloroplasts (Allen and Sanders 1994). The basal bathing medium contained (in mM); 100 KCl, 1 $MgCl_2$, 0.1 EGTA, 20 BTP-Mes, pH 8.0. Pipettes were filled with (in mM); 100 KCl, 1 $MgCl_2$, 0.1 EGTA, pH 6.0. Circuitry, attainment of the whole vacuole configuration and current/voltage (I/V) protocols were as described in Davies et al. (1991). Positive charge flow from the bath (cytosolic membrane face) to the vacuolar lumen was defined as outward current, displayed in the upper half of I/V plots.

Results

Pyrophosphate- and ATP-dependent hydrolytic activities. On transfer of protoplasts to hypotonic Buffer B, 5- and 6-fold increases in total ATPase and PPase activities (respectively) were observed, indicating effective guard-cell lysis (Table 1). Mean (±SE) total PPase hydrolytic activity (in standard basal medium) was 66 ± 7 nmol · mg protein^{-1} · min^{-1} in lysed guard cells (n = 3 independent experiments). Activity was inhibited by 80% with 100 µM AMDP to a mean value of 13 ± 3 nmol · mg protein^{-1} · min^{-1}. This AMDP concentration has been reported to have no effect on soluble PPases (Zhen et al. 1994), suggesting that the majority of total PPase activity here represented that of a membrane-bound PPase. The origin of the AMDP-insensitive activity was unknown (given the incorporation of molybdate in the assay) and has not been explored further. Hydrolytic activity (PPase or ATPase) prior to lysis has not been examined further but could be due to mechanical protoplast rupture. The mean total specific ATPase activity in lysed protoplasts (87 ± 14 nmol · mg protein^{-1} · min^{-1}, n = 3) was inhibited 82% by bafilomycin A_1, suggesting that the greater part of the activity was from membrane-bound V-ATPase.

Effect of total PPi concentration. Pyrophosphate substrate dependency of hydrolytic activity in lysed

Table 1. Hydrolytic activities of PPase and ATPase populations in preparations of intact (pre-lysis) and lysed *Vicia faba* guard-cell protoplasts. The standard basal reaction mixture was used, as described in *Materials and methods*. Either AMDP (100 µM) or bafilomycin A_1 (100 nM) was incorporated as appropriate. Results are the means ± SE of three independent trials

Treatment	Hydrolytic activity, nmol · mg protein^{-1} · min^{-1}	
	+PPi	+ATP
Pre-lysis	11 ± 2	17 ± 2
Lysed	66 ± 7	87 ± 14
Lysed + AMDP	13 ± 3	not tested
Lysed + bafilomycin	not tested	16 ± 2

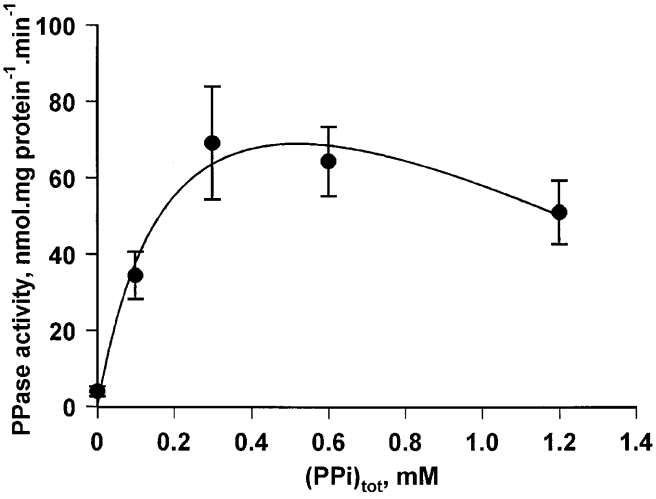

Fig. 1. Total pyrophosphatase hydrolytic activity in lysed guard-cell protoplasts of *Vicia faba* as a function of $(PPi)_{tot}$. Activities were determined using the standard basal assay mixture; total Mg^{2+} was 1.6 mM throughout. Results are means ± SE of three independent experiments

