

# Potassium and voltage dependence of the inorganic pyrophosphatase of intact vacuoles from *Chenopodium rubrum*

Gerhard Obermeyer<sup>\*</sup>, Aniola Sommer, Friedrich-Wilhelm Bentrup

*Institut für Pflanzenphysiologie der Universität, Hellbrunnerstrasse 34, A-5020 Salzburg, Austria*

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## Abstract

The activity and the voltage dependence of the inorganic pyrophosphatase (PPase) was measured on intact vacuoles of *Chenopodium rubrum* cells using the patch-clamp technique. With  $K^+$  at the cytoplasmic side a negative current representing the forward mode of the pump was measured after addition of pyrophosphate ( $PP_i$ ). The pump was reversed and a positive current was detected after addition of orthophosphate ( $P_i$ ) in the presence of  $K^+$  at the vacuolar side when a pH gradient across the tonoplast was applied. The PPase operates as a constant current source, because no voltage dependence was observed (–60 to 60 mV). The  $K^+$  dependence of the  $PP_i$ -induced current was investigated by substitution of cytoplasmic  $K^+$  by other cations. The selectivity sequence was:  $K^+ \geq Rb^+ > NH_4^+ = Cs^+ > Na^+ > Li^+ = \text{choline}^+$ , and was independent of the membrane voltage and  $pH_{\text{cyt}}$ . With  $Cs^+$  or  $Li^+$  in the bath and  $K^+$  inside the vacuole the  $PP_i$ -induced current became voltage-dependent, and positive currents were observed even if the pump was geared to operate in the forward mode. We suggest a ‘tunneling’ effect through a channel-like domain in the PPase molecule which, under defined electrochemical gradient conditions and in the presence of  $PP_i$ , allows  $K^+$  ions to cross the energy barrier usually separating the cytoplasmic from the vacuolar face of the pump.

**Keywords:** Tonoplast; Pyrophosphatase, inorganic; PPase,  $H^+$ -; Proton transport; Potassium ion transport; Electrogenic pump

## 1. Introduction

Two electrogenic proton pumps are responsible for the pH gradient formation across the tonoplast in higher plants: a vacuolar-type  $H^+$ -ATPase (EC 3.6.1.3) and an inorganic pyrophosphatase (PPase, EC 3.6.1.1) [1,2]. The PPase, an ubiquitous protein in the plant vacuolar membrane (tonoplast), has been purified from mung bean [3] and red beet vacuoles [4], and was reconstituted into liposomes [5–7]. The major subunit has a molecular weight ranging from

64.5 to 73 kDa, but the functional molecular size of the enzyme is much larger (135 kDa: [8]; 100–300 kDa: [9]). Also, the sequences of cDNAs encoding the PPase were reported for *Arabidopsis* [10], barley [11], and red beet [12]. In spite of the progress obtained by using biochemical and molecular biological techniques to reveal the structure of the PPase, much less is known about its physiological function including the molecular mechanism of ion transport.

$PP_i$  hydrolysis and  $PP_i$ -dependent  $H^+$ -transport depend on the  $K^+$  concentration of the buffer [13]. Patch-clamp experiments have shown that charge translocation through the PPase depends on the presence of  $K^+$  at the cytoplasmic and at the vacuolar side of the tonoplast for the forward and the backward mode, respectively [14]. Additionally, the reversal potential ( $E_{\text{rev}}$ ) of the PPase proved to be dependent on the pH as well as on the  $K^+$  gradient across the vacuolar membrane and it was thus inferred that

Abbreviations:  $I$ , electrical current (pA);  $i$ , electrical current density ( $\text{mA m}^{-2}$ ); PPase, vacuolar-type inorganic pyrophosphatase (EC 3.6.1.1); BTP, 1,3-bis[tris(hydroxymethyl)methylamino]propane; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid;  $PP_i$ , pyrophosphate.

<sup>\*</sup> Corresponding author. Fax: +43 662 8044619; e-mail: gerhard.obermeyer@sbg.ac.at.

the vacuolar pyrophosphatase transports not only protons but also  $K^+$  [15].

However, this hypothesis is controversial since no evidence for active  $^{42}K^+$  transport has been detected for a purified PPase reconstituted into liposomes [6]. Also, recent experiments using vacuolar membrane vesicles labeled with fluorescent dyes sensitive to  $H^+$ ,  $K^+$ , and membrane voltage, respectively, led to the conclusion that the  $H^+$  transport by the PPase is not necessarily linked to  $K^+$  cotransport [16].

In this study we investigated the cation selectivity and the voltage dependence of the PPase in the forward mode by patch-clamping whole vacuoles of suspension culture cells of *Chenopodium rubrum* and found evidence of an involvement of potassium ions in the  $PP_i$ -energized  $H^+$  transport of the vacuolar inorganic pyrophosphatase.

## 2. Materials and methods

### 2.1. Plant material and isolation of vacuoles

Vacuoles were isolated from cells of a heterotrophic, betalain-containing cell suspension culture of *Chenopodium rubrum* L according to Bentrup et al. [17] with minor modifications. In a first step protoplasts were obtained by digestion of the cell wall. Cells of three-day old suspension culture (1 g fresh weight) were incubated for 3–4 h in isolation medium containing (in mM): 500 mannitol, 25 KCl, 5  $MgCl_2$ , 2  $CaCl_2$ , 20 Hepes/BTP, pH 5, and additionally, 0.35 units cellulase TC and 0.45 units pectinase 5S (Serva, Germany) in a total volume of 10 ml. The suspension was centrifuged for 15 min at  $50 \times g$  and the pellet was washed twice by centrifugation for 10 min at  $50 \times g$  in isolation medium adjusted to pH 6 with BTP (wash medium). A sucrose step gradient was used to collect the protoplasts, which sedimented at the interface of a 250 mM sucrose cushion (10 min,  $50 \times g$ ). Aliquots of purified protoplast suspension were pipetted into a perfusion chamber for electrical recordings (chamber volume of approx. 200  $\mu$ l). Vacuoles were released just before patch-clamp experiments by perfusing the chamber with a hypoosmolar bath solution (standard bath solution,  $B_0$ ) containing (in mM): 250 mannitol, 50 KCl, 2  $MgCl_2$ , 0.5 EGTA, 5 Hepes adjusted to pH 7.5 with BTP.

