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Vacuolar proton-pumping pyrophosphatase in *Beta vulgaris* shows vectorial activation by potassium

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Previous work with membrane vesicles has demonstrated an absolute dependence on K^+ for proton translocation by the inorganic pyrophosphatase (H^+-PPase: EC 3.6.1.1) from the vacuolar membrane (tonoplast) of higher plants. Using intact vacuoles from sugar beet (*Beta vulgaris*) storage tissue, we have monitored PP_i-dependent currents by patch clamp in 'whole vacuole' mode. Serial K^+ substitutions were made at both tonoplast faces. The results show that K^+ activation occurs only at the cytosolic face.

H^+-Pyrophosphatase; Potassium; Tonoplast; Patch clamp; *Beta vulgaris*

1. INTRODUCTION

Two electrogenic, H^+-translocating phosphohydrolases reside in the vacuolar membrane (tonoplast) of higher plants: a V-type ATPase (EC 3.6.1.3) and an inorganic pyrophosphatase (EC 3.6.1.1) [1]. Both transport systems are electrogenic, passing current (positive charge) into the vacuole [2,3]. Nevertheless, structural and mechanistic disparity between these enzymes might be reflected in dissimilar physiological roles [4,5]. Given the primacy of the ATPase [6] in generating a H^+ electrochemical potential gradient for H^+-coupled transport, it is pertinent to question whether the PPase is simply an auxiliary H^+-pump or has a distinct function [1].

Consideration of H^+-PPase energetics allows in principle for PP_i synthesis or hydrolysis in vivo, with physiological poise determined by the stoichiometric ratio of H^+ translocated : PP_i hydrolysed [1]. If synthetic, the H^+-PPase (driven by the ATPase-generated H^+ gradient) could act to stabilize cytosolic PP_i. However, recent thermodynamic analysis of PP_i-driven H^+ translocation in isolated intact vacuoles [7] has suggested that the stoichiometric ratio is unity, which implies an in vivo poise in the direction of H^+ pumping.

Proton pumping by the H^+-PPase exhibits an obligatory dependence on the presence of K^+ [8]. This observation raises the possibility that, rather than merely acting as a supplementary H^+ pump, the H^+-PPase serves also to catalyze K^+ translocation across the tonoplast. In K^+-replete tissue, the vacuolar K^+ activity may exceed the K^+ activity in the cytosol [9], and the trans-tonoplast electrical potential difference is generally thought to be in the region +20 to +50 mV referenced to the cytosolic side [2,10]. Therefore a mechanism for energization of K^+ uptake into the vacuole is required. Were the H^+-PPase to fulfil this role, it would be predicted that the K^+ binding site which stimulates hydrolysis resides on the cytosolic side of the membrane.

The aim of the present work is to determine whether the H^+-PPase is vectorially activated by K^+, and, if so, on which side of the membrane activation occurs. The use of tonoplast vesicles for such studies is confounded by inherent membrane leakiness to K^+. However, 'whole vacuole'-mode patch clamp [11] offers the possibility of K^+ substitution selectively on either side of the membrane as any concentration changes effected by channel-mediated leakage of K^+ across the tonoplast are minimized by the relatively large volumes of the bathing medium and the patch pipette/intravacuolar continuum. The results demonstrate clearly that the H^+-PPase is activated selectively by cytosolic K^+. While it cannot be considered as evidence for H^+-PPase-mediated K^+ translocation, this finding is consistent with the vectorial properties anticipated a priori for such a transport system.

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Abbreviations: EGTA, ethyleneglycol-bis-(aminomethylether)-*N,N,N',N'*-tetraacetic acid; Mes, 2-[*N*-morpholino]ethanesulfonic acid; H^+-PPase, proton-translocating inorganic pyrophosphatase; PP_i, inorganic pyrophosphate

2. MATERIALS AND METHODS

2.1. *Preparation of vacuoles*

Sugar beet (*Beta vulgaris* var. Regina M49) was grown in John In-

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nes no. 2 potting compost under greenhouse conditions, with temperature in the range 15–18°C and lighting supplemented during winter for 16 h/day by metal halide lamps. Vacuoles (from 400 to 500 g tap roots) were isolated directly into the recording chamber [12]. The standard composition of the bathing medium was 50 mM KCl, 3.6 mM $MgCl_2$, 0.1 mM EGTA, 20 mM Tris-Mes, pH 7.5. In K^+-substitution experiments KCl was replaced by 50 mM choline chloride. Vacuoles were isolated in either standard or choline medium. Osmolarity of the bathing medium was determined with a freezing point osmometer (Mod 200, Camlab) and was adjusted to 100–150 mOsm above that of the tissue with D-sorbitol. Debris in the recording chamber was removed by perfusion and vacuoles adhering to the chamber floor were used for experimentation. Fresh vacuoles were prepared for each experiment.