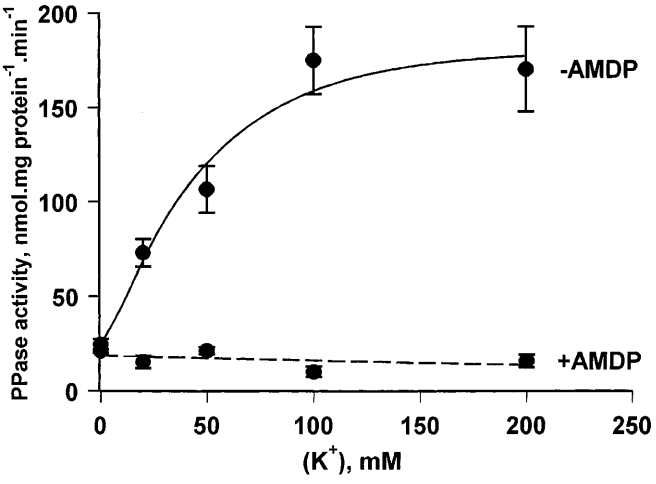

Fig. 2. Dependence of pyrophosphatase hydrolytic activity in lysed *V. faba* protoplasts on K^+. The standard basal assay was modified with KCl/choline-Cl as described in the text. The V-PPase inhibitor AMDP was added at 100 μM (*dashed line*). Results are the means ± SE of three independent assays

protoplasts is shown in Fig. 1. At a fixed total Mg^{2+} $[(Mg)_{tot}]$ concentration of 1.6 mM, peak measured total PPase activity (69 ± 15 nmol · mg protein^{-1} · min^{-1}, $n = 3$) occurred at 0.3 mM total PPi $[(PPi)_{tot}]$. Variation of PPi-dependent activity with $(PPi)_{tot}$ exhibited apparent substrate inhibition when $(PPi)_{tot}$ exceeded 0.6 mM, an observation which qualitatively agrees with those on non-stomatal preparations (Leigh et al. 1992; White et al. 1990). The low activity (representing approximately 3 μM Pi) observed with ostensibly no PPi in the assay is not readily explained given that controls containing lysed protoplasts but no added substrate were run to compensate for possible contamination by cytoplasmic PPi and Pi, released upon protoplast lysis.

Stimulation of pyrophosphatase hydrolytic activity by K^+. A characteristic of tonoplast V-PPase activity is its complete dependence on K^+ (Wang et al. 1986). In lysed guard-cell protoplasts, hydrolytic activity elicited by 0.6 mM $(PPi)_{tot}$ in the presence of 1.6 mM $(Mg)_{tot}$ increased with increasing K^+ concentration (Fig. 2), saturating at approximately 200 mM. Some limited activity was apparent with no added K^+ (mean, 24 nmol · mg protein^{-1} · min^{-1}; $n = 3$) which might be explained by assay contamination with cytosolic K^+. However, AMDP (100 μM) inhibited K^+-stimulated activity to levels similar to those recorded in ostensibly K^+-free conditions, strongly suggesting that the former was generated by a membrane-bound V-PPase population. A K_m for K^+ of 51 mM was estimated using a Lineweaver-Burke plot for the K^+-stimulated, AMDP-sensitive activity (i.e. that representing the difference between control and AMDP-inhibited activity). The origin of the AMDP-insensitive activity was not investigated.