### 2.2. Electrical measurements

Patch pipettes with a tip diameter of 1–1.5  $\mu$ m were pulled from borosilicate glass capillaries, heat polished and finally coated with bees wax or Sigmacote (Sigma, Austria). Unless otherwise stated, both the reference electrode

and the patch pipettes (typical resistances of 7–12  $M\Omega$ ) were filled with standard bath solution ( $B_0$ ). Seal resistances between the tonoplast and the pipette tip were of 5–10  $G\Omega$ . All measurements were performed in the 'whole-vacuole' configuration which was obtained from the vacuole-attached configuration either by applying a gentle suction to the pipette or by a 'zap' pulse ( $\tau = 0.5$ –5 ms,  $V_{DC} = 1.3$  V) delivered from a patch-clamp amplifier (Axopatch 200A, Axon Instruments, USA). Equilibration of the vacuolar contents with the pipette solution took 20–40 min and was detected as a current stabilization (ca. 0 pA) and a loss of the pink pigmentation (betalains) of the vacuole. The forward mode of the pump was investigated by recording the current evoked after perfusing the chamber with  $B_0$  that additionally contained 5–100  $\mu$ M BTP- $PP_i$  while clamping the membrane voltage at 0 mV. The pump was reversed by using a  $H^+$  gradient across the tonoplast:  $B_0$ , adjusted to pH 5.5, as pipette solution and  $B_0$ , pH 7.5 as bath solution. Addition of 5–75 mM BTP- $P_i$  generated a positive current corresponding to positive charges which were transported out of the vacuole at  $V_m = 0$  mV.

For current–voltage relations ( $I$ – $V$  curves), voltage step pulses with a duration of 2.8 s were applied to the vacuole while continuously perfusing the chamber with the appropriate test solution. The resulting currents were recorded using an A/D converter (Digidata 1200, Axon Instruments) and the pClamp software (vers. 5.5, Axon Instruments).  $I$ – $V$  curves were calculated by averaging the steady-state current of the last 1.2 s of the voltage pulse and plotting the mean current ( $I$ ) against the pipette voltage ( $V$ ). The current density ( $i$  in  $mA\ m^{-2}$ ) was calculated using the mean steady-state current and the surface area of the respective vacuole (the diameter was measured under the microscope at the beginning and the end of an experiment). Pyrophosphate- and  $P_i$ -dependent  $i$ – $V$  curves are difference curves which have been obtained by subtracting the  $i$ – $V$  curve lacking the substrate ( $PP_i$  or  $P_i$ ) from the  $i$ – $V$  curves measured in the presence of the respective substrate.

Ion selectivity of the PPase was investigated by perfusing the chamber with  $B_0$  containing 50 mM chloride salt of the respective  $K^+$  substitute (alkali ions,  $NH_4^+$ , and choline $^+$ ) and measuring the pump currents after addition of 50  $\mu$ M  $PP_i$ . The measurements were performed as follows: a patch-clamped vacuole was first bathed in standard bath solution (which was also used as pipette solution), then 50  $\mu$ M BTP- $PP_i$  were added to the bath (corresponding to the cytoplasmic side) and the pump current was recorded. The substrate was washed out with  $B_0$ , then the vacuole was exposed to  $B_0$  with a  $K^+$  substitute, and subsequently bathed in the same solution plus 50  $\mu$ M BTP- $PP_i$ . Pump currents were recorded and normalized to

the current measured in the presence of  $K^+$ . For each ion, the absolute pump currents, current densities, and the relative currents ( $i_{\text{cation}}/i_{K^+}$ ) were averaged over at least five individual experiments.  $PP_i$ -dependent  $i$ - $V$  curves with different cations at the cytoplasmic side of the vacuole were measured and calculated as described above.

Membrane voltages were corrected for liquid junction potentials (LJP) according to Neher [18] and Barry and Lynch [19]. Calculations using the generalized Henderson equation gave LJP's up to 16 mV for experiments on cation selectivity. Nevertheless, pump  $i$ - $V$  characteristics were unaffected by these corrections because LJP's were compensated by subtracting the  $i$ - $V$  curves corresponding to the presence and absence of the substrate (see above). Where appropriate, data are given as mean  $\pm$  standard deviation (S.D.) of  $n$  independent experiments. All experiments were performed at room temperature (21–23°C).

### 2.3. Sign convention

In all experiments the pipette potential equals the potential inside the vacuole and was referred to a potential of 0 mV of the bath. This implies that negative currents showed as downward currents represent an influx of cations into the vacuole, i.e. the forward mode of the pump. This straightforward sign convention is thus opposite to the sign convention proposed by Bertl et al. [20] which relates inward and outward to the cytoplasm, and is misleading if used for work on isolated vacuoles.

## 3. Results

### 3.1. Substrate dependence

With both vacuolar and cytoplasmic side of the vacuoles exposed to solution  $B_o$ , no current was detected while clamping the tonoplast at 0 mV. Addition of 50  $\mu$ M  $PP_i$ -BTP to the bath (cytoplasmic side of the vacuole) caused a flow of positive charges into the vacuole corresponding to an influx of cations, whereas addition of 20 mM  $P_i$ -BTP evoked a current of opposite direction (Fig. 1A). The current densities measured in the forward mode (after  $PP_i$  addition) and in the backward mode ( $P_i$  addition) showed a large variation, reflecting the variability of the vacuoles. An average current density of  $-2.7 \pm 1.3$  mA  $m^{-2}$  ( $n = 42$ , max =  $-5.7$  mA  $m^{-2}$ , min =  $-0.36$  mA  $m^{-2}$ ) for the  $PP_i$ -dependent current was measured. Fig. 1B shows the distribution of these currents. In spite of the large variation of the current densities no correlation to vacuole size, age of the isolated protoplasts or age of the culture cells was observed (data not shown).

