Patch-clamp studies of ion transport in isolated plant vacuoles

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Plant cell vacuoles act as storage depots for substances such as sugars, organic and inorganic ions, many of which are commercially important. We have used the patch-clamp technique to characterize an ATP-dependent proton pump and a non-selective channel in the vacuolar membrane in single isolated barley leaf vacuoles. The large (60 to 80 pS) ion channel shows inward rectification, and carries both K^+ or malate ions driven by their concentration gradient as well as the electrical gradient established by the ATP-dependent proton pump.

Patch-clamp *Vacuole* *Electrogenic pump* *Ion channel*

1. INTRODUCTION

The vacuole accounts for up to 90% of a plant cell's volume, functioning as a storage depot for sugars, organic and inorganic ions [1–4]. Barley leaf vacuoles have been widely used as an example for the storage and release of plant cell metabolites. Studies on the transport mechanisms of the vacuolar membrane (tonoplast) have been performed using whole leaves or large populations of isolated vacuoles; however, these studies cannot reliably distinguish between various transport mechanisms such as carriers, channels and pumps. These limitations have left many questions open regarding the nature of tonoplast transport mechanisms. As we show here, the patch-clamp technique, applied to single, isolated vacuoles is a powerful tool, able to distinguish the various transport mechanisms, thus opening up the possibility for a detailed understanding of their nature and regulation.

During photosynthesis, barley leaf vacuoles accumulate malate against a gradient [4]. At night malate is released again into the cytosol, presumably supplying the mitochondria with metabolites in order to maintain the energy status of the cell [4]. In addition to malate, H^+ and K^+ also cross the membrane, preserving electroneutrality and isoosmolarity between a vacuole and a cytosol [5–7]. In this work we attempt an identification of the transport systems responsible for the movement of these ions through the tonoplast.

2. RESULTS AND DISCUSSION

Vacuoles were isolated from barley mesophyll protoplasts [3]; ionic currents and membrane potentials were measured by sealing a patch pipette [8] against the vacuole and breaking the underlying membrane by a pulse of hyperpolarizing voltage (−200 mV). This allows exchange between the vacuole and pipette solution. Fig.1A shows typical ionic currents recorded in 'whole-vacuole' (analogous to 'whole-cell' [8]) voltage clamp in symmetric 50 mM KCl. Hyperpolarizing (inside negative) voltage steps evoked large inward currents, whereas depolarizing steps evoked no current, suggesting the presence of inwardly rectifying ion channels. To determine whether K^+ and malate pass through the inward rectifier channels, the

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vacuoles were exposed to solutions containing different concentrations of K^+ and malate. Fig.1B shows the current-voltage relationships obtained with 50 mM K_2malate, pH 7.3, in the bath and 50 mM K_2malate, pH 5.5, in the pipette (fig.1B, closed symbols). The *I-V* relationship in these solutions revealed only an inwardly rectifying current with a zero-current potential of 0 mV. Under these conditions, activation of inward current began at about −15 mV.