Effect of divalent ions on hydrolytic activity. Previous work on *Beta vulgaris* storage root tissue V-PPase by Rea et al. (1992) showed that the enzyme was acutely sensitive to $(Ca^{2+})_f$ at a set $(Mg^{2+})_f$ of 0.27 mM. Here, the assay conditions used by Rea et al. (1992) for free divalent-ion and K^+ concentrations, pH and $(PPi)_{tot}$ have been duplicated to permit direct comparison. Pyrophosphatase activity was assayed at $(Mg^{2+})_f$ of 0.27 or 3.0 mM. With no Ca^{2+} present (Fig. 3), *V. faba* guard cell PPase activity was higher at 0.27 mM $(Mg^{2+})_f$ (56 ± 3 nmol · mg protein^{-1} · min^{-1}; $n = 3$) than 3.0 mM (38 ± 9 nmol · mg protein^{-1} · min^{-1}; $n = 3$). This is in contrast to *B. vulgaris*, where activity was higher at 3.0 mM $(Mg^{2+})_f$. However, as with

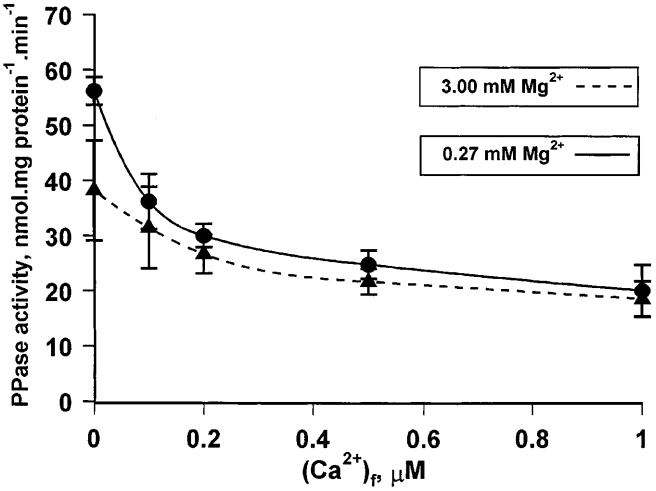

Fig. 3. Variation of pyrophosphatase hydrolytic activity at 0.27 and 3.0 mM free Mg^{2+} as a function of free Ca^{2+}. Assay conditions are described in the text. Total Ca^{2+} ranged from 0 to 4.81 mM at $(Mg^{2+})_f$ 0.27 mM (*circles*, *solid line*) and 0 to 4.75 mM at $(Mg^{2+})_f$ 3 mM (*triangles*, *dashed line*). Results are the means ± SE of three independent experiments

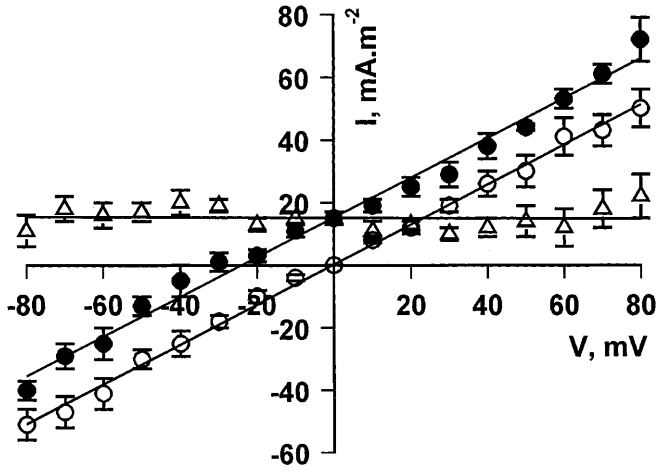

Fig. 4. Mean I/V relationships from five *V. faba* vacuoles. Data were acquired as described in the text. *open circles*, control relationship; *closed circles*, +100 μM $(PPi)_{tot}$; *open triangles*, difference representing PPi-dependent current

B. vulgaris, total *V. faba* PPase activity was inhibited by Ca^{2+} at both $(Mg^{2+})_f$; Fig. 3 illustrates this inhibition, considered as the consequence of $(Ca^{2+})_f$ increasing from 0 to 1 μM. Mean estimated $K_{0.5}$ values (where $K_{0.5}$ is the level of $(Ca^{2+})_f$ required for 50% maximal inhibition; Rea et al. 1992) were 80 nM and 160 nM at 0.27 and 3.0 mM $(Mg^{2+})_f$ respectively. This compares with respective values of 2.9 μM and 2.2 μM for *B. vulgaris* V-PPase (Rea et al. 1992).