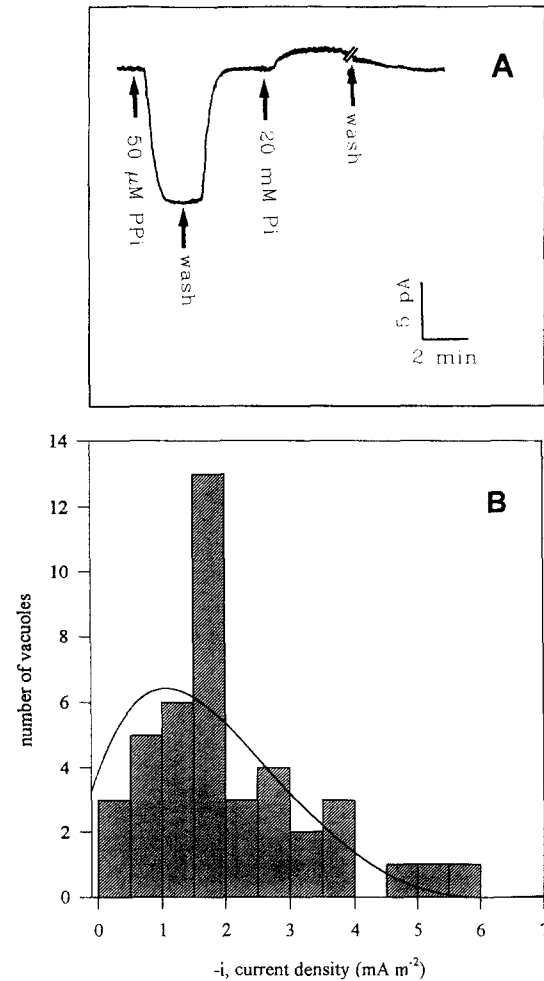


Fig. 1. Representative pump currents, measured in the presence of appropriate substrates. (A) Typical current recording showing both pump modes. Addition of 50  $\mu$ M  $PP_i$  evoked a downward deflection corresponding to a flux of positive charges into the vacuole, while addition of 20 mM  $P_i$  induced an upward deflection (positive charges flow out of the vacuole).  $V_m$  was clamped to 0 mV,  $pH_{\text{vac}} = 5.5$ ,  $pH_{\text{cyt}} = 7.5$ ,  $[K^+]_{\text{vac}} = [K^+]_{\text{cyt}} = 50$  mM. (B) Distribution of the pump current densities measured after exposing vacuoles to 50  $\mu$ M  $PP_i$  ( $pH_{\text{vac}} = pH_{\text{cyt}} = 7.5$ ,  $[K^+]_{\text{vac}} = [K^+]_{\text{cyt}} = 50$  mM,  $V_m = 0$  mV). Data were collected from 42 experiments.

The dependence of the inward current on the substrate concentration was measured by adding 5–100  $\mu$ M BTP- $PP_i$  to the bath medium, and gave current densities in the range of  $-0.03$  to  $-5.73$  mA  $m^{-2}$  ( $n = 60$ , Fig. 2A). In these experiments pH and  $K^+$  concentration of pipette and bath solution were the same. A Hanes plot was drawn from these data, and  $K_M$  was calculated as 18  $\mu$ M and the maximum pump current density ( $i_{\text{max}}$ ) was  $-2.1$  mA  $m^{-2}$ .

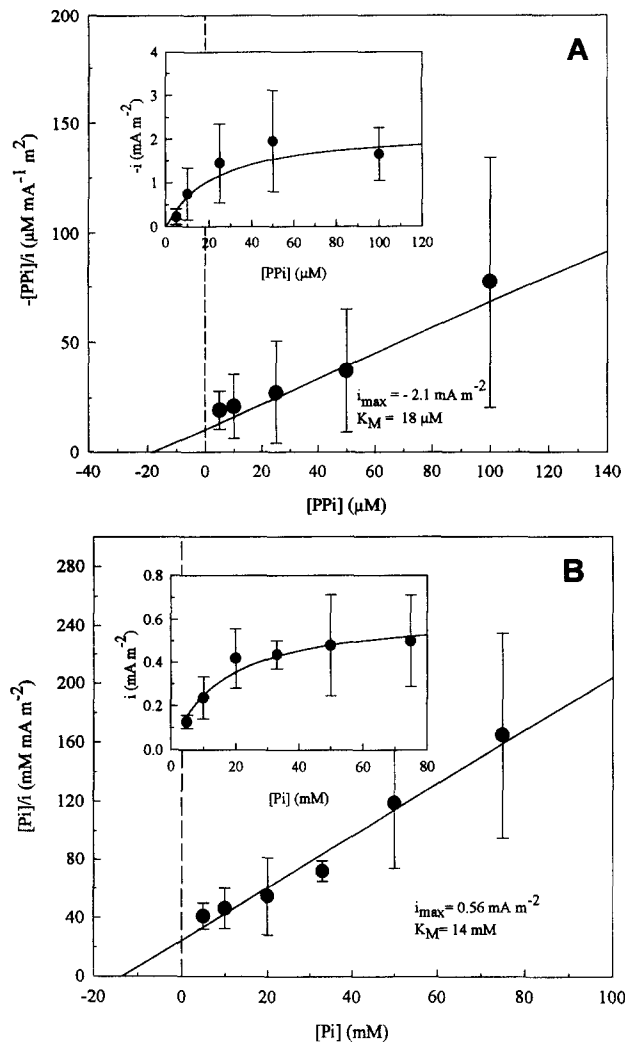


Fig. 2.  $\text{PP}_i$ - and  $\text{P}_i$ -dependent currents (current density,  $-i$ ) reflecting the pump activity in the forward and backward mode, respectively. The  $i_{\max}$  and  $K_M$  values were determined from the regression curve of the Hanes plots and were used to generate the solid lines in the insets. (A) Hanes plot of the pyrophosphatase activity with BTP- $\text{PP}_i$  as substrate ( $\text{pH}_{\text{vac}} = \text{pH}_{\text{cyt}} = 7.5$ ,  $[\text{K}^+]_{\text{vac}} = [\text{K}^+]_{\text{cyt}} = 50 \text{ mM}$ ). Inset: current densities measured for various substrate concentrations. (B) Hanes plot of the pyrophosphatase activity in the backward mode (protons pumped out of the vacuole). Inset: current density values corresponding to different concentrations of the substrate BTP- $\text{P}_i$ ,  $\text{pH}_{\text{vac}} = 5.5$ ,  $\text{pH}_{\text{cyt}} = 7.5$ ,  $[\text{K}^+]_{\text{vac}} = [\text{K}^+]_{\text{cyt}} = 50 \text{ mM}$ .

A current in the opposite direction (out of the vacuole), generated after addition of BTP- $\text{P}_i$  to the bath was measured when a proton gradient across the tonoplast was applied ( $\text{pH}_{\text{vac}} = 5.5$ ,  $\text{pH}_{\text{cyt}} = 7.5$ ). The  $\text{K}^+$  concentration in the bath and pipette solution was the same. With 5 to 75 mM BTP- $\text{P}_i$  in  $\text{B}_0$ , the pump current densities were in the range of 0.1 to 0.75  $\text{mA m}^{-2}$  ( $n = 22$ , Fig. 2B). The

corresponding  $K_M$  for the backward mode was 14 mM and  $i_{\max} = 0.56 \text{ mA m}^{-2}$ . To observe the currents generated by the pump in the forward mode it was necessary for  $\text{K}^+$  to be present at the cytoplasmic and for the backward mode at the vacuolar side (data not shown).