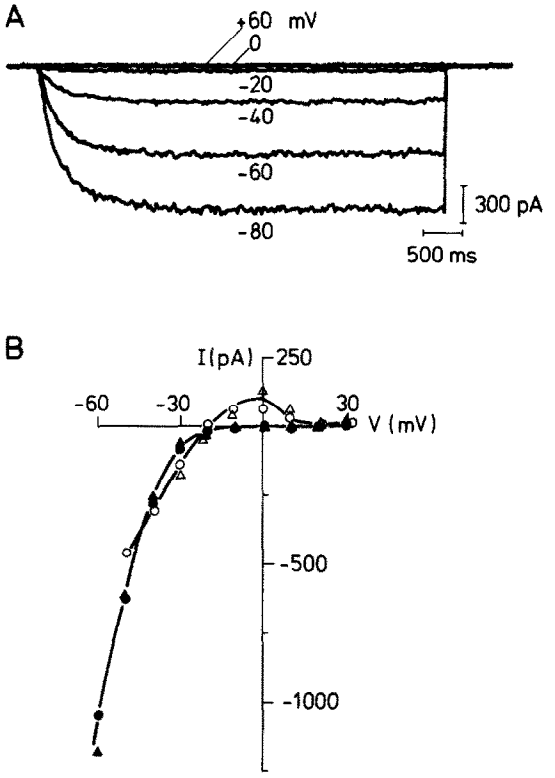

Fig.1. (A) Whole-vacuole currents in response to a series of voltage steps from a holding potential of 0 mV. The solutions were: 50 mM KCl, 1 mM $CaCl_2$, 50 mM Hepes/imidazole (pH 7.3) in the bath and 50 mM KCl, 1 mM $CaCl_2$, 50 mM Hepes/Mes (pH 5.5) in the pipette. (B) Current-voltage relationship of the vacuolar membrane measured in symmetric 50 mM K_2malate solutions (closed symbols) and after reducing the external concentration of K^+ and malate to 20 and 5 mM, respectively (open symbols). The ionic currents were measured at steady state. Zero-current potential was determined by reversal of the tail currents (fig.2A). The solutions were: external, 50 mM K_2malate, 1 mM $CaCl_2$, 50 mM Hepes/imidazole (pH 7.3) and internal (pipette), 50 mM K_2 malate, 1 mM $CaCl_2$, 50 mM Hepes/Mes (pH 5.5). K^+ was always added as KOH. The external solution with a reduced concentration of K_2malate was readjusted with D-sorbitol in order to maintain the osmolarity of 600 mosmol.

After the bath solution was changed to 20 mM K^+, 5 mM $malate^{2-}$ (about the cytoplasmic malate concentration [5]) the zero-current potential shifted to −17 mV (fig.1B, open symbols). The activation threshold also changed to about 15 mV. The shift in the reversal potential could be attributed neither to K^+ (E_{K^+} = −40 mV) nor to malate ($E_{mal^{2-}}$ = 20 mV, E_{mal^-} = 150 mV) alone, but rather to both ions. (A significant contribution by Ca^{2+} or Cl^- is ruled out due to their low concentrations (see fig.1B legend.)) Considering K^+, malate $^{2-}$ and $malate^-$ as the main ion carriers and using the general current equation derived from the constant field theory [9] a permeability ratio can be calculated:

$$\frac{P_{mal^{2-}} + \frac{P_{mal^-}}{2.43}}{P_{K^+}} = 0.49$$

The shift in the activation threshold (to more positive potentials) could be due to the presence of surface charges that became unshielded when the ionic strength of the extravacuolar solution is reduced (as in the case of fig.1B).

Fig.2A shows the time course of the decay of the macroscopic current at different voltages in a 'tail-current' experiment using two voltage levels. The first level (−80 mV) was chosen to produce a large inward current. Following the second step to different levels (20 to −20 mV) the currents ('tails') decreased with a voltage-dependent time course, reversing direction at the zero-current potential. The decrease in current was much faster at positive than at negative potentials as expected from the strong voltage dependence of the membrane currents.

To determine whether the macroscopic (whole-vacuole) current could be resolved as unitary single-channel currents, we used outside-out isolated patches [8] and a similar voltage protocol to that shown in fig.2A. The excised patches were held at a hyperpolarized potential (−40 mV) in order to activate the inwardly rectifying channels. Fluctuations of many unitary channel currents were observed (fig.2B). When the membrane