Pyrophosphatase-dependent currents from guard-cell tonoplasts. With tonoplast voltage clamped at 0 mV in "whole vacuole" mode, addition of 100 μM $(PPi)_{tot}$ to the bathing medium resulted in an outward current (i.e. positive charge translocation into the vacuolar lumen) of mean (±SE) density 15.4 ± 1.8 mA · m^{-2} (results from five separate vacuoles). The current evoked by 100 μM $(PPi)_{tot}$ varied in density from 11 to 21 mA · m^{-2}. These values are in the range reported for V-PPase from other cell types (Hedrich et al. 1989; Davies et al. 1991; Obermeyer et al. 1996). The mean whole membrane I/V relationships and mean PPi-dependent current (derived by subtraction of control I/V relationship from that obtained in the presence of PPi) are presented in Fig. 4. The I/V relationship of the PPi-dependent current is consistent with the operation of a V-PPase in its forward mode with PPi hydrolysis coupled to H^+ translocation into the lumen (Davies et al. 1991). The effect of $(PPi)_{tot}$ on the magnitude of evoked current at 0 mV is shown in Table 2, together with estimates of (Mg_2PPi) (the putative substrate of V-PPase; Leigh et al. 1992, Gordon-Weeks et al. 1996), (MgPPi) and $(Mg^{2+})_f$ (a putative activator species; Leigh et al. 1992; Rea et al. 1992).

Discussion

Appreciable PPi hydrolysis by lysed *V. faba* guard-cell protoplasts was observed in this study. The detection of bafilomycin-sensitive ATP hydrolysis (indicating V-ATPase activity) tends to confirm that lysis was effective in exposing the tonoplast. That the majority of PPase activity was inhibited by an AMDP concentration shown previously to be specific to V-PPase (Zhen et al. 1994) suggests the existence of a membrane-bound PPase (i.e. V-PPase) population.

The location of the PPase activity cannot be concluded to be at the tonoplast simply through coincident observation of a bafilomycin-sensitive ATPase activity. Protoplast lysis would also have exposed the cytosolic face of the plasma membrane. Recent studies have shown that higher-plant V-PPase may also be located in that membrane (e.g. Long et al. 1997). The possibility of plasma-membrane V-PPase residence was not addressed here. However, immunofluorescence studies on wheat leaf tissue (using an antibody directed against a synthetic oligopeptide corresponding to the putative hydrophilic loop IV of *Arabidopsis thaliana* V-PPase; Sarafian et al. 1992) have not supported the existence of V-PPase polypeptide in guard-cell plasma membrane (Darley 1997). Rather, unequivocal localisation of PPase to the *V. faba* guard-cell tonoplast has been afforded by detection of PPi-dependent electrogenic activity. Taken together, the patch-clamp results and AMDP-sensitivity of hydrolytic activity point to the presence of a functional V-PPase in guard-cell tonoplast.

In addition to AMDP susceptibility, the PPase hydrolytic activity showed other hallmarks of higher-plant tonoplast V-PPase. Firstly, variation of activity with $(PPi)_{tot}$ qualitatively resembled the characteristic non-Michaelis Menten response shown previously by V-PPase from *Avena sativa* roots (Leigh et al. 1992), *Kalanchoë daigremontiana* mesophyll (White et al. 1990) and *Vigna radiata* hypocotyls (Gordon-Weeks et al. 1996). This apparent inhibition by $(PPi)_{tot}$ as a "gross" substrate was also observed in the patch-clamp studies of current density at 0 mV as a function of $(PPi)_{tot}$ (Table 2). It is now thought that Mg_2PPi (rather than MgPPi) is the true V-PPase substrate and also effects non-competitive inhibition at high concentrations (Leigh et al. 1992; Gordon-Weeks et al. 1996). The