To further characterize the functioning of the pyrophosphatase, we investigated the voltage dependence of the currents induced by perfusing the recording chamber first with 100  $\mu\text{M}$   $\text{PP}_i$  and thereafter with 10 mM  $\text{P}_i$  according to Fig. 1A. The pipette solution was  $\text{B}_0$  adjusted to pH 5.5, and the bath solution at pH 7.5. We thus obtained the  $i$ - $V$

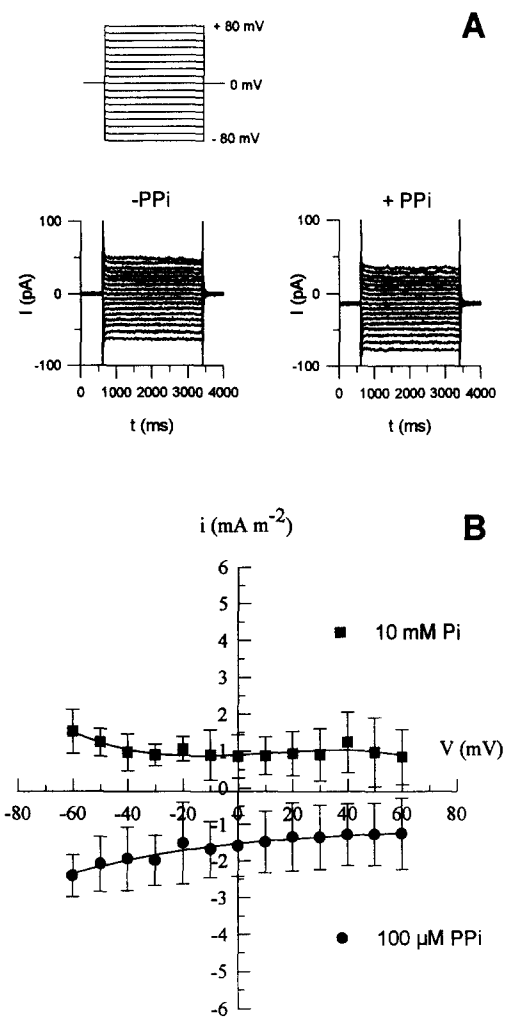


Fig. 3. Voltage dependence of the forward and backward mode of the pyrophosphatase. (A) Voltage pulse protocol is shown together with the corresponding currents measured in the presence and in the absence of  $\text{PP}_i$ . (B)  $i$ - $V$  characteristics (difference curves) of the pyrophosphatase operating in the forward mode (with 100  $\mu\text{M}$   $\text{PP}_i$  as substrate) and in the backward mode (with 10 mM  $\text{P}_i$  as substrate), respectively.  $\text{pH}_{\text{vac}} = 5.5$ ,  $\text{pH}_{\text{cyt}} = 7.5$ ,  $[\text{K}^+]_{\text{vac}} = [\text{K}^+]_{\text{cyt}} = 50 \text{ mM}$ .

Table 1  
Cation selectivity of the pyrophosphatase

Cation (number of vacuoles)		$i$ (mA m <sup>-2</sup> )	$i_{\text{cation}} / i_{\text{K}^+}$ (%)
K <sup>+</sup>	pH 6.5 (6)	0.80 ± 0.52	100
	pH 7.5 (42)	2.07 ± 1.27	100
	pH 8.5 (6)	1.15 ± 1.11	100
Rb <sup>+</sup> (6)		2.04 ± 1.01	89 ± 12
Cs <sup>+</sup>	pH 6.5 (5)	0.54 ± 0.36	84 ± 20
	pH 7.5 (10)	0.76 ± 0.53	54 ± 9
	pH 8.5 (4)	0.45 ± 0.23	48 ± 2
NH <sub>4</sub> <sup>+</sup> (5)		1.87 ± 1.39	54 ± 14
Na <sup>+</sup> (5)		0.95 ± 0.42	34 ± 8
Li <sup>+</sup>	pH 6.5 (5)	0.15 ± 0.11	26 ± 14
	pH 7.5 (9)	0.22 ± 0.19	10 ± 6
	pH 8.5 (4)	0.13 ± 0.09	7 ± 2
Chl <sup>+</sup> (4)		0.14 ± 0.08	7 ± 5

Pump activity is expressed as pump current density (mA m<sup>-2</sup>). Values are given as mean ± S.D. Measurements were done under bi-ionic conditions, with K<sup>+</sup> inside the vacuole and the respective substitute added in the same concentration at the cytoplasmic side of the same vacuole. Relative currents were calculated for each vacuole and then averaged. Cations were used as 50 mM chloride salts, and the pH at both sides of the tonoplast was 7.5. For K<sup>+</sup>, Cs<sup>+</sup> and Li<sup>+</sup> pump currents were also measured at pH 6.5 and pH 8.5 (pH<sub>vac</sub> = pH<sub>cyt</sub>).

characteristics of the pump working in the forward and in the backward mode, respectively, from the same vacuole. Fig. 3A shows the voltage pulse protocol together with the

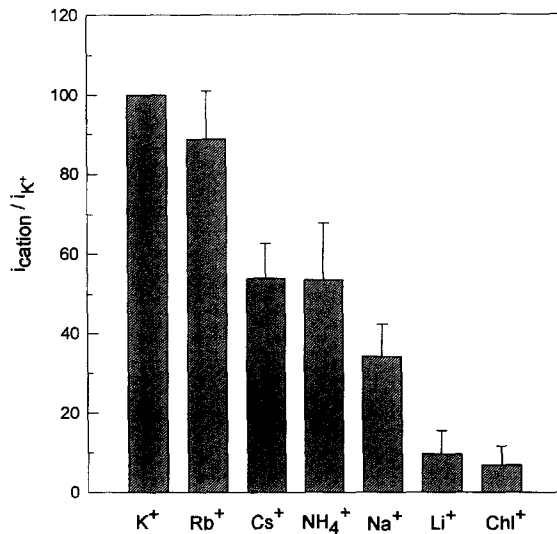


Fig. 4. Normalized values ( $i_{\text{cation}} / i_{\text{K}^+}$ ) of the PP<sub>i</sub>-induced current measured after replacing K<sup>+</sup> of the bath solution. All cations were added as 50 mM chloride salts, the pH was 7.5 at both sides of the tonoplast and the membrane voltage was clamped at 0 mV. ( $n = 81$ ).