2.2. *Patch clamp protocol*

Patch pipettes were fabricated from Kimax-51 borosilicate capillaries, coated and fire polished. Pipettes (5–15 MΩ) were filled with a standard intravacuolar solution comprising 50 mM KCl, 1 mM $CaCl_2$, 3.6 mM $MgCl_2$, 20 mM Tris-Mes, pH 5.5. Osmotic potential was matched to the bathing medium with D-sorbitol. In K^+ substitution experiments, 50 mM choline chloride replaced KCl. For vacuoles of diameter 15–25 μm, seal resistances between the pipette tip and the tonoplast were in the region 4–20 GΩ.

Membrane breakdown at the tip was achieved using bipolar voltage pulses of 400–600 mV amplitude and 3–5 ms duration, generated from a microcomputer. Vacuoles with a membrane capacitance of 20–30 pF were selected for use. After equilibration of vacuolar and pipette contents, membrane potential was clamped to 0 mV with a patch clamp amplifier (EPC-7, List Electronic). Tris-PP_i (from a 28 mM stock solution), present at a final concentration of 100 μM in the appropriate bathing medium, was then delivered to the chamber by perfusion (Gilson Minipuls 2 peristaltic pumps). Delivery time was 25 s from the reservoir, flow rate was 0.3 ml·min^{-1} and chamber clearance time, approximately 45 s. Recordings were filtered at 0.2–3 kHz, digitized and stored on video tape.

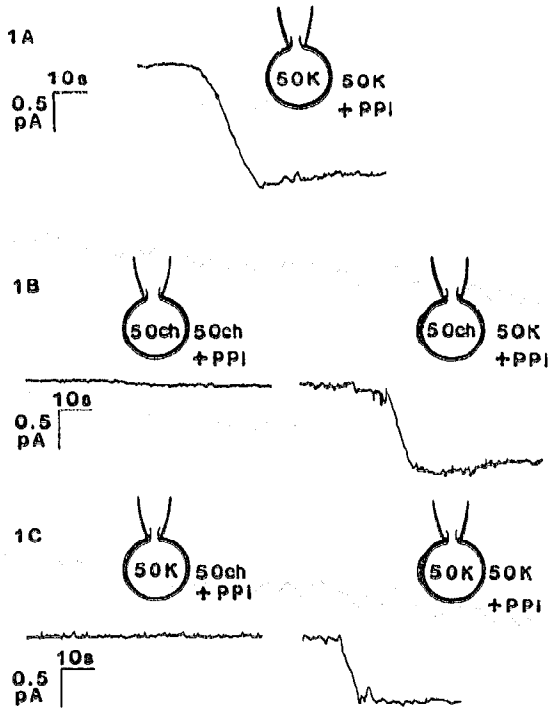

Fig. 1. Responses to PP_i at the cytoplasmic face with ionic conditions as indicated in inset. All traces filtered at 10 Hz. (A) Response lag time 94 s from start of perfusion. (B) (Left) Absence of PP_i-dependent current with choline on both sides of the vacuolar membrane. (Right) PP_i dependent current restored in the same vacuole after selective provision of K^+ on the cytosolic side. (C) (Left) With K^+ on the cytosolic side selectively replaced by choline, no PP_i-dependent current is apparent. (Right) Appearance of PP_i-dependent current in the same vacuole when K^+ is restored to the cytosolic side. A downward deflection indicates the entry of positive charge into the vacuole.

2.3. *Current/voltage relationships*

Membrane current-voltage (I–V) relationships were obtained by applying voltage-clamping pulses in 10 mV increments as a bipolar staircase from a 0 mV baseline. Pulse duration was 10 s. Current was sampled in the last 2 s of the pulse, filtered at 10 Hz and analyzed using commissioned software. All I–V recordings were performed during perfusion.

3. RESULTS AND DISCUSSION

With K^+ present at both cytoplasmic and vacuolar faces of the tonoplast, addition of 100 μM Tris-PP_i to the bathing medium evoked an inwardly-directed current (Fig. 1A). This result can be interpreted as the ionic current mediated by the H^+-PPase. In a sample of 8 vacuoles, the PP_i-dependent current ranged from 0.1 to 11.3 mA·m^{-2}, with a mean ± SE of 3.0 ± 0.7 mA·m^{-2} (Table I). These values are within the range previously reported for PP_i-dependent currents in higher plant vacuoles [2].