Table 2. Outward PPi-dependent current density at a membrane voltage of 0 mV, generated by the addition of varying $(PPi)_{tot}$. Results are expressed as mean ± SE (*n* independent trials). Estimates of species were made using the SOLCON program (see *Materials and methods*)

$(PPi)_{tot}$, μM	I, mA · m^{-2}	(Mg_2PPi), μM	(MgPPi), μM	$(Mg^{2+})_f$, mM
100	15.4 ± 1.8 (5)	12.00	75.18	0.74
200	24.6 ± 4.2 (5)	22.79	150.82	0.66
500	6.8 ± 1.7 (5)	35.98	372.76	0.44

results shown in Table 2 do not allow discrimination between the effects on activity of these two PPi complexes. The observed near 2-fold increase in current density between 100 and 200 μM $(PPi)_{tot}$ was matched by an estimated 2-fold increase in both (Mg_2PPi) and (MgPPi). As activity fell at 500 μM $(PPi)_{tot}$, estimated concentrations of both complexes increased. Further kinetic studies are now required to identify the guard-cell tonoplast V-PPase substrate and ascertain the degree of reaction-scheme similarity to that of other V-PPases.

The marked dependence of AMDP-sensitive PPase hydrolytic activity on K^+ is also indicative of a tonoplast V-PPase population (Wang et al. 1986). The K_m for K^+ stimulation (51 mM) is higher than that reported for other V-PPase activities; e.g. 40 mM, *Vigna radiata* hypocotyls (Baykov et al. 1993); 11 mM, *Chenopodium rubrum* suspension cells (Obermeyer et al. 1996). The value estimated here may well be an overestimate because BTP was used as a buffer; Gordon-Weeks et al. (1997) have recently discovered that BTP can inhibit K^+-stimulated V-PPase activity, resulting in overestimates of K_m of an order of magnitude. A full characterisation of guard-cell V-PPase K^+ relations was beyond the aim of this study but, having demonstrated hydrolytic K^+ depedendency, future work must now relate V-PPase activity to guard-cell K^+ status, which is known to fluctuate with stomatal movement (Assmann 1993).

Inhibition by Ca^{2+} is a characteristic of both soluble and membrane-bound PPases, and for the V-PPase of *Beta vulgaris* inhibition is most likely effected by the free ion (Rea et al. 1992). Here, when the effect of $(Ca^{2+})_f$ on guard-cell PPase hydrolytic activity was considered, the enzyme appeared far more sensitive to $(Ca^{2+})_f$ at low $(Mg^{2+})_f$ than *B. vulgaris*. Also, although the *V. faba* preparation was crude, its PPase activity decreased with increasing $(Mg^{2+})_f$ at zero $(Ca^{2+})_f$, whereas that of *B. vulgaris* increased (Rea et al. 1992). Moreover (and in contrast to *B. vulgaris*), the *V. faba* $K_{0.5}$ for $(Ca^{2+})_f$ increased with $(Mg^{2+})_f$ (see *Results*). For *B. vulgaris* the relative insensitivity of $K_{0.5}$ for $(Ca^{2+})_f$ to changes in $(Mg^{2+})_f$ led (in part) to the conclusion that free Ca^{2+} can bind to a specific inhibitory site on the V-PPase whilst free Mg^{2+} can occupy a discrete activator site. The finding that for *V. faba* the $K_{0.5}$ for free Ca^{2+} increases with $(Mg^{2+})_f$ indicates that a simpler model of two free ions competing for a single binding site may be applicable. Again, further kinetic studies are now required to assess the likelihood of free Ca^{2+} being the inhibitory species of the guard-cell V-PPase and to establish a reaction scheme.