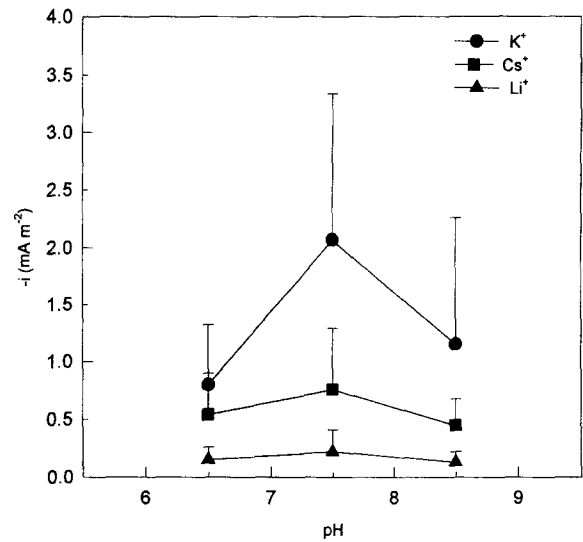


Fig. 5. pH dependence of the PP<sub>i</sub>-induced current densities in the presence of K<sup>+</sup>, Cs<sup>+</sup> or Li<sup>+</sup>. Cations were added as 50 mM chloride salts and pH was the same at both sides of the tonoplast. ( $n = 40$ ).

corresponding currents measured in the presence and the absence of PP<sub>i</sub>. On adding PP<sub>i</sub>, a negative current was measured while the membrane potential was clamped to 0 mV. Note the negative shift in the baseline with the addition of PP<sub>i</sub>. The  $i$ - $V$  characteristics of the PPase in the

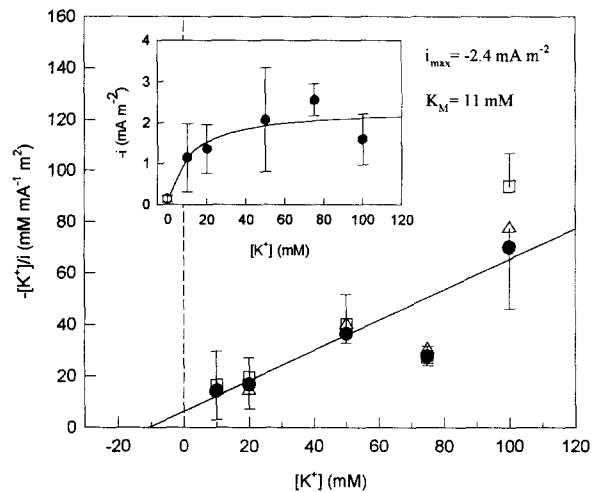


Fig. 6. Hanes plot showing the dependence of the pyrophosphatase activity on K<sup>+</sup> concentration. The  $i_{\text{max}}$  and  $K_{\text{M}}$  values were determined from the regression curve (solid line) of the Hanes plot and were used to generate the solid line in the inset. K<sup>+</sup> concentration and pH (7.5) were the same in the bath and in the pipette solution. Filled circles represent the data at  $V_{\text{m}} = 0$  mV whereas the open symbols show the current at +40 mV (squares, upward error bars) and -40 mV (triangles, downward error bars). ( $n = 61$ ). The open circle in the inset represents the data at zero K<sup>+</sup> concentration but in the presence of 50 mM chl<sup>+</sup> (Table 1).

forward as well as in the backward mode from 5 independent experiments are presented in Fig. 3B. Both pump modes showed a poor voltage dependence in the investigated voltage range, suggesting that the pump acts as a constant current source. No activation of the ubiquitous  $\text{sv}$  channel was measured under these experimental conditions, because calcium ions of the bath and pipette solution possibly activating this channel were complexed by addition of EGTA [21]. This precaution was important, because otherwise the large channel currents would have superimposed the much smaller pump currents.

### 3.2. Selectivity for monovalent cations

The measurements shown in Figs. 1–3 were conducted with  $\text{K}^+$  present in equal concentrations at both sides of the vacuole. However, a very interesting and at present

disputed feature of the vacuolar pyrophosphatase is its requirement for potassium. It is yet not clear if  $\text{K}^+$  is just stimulating the activity of this pump or if it is actually cotransported together with  $\text{H}^+$ . To address this question, we analysed the PPase acting in the forward mode when  $\text{K}^+$  in the bath was substituted by 50 mM of the following cations:  $\text{Li}^+$ ,  $\text{Na}^+$ ,  $\text{Cs}^+$ ,  $\text{Rb}^+$ ,  $\text{NH}_4^+$ , and choline $^+$ . The results are presented in Table 1 and Fig. 4. In the presence of  $\text{LiCl}$  or choline chloride the pump currents were considerably lower, while  $\text{Cs}^+$  and  $\text{NH}_4^+$  reduced the current to about half its value.  $\text{Rb}^+$ , on the other hand, proved to be a good substitute for  $\text{K}^+$ . A relative selectivity sequence of  $\text{K}^+ \geq \text{Rb}^+ > \text{NH}_4^+ = \text{Cs}^+ > \text{Na}^+ > \text{Li}^+ = \text{choline}^+$  was obtained at  $V_m = 0$  mV. This sequence did not change at different membrane voltages ( $-60$  mV  $< V_m < +60$  mV). Such a sequence is similar to the Eisenman sequence no. IV corresponding to a rather weakly charged binding site [22].