Substituting choline for K^+ on both sides of the tonoplast did not affect sealing or achievement of the whole vacuole configuration. However, PP_i-dependent currents were never observed in these conditions (n = 7: Fig. 1B and Table I). This result can be attributed to the absence of K^+ rather than the presence of choline, since choline did not inhibit PP_i hydrolysis in tonoplast

Table I

PP_i-dependent currents with K^+ selectively present at cytoplasmic and/or vacuolar faces of the tonoplast

	Current (mA·m^{-2})				
Vacuolar side:	K^+	Choline		Choline	
Cytosolic side:	K^+	Choline	→ K^+	Choline	→ K^+
	11.3	0	5.7	0	0.4
	2.8	0	1.3	0	1.4
	0.5	0	6.0	0	0.8
	4.1	0	0.9	0	3.5
	0.1	0	0.7		
	4.1	0	4.5		
	0.8	0	(NT)		
	0.3				
Mean	3.0	0	3.2	0	1.5
SE	0.7	0	0.6	0	0.6

Data from individual vacuoles, with columns 3 and 5 reporting PP_i-dependent currents after provision of K^+ in the bathing medium. NT, not tested.

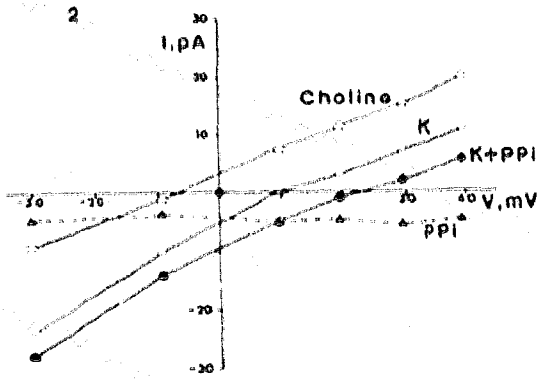

Fig. 2. Current/voltage relationship of a single vacuole (contents set with choline-based pipette medium) in successive choline (□), K^+ (○) and K^+ + PP_i (●) bathing media. See section 2.2 for details of perfusion. Membrane potential was clamped at 0 mV and voltage-clamping pulses were applied as given in Section 2.3.

vesicles when assayed in the presence of K^+ (data not shown). Thus, previous results demonstrating an obligatory dependence on K^+ for PP_i-dependent H^+ pumping in tonoplast vesicles are confirmed using electrophysiological techniques on intact vacuoles.

After flushing with control choline bathing medium for up to 5 min, the chamber was perfused with standard (50 mM K^+) solution. Once equilibrated, the vacuole was presented with 100 μM Tris-PP_i. All vacuoles then tested for a response (now with K^+ selectively at the cytoplasmic face) showed a PP_i-dependent current (Fig. 1B and Table I) with a mean value (3.2 ± 0.6 $mA \cdot m^{-2}$) which agrees well with the control. Fig. 2 shows the $I-V$ relationship of such a vacuole: with K^+ on the cytosolic side (and choline on the vacuolar) addition of PP_i produced a hyperpolarizing shift in membrane potential of 12.5 mV. The PP_i-dependent $I-V$ difference curve runs parallel to the voltage axis, with no sign of a reversal potential. This result is anticipated, since the bathing medium was nominally orthophosphate-free.

The notion that H^+-PPase activity is specifically dependent on the presence of K^+ on the cytosolic side, but is indifferent to intravacuolar K^+, is confirmed in the reciprocal experiment. With K^+ selectively at the vacuolar face, a PP_i-dependent current could not be elicited in the absence of K^+ in the bathing medium (n = 4: Fig. 1C and Table I). Provision of bathing medium K^+ allowed PP_i-dependent current to be measured from the same vacuole (Fig. 1C and Table I). The mean current in symmetrical K^+ is roughly half that of the control set, although the individual values are well within the range encountered in control conditions and variability between different vacuoles precludes quantitative comparisons with small sample size. More importantly, it is clear that the presence of K^+ solely in the vacuole is insufficient to evoke a PP_i-dependent current.

At present, direct evidence that the H^+-PPase of higher plant tonoplast actually translocates K^+ is lacking. However, the present identification of a stimulatory site for K^+ selectively on the cytosolic face of the tonoplast is certainly in accord with expectations based on the in vivo direction of the trans-tonoplast electrochemical gradient for K^+. A K^+ transport function for the H^+-PPase could, furthermore, have been eliminated were the K^+ stimulatory site to have been located on the vacuolar side, since outward transport of K^+ is not consistent both with a H^+ : PP_i stoichiometry of unity and a PP_i-driven inward current. Finally, it should be noted that, while H^+ gradient-driven transport systems have been extensively characterized for other cations which are accumulated in the vacuole (Na^+: [13,14]; Ca^{2+}: [15,16]) there are, to date, no reports of H^+ gradient-driven K^+ transport at the tonoplast. This perhaps strengthens the need to consider the possibility that energization of K^+ transport is achieved via a primary pump.

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