It is perhaps the sensitivity of the guard-cell V-PPase to Ca^{2+} that is of greatest interest in terms of stomatal function, given the pivotal role of free Ca^{2+} in guard-cell intracellular signalling (Assmann 1993). Placing the observed V-PPase responses into a precise physiological context is not feasible at this time but the following points should be considered. Firstly, studies on *C. communis* guard-cell tonoplast V-ATPase suggest a low sensitivity to Ca^{2+} (Willmer et al. 1995), which is in contrast to the present findings on the guard-cell V-PPase. Such results may be indicative of differential control of the tonoplast H^+-pumps by free Ca^{2+}. Further, if it were borne out that free Ca^{2+} were the V-PPase inhibitory species, an inhibitor constant for free Ca^{2+} of 80 nM at 0.27 mM $(Mg^{2+})_f$ (which is close to the putative physiological $(Mg^{2+})_f$ of 0.4 mM; Yazaki et al. 1988) would imply that pump activity would be approximately half-maximal even in the stomatal open state [when cytosolic $(Ca^{2+})_f$ is at or lower than 100 nM; Gilroy et al. 1991; McAinsh et al. 1992)]. Elevation of cytosolic free Ca^{2+} to micromolar levels in the advent of closure could effectively render the V-PPase inactive. This suggests that in guard cells the V-ATPase would have the primary role in tonoplast energisation.

Where could the physiological role of the guard-cell V-PPase lie? It is premature to speculate on a role in stress physiology but again, the in vitro Ca^{2+} response may provide an answer. The dark-to-light transition would lower guard-cell cytosolic free Ca^{2+} and also activate photosynthesis. Light-opening of *V. faba* stomata is accompanied by photosynthetic sucrose synthesis (Talbott and Zeiger 1993) and sucrose may be a predominant stomatal osmoticum, depending on environmental conditions (Talbott and Zeiger 1996). The tonoplast V-PPase has previously been considered to be a route for hydrolytic disposal of the increased PPi produced during photosynthetic sucrose synthesis, ensuring that production of the sugar continues (Quick et al. 1989). Perhaps the idea put forward by Rea et al. (1992) that decreasing free Ca^{2+} releases V-PPase inhibition, hence permitting sucrose accumulation (through control of cytosolic PPi), is applicable and highly relevant to guard cells. The tonoplast V-PPase reported in this study may therefore provide a link between photosynthetic metabolism and stomatal opening via changes in cytosolic free Ca^{2+}. Further studies are now required to test this tentative model, applying the full range of techniques which have been used for exploring the role of non-stomatal V-PPase.

The authors gratefully acknowledge A. Pope (SB Pharmaceuticals) for provision of bafilomycin A_1 and P.A. Rea (University of Pennsylvania, USA) for AMDP. The authors thank G. Allen for advice on protoplast preparation and F. Lemtiri-Chlieh for help with figures. This work was supported by a Biotechnology and Biological Sciences Research Council grant to D.S. (No. 87/PO4043) and post-graduate research studentship to C.P.D., a Royal Society Research Fellowship to J.M.D. and the University of Cambridge.

References

Allen GJ, Sanders D (1994) Two voltage-gated, calcium release channels coreside in the vacuolar membrane of broad bean guard cells. Plant Cell 6: 685–694

Assmann SM (1993) Signal transduction in guard cells. Annu Rev Cell Biol 9: 345–375

Baykov AA, Dubnova 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

Bowman EJ, Siebers A, Altendorf K (1988) Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells and plant cells. Proc Natl Acad Sci USA 85: 7972–7976

Carystinos GD, MacDonald HR, Monroy AF, Dhindsa RS, Poole RJ (1995) Vacuolar H^+-translocating pyrophosphatase is induced by anoxia and chilling in seedlings of rice (*Oryza sativa* L.). Plant Physiol 108: 641–649