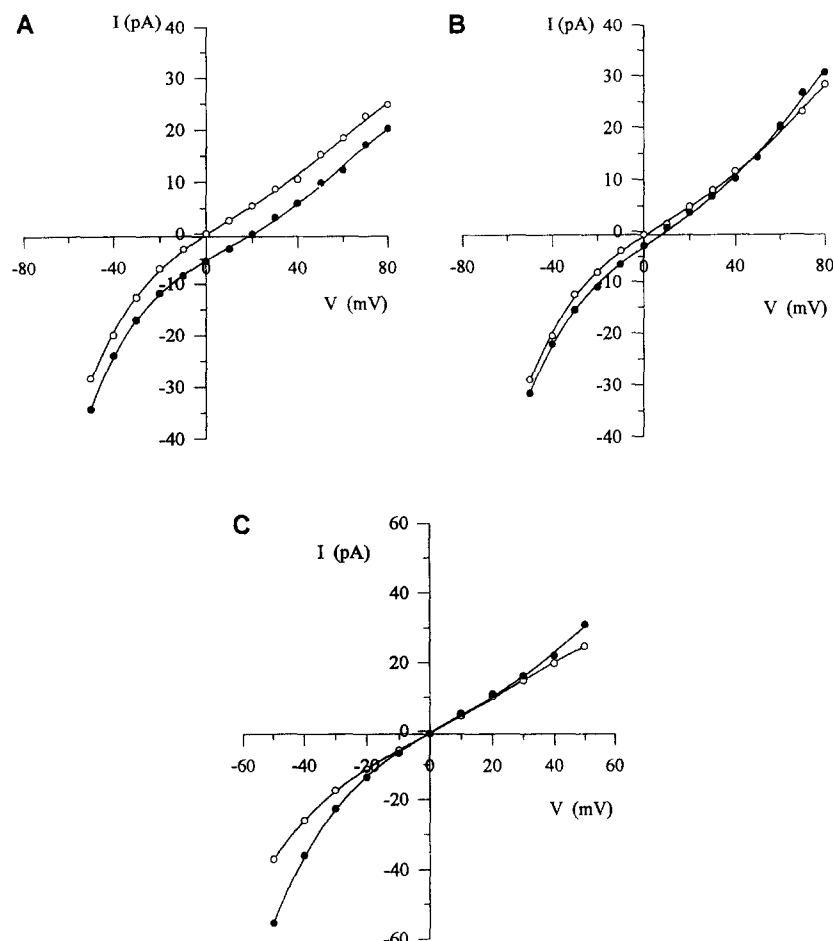


Fig. 7. Representative current–voltage characteristics from one single vacuole obtained in the absence (open circles) and in the presence (filled circles) of 50  $\mu\text{M}$   $\text{PP}_i$  ( $\text{pH}_{\text{vac}} = \text{pH}_{\text{cyt}} = 7.5$ ,  $[\text{K}^+]_{\text{vac}} = 50$  mM). The cytoplasmic side of the vacuole was perfused with standard bath solution containing 50 mM  $\text{K}^+$  (A),  $\text{Cs}^+$  (B) or  $\text{Li}^+$  (C), respectively.

If such weakly charged binding sites were involved in the binding of  $K^+$  we would expect the binding to be influenced by pH. Thus, the  $K^+$  dependence of the PPase was measured at different cytosolic pH (Table 1, Fig. 5,  $pH_{\text{cyt}} = pH_{\text{vac}}$ ).  $Li^+$  and  $Cs^+$  were chosen to substitute for  $K^+$  because of their extremely different charge densities. The same selectivity sequence,  $K^+ > Cs^+ > Li^+$ , was observed at pH 6.5, 7.5 and 8.5, but the current density was always highest at pH 7.5 for all three alkali ions (Fig. 5). At acidic pH the  $Cs^+$ -dependent currents were about 84% of the respective  $K^+$ -dependent current, whereas under standard conditions (pH 7.5) and at pH 8.5 the  $Cs^+$ -depen-

dent current was approximately 50% indicating that  $K^+$  stimulation and/or  $K^+$  binding of the PPase may be influenced by cytoplasmic pH.

### 3.3. Potassium and voltage dependence

To further address the  $K^+$  binding by the PPase, pump currents in the forward mode were measured at different  $K^+$  concentrations of the bath solution. The  $K_M$  for  $K^+$  was determined as being 11 mM and  $i_{\text{max}} = -2.4 \text{ mA m}^{-2}$  at  $V_m = 0 \text{ mV}$  (Fig. 6). However, the binding of  $K^+$  and consequently  $K_M$  and  $i_{\text{max}}$  may depend on membrane voltage. Therefore, these parameters were also determined at  $-40$  and  $40 \text{ mV}$  (Fig. 6). No difference in the  $K^+$ -dependence of the PPase was detected, indicating that the  $K^+$  binding is voltage independent.

Current–voltage relations in the presence and in the absence of  $PP_i$  were measured when  $K^+$ ,  $Cs^+$  or  $Li^+$  were added at the cytoplasmic side and  $K^+$  at the vacuolar side (Fig. 7,  $pH_{\text{cyt}} = pH_{\text{vac}} = 7.5$ ). With  $K^+$  at both sides of the tonoplast, the current curves in the absence and in the presence of  $PP_i$  are parallel over the whole investigated voltage range, indicating that the  $PP_i$ -evoked current is voltage-independent (Fig. 7A). On the contrary, in the presence of  $Cs^+$  or  $Li^+$  the whole-vacuole current curves measured before and after  $PP_i$  addition crossed at positive voltages (Fig. 7B and C) suggesting that in these cases the pump current becomes voltage-dependent. To get further insight into this surprising behaviour, we performed similar measurements at different pH values and we analysed the pump currents in the voltage range ( $-50 \text{ mV}$ ,  $+50 \text{ mV}$ ). With  $Cs^+$  or  $Li^+$  at the cytoplasmic side a slight voltage dependence was indeed observed (Fig. 8A) at pH 7.5, whereas similar to Fig. 3 no voltage dependence was detected in the forward mode of the PPase with  $K^+$  in the bath medium. Surprisingly, the current reversed at around  $V_m = 40 \text{ mV}$  when  $Cs^+$ , and at approximately  $0 \text{ mV}$  when  $Li^+$  was replacing  $K^+$  (Fig. 8A). Positive currents corresponding to an efflux of cations out of the vacuole were now detected in the forward mode. Such positive currents were never observed, if  $K^+$  of the bath solution was substituted by  $Rb^+$  (data not shown), or with equal  $K^+$  concentrations on both sides of the tonoplast. Note that only  $PP_i$ , no  $P_i$  had been added to the bath solution and intersections of the  $i$ - $V$  curve with the voltage axis, therefore, are no true reversal potentials in the meaning that the pump changed its mode of operation. At more alkaline pH (8.5, Fig. 8B) the  $Cs^+$  dependent current reversed at less positive voltages ( $V_m = 20 \text{ mV}$ ), whereas with  $Li^+$  the current reversed at approximately  $0 \text{ mV}$ . However, pump currents in the presence of  $Li^+$  were very