Darley CP (1997) The physiological roles of vacuolar proton-pumping inorganic pyrophosphatase. PhD thesis, Univeristy of York

Darley CP, Davies JM, Sanders D (1995) Chill-induced changes in the activity and abundance of the vacuolar proton-pumping pyrophosphatase from mung bean hypocotyls. Plant Physiol 109: 659–665

Davies JM (1997) Vacuolar energization: pumps, shunts and stress. J Exp Bot 48: 633–641

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

Davies JM, Hunt IE, Sanders D (1994) Vacuolar H^+-pumping ATPase variable transport coupling ratio controlled by pH. Proc Natl Acad Sci USA 91: 8547–8551

Fricker MD, Willmer CM (1990) Nitrate-sensitive ATPase activity and proton-pumping in guard cell protoplasts of *Commelina*. J Exp Bot 41: 193–198

Gilroy S, Fricker MD, Read ND, Trewavas AJ (1991) Role of calcium in signal transduction of *Commelina* guard cells. Plant Cell 3: 333–344

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

Gordon-Weeks R, Korenkov VD, Steele SH, Leigh RA (1997) Tris is a competitive inhibitor of the vacuolar H^+-pumping pyrophophatase. Plant Physiol 114: 901–905

Hedrich R, Barbier-Brygoo H, Felle H, Flügge UI, Lüttge U, Maathuis FJM, Marx S, Prins HBA, Raschke K, Schnable H, Schroeder JI, Struve I, Taiz L, Ziegler P (1988) General mechanisms for solute transport across the tonoplasts of vacuoles: a patch clamp survey of ion channels and proton pumps. Bot Acta 101: 7–13

Hedrich R, Kurkdjian A, Guern J, Flügge UI (1989) Comparative studies on the electrical properties of the H^+-translocating ATPase and pyrophosphatase of the vacuolar lysosomal compartment. EMBO J 8: 2835–2841

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

Long AR, Hall JL, Williams LE (1997) Chromatographic resolution and purification of H^+-PPase and H^+-ATPase from *Ricinus communis*. J Plant Physiol 151: 16–24

McAinsh MR, Brownlee C, Hetherington AM (1990) ABA-induced elevation of guard cell Ca^{2+} precedes stomatal closure. Nature 343: 186–188

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

Quick P, Neuhaus E, Feil R, Stitt M (1989) Fluoride leads to an increase of inorganic pyrophosphate and an inhibition of photosynthetic sucrose synthesis in spinach leaves. Biochim Biophys Acta 973: 263–271

Rea PA, Britten CJ, Jennings IR, Calvert CM, Skiera LA, Leigh RA (1992) Regulation of the vacuolar H^+-pyrophosphatase by free calcium. Plant Physiol 100: 1706–1715

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

Talbott LD, Zeiger E (1993) Sugar and organic acid accumulation in guard cells of *Vicia faba* in response to red and blue light. Plant Physiol 102: 1163–1169

Talbott LD, Zeiger E (1996) Central roles for potassium and sucrose in guard cell osmoregulation. Plant Physiol 111: 1051–1057

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

Willmer CM, Grammatikopoulos G, Lascève G, Vavasseur A (1995) Characterization of the vacuolar-type H^+-ATPase from guard cell protoplasts of *Commelina*. J Exp Bot 46: 383–389

Yazaki Y, Asukagawa N, Ishikawa Y, Ohta E, Sakata M (1988) Estimation of cytoplasmic free Mg^{2+} levels and phosphorylation potentials in mung bean root tips by in vivo ^{31}P NMR spectroscopy. Plant Cell Physiol 29: 919–924

Zhen R-G, Baykov AA, Bakuleva NP, Rea PA (1994) Aminomethylenediphosphonate: a potent type-specific inhibitor of both plant and phototrophic bacteria pyrophosphatases. Plant Physiol 104: 153–156