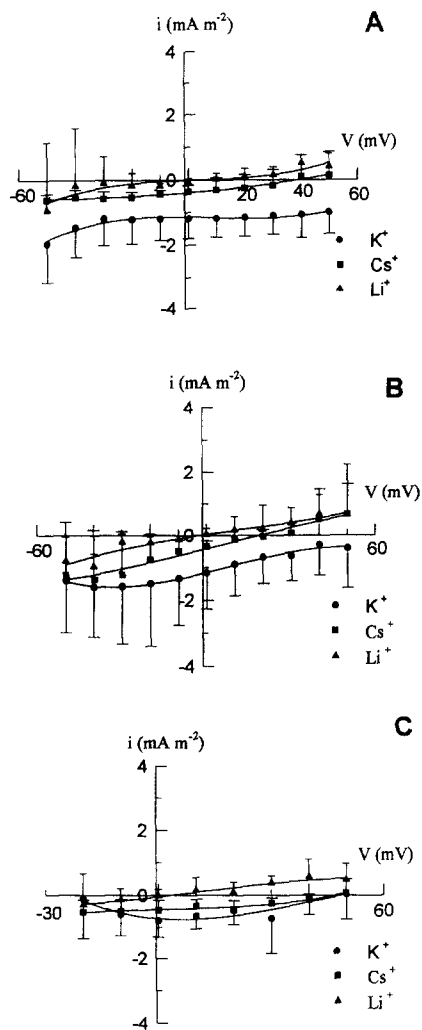


Fig. 8. Current–voltage characteristics of the pyrophosphatase in the presence of  $K^+$ ,  $Cs^+$  or  $Li^+$ . Cations were added as 50 mM chloride salts. The pH was always the same at both sides of the tonoplast: (A) pH = 7.5, (B) pH = 8.5, (C) pH = 6.5. The average of at least four  $i$ - $V$  curves is shown. The downward error bars belong to the  $K^+$  data, the upward error bars to the  $Cs^+$  experiments and the upward bars with the smaller cap width to the  $Li^+$  experiments.

small in the measured voltage range and therefore not satisfactorily reliable. All currents became more voltage-dependent at pH 8.5 than at pH 7.5. In Fig. 8C the  $i$ - $V$  curves at acidic pH ( $\text{pH}_{\text{cyt}} = \text{pH}_{\text{vac}} = 6.5$ ) are shown. Generally, the currents for all tested alkali ions were much lower at acidic pH than at alkaline pH, therefore the intersection potentials could not be determined with accuracy. At voltages more negative than  $-20$  mV the current could not be analysed because under these conditions the current noise increased, so that the difference between the current measured in the presence and absence of  $\text{PP}_i$  could not be determined reliably.

#### 4. Discussion

A  $\text{PP}_i$ - and a  $\text{P}_i$ -dependent current representing the forward and backward mode of the  $\text{H}^+$  pumping PPase, respectively, were measured on intact vacuoles of *Chenopodium rubrum* culture cells using the whole-cell configuration of the patch-clamp technique. The  $K_M$  for the  $\text{PP}_i$ -generated current ( $18 \mu\text{M}$ ) obtained with the patch-clamp method was similar to the  $K_M$  values determined by measuring the  $\text{PP}_i$  hydrolysis ( $12 \mu\text{M}$ ) and the  $\text{H}^+$  transport activity ( $9 \mu\text{M}$ ) of a tonoplast vesicle preparation from *Chenopodium* cells [23,24]. With appropriate thermodynamic conditions it should be possible to reverse the PPase so that in the presence of orthophosphate ( $\text{P}_i$ ) a current in the opposite direction (positive charges flowing out of the vacuole) will be generated. Thus, a  $\text{H}^+$  gradient of  $\Delta\text{pH} = 2$  should suffice to energize this backward mode current implying synthesis of  $\text{PP}_i$ . (Unfortunately, however, this postulated  $\text{PP}_i$  synthesis could not be measured, because the amount of  $\text{PP}_i$  synthesized from a single vacuole would have been undetectably small). The  $K_M$  of  $\text{P}_i$  for the backward mode of  $14 \text{ mM}$  was well in the concentration range used to reverse the PPase of red beet vacuoles [15]. Both the  $\text{PP}_i$ - and the  $\text{P}_i$ -dependent currents showed large deviations from the average current density (Fig. 1B and 2B). A similar large range of current densities was measured with red beet vacuoles ( $0.1$ – $11.3 \text{ mA m}^{-2}$ , [15]). This notable variability may be due to unknown cytoplasmic factors which stimulate the PPase and are not completely removed during isolation of the vacuoles. For instance, D-sphingosine was shown to stimulate the PPase [24], whereas no effect was measured with calmodulin antagonists [25].

##### 4.1. Potassium and voltage dependence

The  $\text{K}^+$  dependence of the pump current and the  $\text{K}^+$  dependent shift of the reversal potential ( $E_{\text{rev}}$ ) of the PPase

have been interpreted to imply that the  $\text{H}^+$  PPase cotransports  $\text{K}^+$  [15]. But evidence for  $\text{K}^+$  transport by the PPase reconstituted into liposomes using  $^{42}\text{K}^+$  [6], or by using tonoplast vesicles loaded with a  $\text{K}^+$ -sensitive fluorescent dye [16] is lacking. Therefore, it may be concluded that the PPase does not carry  $\text{K}^+$  by itself, but that the PPase-generated  $\text{H}^+$ -gradient energizes a secondary  $\text{H}^+/\text{K}^+$  antiport transporting  $\text{K}^+$  into the vacuole.

We have observed that in *Chenopodium* vacuoles  $\text{K}^+$  has to be present at the cytoplasmic side to measure  $\text{PP}_i$ -dependent currents and at the vacuolar side of the tonoplast to detect  $\text{P}_i$ -dependent currents, insofar confirming the data of Davies et al. [14,15] on red beet vacuoles. The calculated  $K_M$  for  $\text{K}^+$  ( $11 \text{ mM}$ ) in the forward mode determined by patch-clamp experiments with intact vacuoles notably was in the same range as the  $K_M$  for  $\text{K}^+$  determined by measuring  $\text{PP}_i$  hydrolysis ( $5 \text{ mM}$ ) and  $\text{H}^+$ -transport activity ( $6 \text{ mM}$ ) of tonoplast vesicles from *Chenopodium* [23,24]. The  $K_M$  for  $\text{K}^+$  in the present study (Fig. 6) was independent of the membrane voltage indicating that the binding of  $\text{K}^+$  may not be involved in an electrogenic reaction step of the pump (cf. Lauser [26]).

Potassium may be substituted by other alkali ions and similar ionic selectivity sequences were found regardless of the method used to obtain the data [23,27–30]. In our study, substitution of  $\text{K}^+$  by  $\text{Cs}^+$  or  $\text{Li}^+$  reduced the PPase current, which was measured at  $V_m = 0 \text{ mV}$  and pH 7.5 of the bath solution (Fig. 4). At more acidic pH (pH 6.5) at both sides of the tonoplast, the PPase still shows the normal selectivity sequence ( $\text{K}^+ > \text{Cs}^+ > \text{Li}^+$ ), but the stimulatory effect of  $\text{K}^+$  is not much higher than stimulation by  $\text{Cs}^+$  (Fig. 5). Therefore, the PPase seems to lose its ability to discriminate between  $\text{K}^+$  and  $\text{Cs}^+$  at lower cytoplasmic pH. This observation is consistent with recent findings where an acidic amino acid of the PPase molecule was exchanged. The aspartic acid (Asp-504) was substituted by asparagine (Asn), and the mutant PPase showed less than 10% of the wild-type hydrolytic and  $\text{H}^+$ -transport activity (Zhen, R.-G., Kim, E.J. and Rea, P.A., unpublished results). Also, the recombinant PPase lost its ability to distinguish between  $\text{K}^+$  and  $\text{Cs}^+$ . The stimulation by  $\text{Cs}^+$  was even higher than the  $\text{K}^+$  stimulation (Zhen, R.-G. and Rea, P.A., personal communication). One may therefore infer that the negative charge of the Asp-504 is important for the specific binding of  $\text{K}^+$ , and therefore the ability of  $\text{K}^+$  to stimulate the PPase is lost if the Asp-504 carboxy group is partially protonated at acidic pH.

The voltage dependence of the pump was affected by the ionic composition of the bath solution. In the presence of  $\text{K}^+$  the PPase acts as a constant current source in the forward and in the backward mode showing no voltage dependence in the measured voltage range (Fig. 3), whereas



in the presence of  $\text{Cs}^+$  or  $\text{Li}^+$  the pump current became voltage-dependent (Figs. 7 and 8). With  $\text{Cs}^+$  or  $\text{Li}^+$  in the bath solution and at positive voltages an efflux of positive charges out of the vacuole occurs in the presence of  $\text{PP}_i$  concentrations usually inducing a current of opposite direction. The intersections of the  $\text{PP}_i$ -induced  $i$ - $V$  curves (Fig. 8) do not represent reversal potentials, because the pump is still operating in the forward mode. Clearly, no  $\text{P}_i$  or pH gradient were present to drive the backward mode. These intersection potentials ( $E_{\text{int}}$ ) thus are to be distinguished from pump reversal potentials at which a pump is forced to change its mode of operation. With  $\text{Cs}^+$  in the bath solution (pH 7.5) and voltages below  $E_{\text{int}}$  (40 mV in Fig. 8A), the pump operates in the forward mode generating an inward current. At the 'intersection potential' an outwardly directed current equals the inward pump current, and at  $V_m > E_{\text{int}}$  the outward current finally exceeds the inward pump current resulting in an overall outward current. At pH 8.5 (Fig. 8B) the  $\text{PP}_i$ -induced,  $\text{Cs}^+$ -stimulated current reversed already at lower voltages (20 mV), presumably because less protons for the PPase forward mode (inward current) are available than at pH 7.5. Therefore, the outward current exceeds the inward pump current at lower  $E_{\text{int}}$ .

A possible explanation for the positive,  $\text{PP}_i$ -dependent current involves the action of two or three different ion transporters, i.e. the PPase pumping exclusively  $\text{H}^+$  into the vacuole, a  $\text{H}^+/\text{K}^+$  antiporter and a  $\text{K}^+$  channel. If a putative  $\text{H}^+/\text{K}^+$  antiporter or a  $\text{K}^+$  channel were responsible for the observed outward current, they both would have to be voltage-dependent and be activated by  $\text{PP}_i$  directly or indirectly via the PPase. However, this explanation is not very likely, because both transporters will be present in all vacuoles and therefore positive,  $\text{PP}_i$ -dependent currents should have also been measured with  $\text{K}^+$  at both sides of the tonoplast.

According to Davies and Sanders [31], addition of ATP to red beet vacuoles revealed voltage-dependent 'leaks' (shunt conductance) and the same may occur in *Chenopodium* vacuoles after adding  $\text{PP}_i$ . Such a  $\text{PP}_i$ -inducible 'leak' may be responsible for the positive currents at positive voltages when  $\text{Cs}^+$  or  $\text{Li}^+$  substitute for  $\text{K}^+$ . But, as shown in Fig. 3A, we did not observe an additional voltage-dependent current after addition of  $\text{PP}_i$ , just a constant shift of the current at any given voltage.

Rather, we argue that the  $\text{PP}_i$ -dependent outward current flows through a  $\text{K}^+$ -permeable, channel-like domain within the PPase molecule itself. At voltages higher than the  $E_{\text{int}}$  and in the presence of  $\text{Cs}^+$  or  $\text{Li}^+$  in the bath solution the electrochemical potential gradient for  $\text{K}^+$  becomes so high that vacuolar  $\text{K}^+$  ions may cross a particular energy barrier inside the PPase molecule that usually separates the vacuo-

lar from the cytoplasmic face of the pump. Potassium ions may cross the energy barrier inside the PPase molecule when the pump operates in the forward mode, i.e. after addition of  $\text{PP}_i$ . Such a 'tunneling' effect [32,26] has been reported for a number of ion transporters, including the band-3 protein from erythrocytes [33], the  $\text{Na}^+,\text{K}^+$ -ATPase [34], and the  $\text{Ca}^{2+}$ -ATPase [35].

Clearly, a 'tunneling' mode in the PPase transport mechanism would explain the observed voltage and potassium dependence of the PPase. Although the question is still open whether the PPase indeed translocates  $\text{K}^+$ , our data support the idea that  $\text{K}^+$  ions are involved in the generation of the pump current in the forward mode by binding to a specific binding site and secondly, that a channel-like pathway exists in the PPase to facilitate a  $\text{PP}_i$ -dependent permeation of  $\text{K}^+$  through the PPase.

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