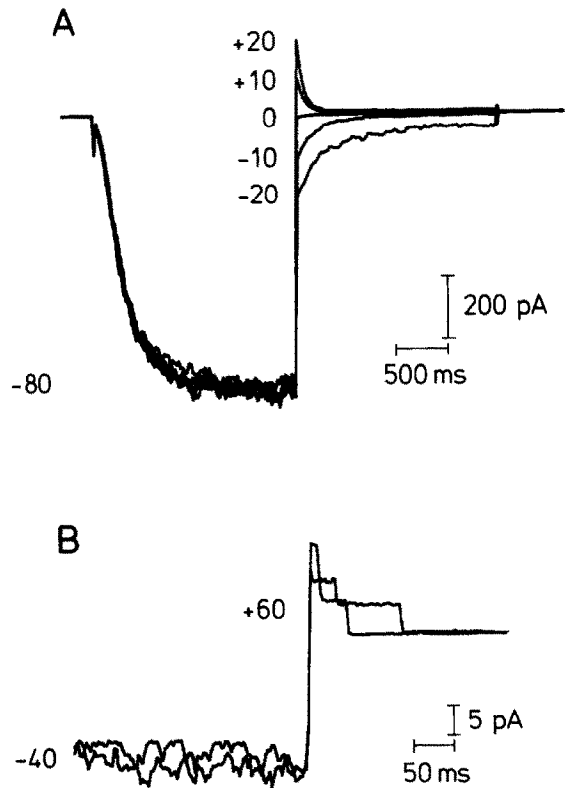

Fig.2. (A) Membrane currents during a double-pulse sequence. The membrane potential was stepped from a holding potential of 0 mV to a prepulse level of −80 mV and then to various depolarizing levels as shown. Five current traces are superimposed. During the test pulse the macroscopic currents (tails) declined with a voltage-dependent time course and reversed their direction near 0 mV. (B) Currents recorded from an outside-out patch from the vacuolar membrane, held at a hyperpolarizing potential (−40 mV). The fluctuations of several channels can be seen. Upon stepping the membrane potential to depolarizing values (60 mV) the channels closed (two superimposed records) showing similar time course and voltage dependence to the macroscopic tails shown in A. Solutions in A and B were 50 mM K_2malate, 50 mM Hepes/imidazole (pH 7.3), 1 mM $CaCl_2$ in the bath and 50 mM K_2malate, Hepes/Mes (pH 5.5), 1 mM $CaCl_2$ in the pipette.

potential was stepped to 60 mV, the individual channels closed, in agreement with the voltage dependence exhibited by the macroscopic current. The unitary conductance of these channels was 60–80 pS in symmetric 50 mM K_2malate. Similar ion channels were also observed in the vacuole-attached (analogous to cell-attached [8]) configuration.

In symmetric *N*-methylglucamine-glutamate solutions (NMG is an impermeable cation), the vacuolar resting potential was zero and no currents were measured under voltage clamp at both depolarizing and hyperpolarizing potentials. After the application of 1 mM ATP to the extravacuolar medium, a rapid depolarization from 0 to 50 mV was observed (fig.3A), indicating the presence of an electrogenic pump energized by ATP. Addition of 1 μM tributyltin, a known inhibitor of H^+-ATPases [6,10–12] totally abolished the membrane potential (fig.3A).

The ATP-induced shift of the membrane potential to positive values was generated by inward currents (fig.3B). When the ATP concentration was increased stepwise by bath perfusion, the pump currents also increased stepwise, reaching 65 pA at 10 mM Mg-ATP. When ATP was removed from the bath, the inward current declined to zero. The Lineweaver-Burk plot gave an apparent K_m for ATP of 0.8 mM and a maximal current of 50–85 pA (fig.3C).

In contrast to previous findings [8], the K_m of this ATPase is close to the ATP concentration measured in the cytosol [13]. Our data suggest that at this concentration of ATP changes in the cytosolic energy supply will affect the activity of the pump, although other regulatory mechanisms might also be involved in controlling H^+ transport.

Under physiological conditions the observed cytoplasmic ATP concentration was about 1 mM [13]. The pH is about 7–8 in the cytoplasm and 5–6 in the vacuole [16–18]. Our results suggest that these conditions will produce a pump current of 50 pA/vacuole. Assuming that anionic malate short-circuits the H^+ current by following H^+ passively, vacuoles could accumulate malate under these conditions at a rate of 12.5 fmol/vacuole per s [7]. If we assume that the pump current is entirely due to H^+, then two H^+ are pumped into the vacuole per $malate^{2-}$ accumulated.

It has been suggested than an electrogenic proton pump is responsible for solute accumulation in the vacuolar space [14,15], however, direct evidence is still lacking. Our results demonstrate the presence of such an electrogenic pump in single isolated vacuoles and show that it provides enough current to drive the accumulation of malate during the

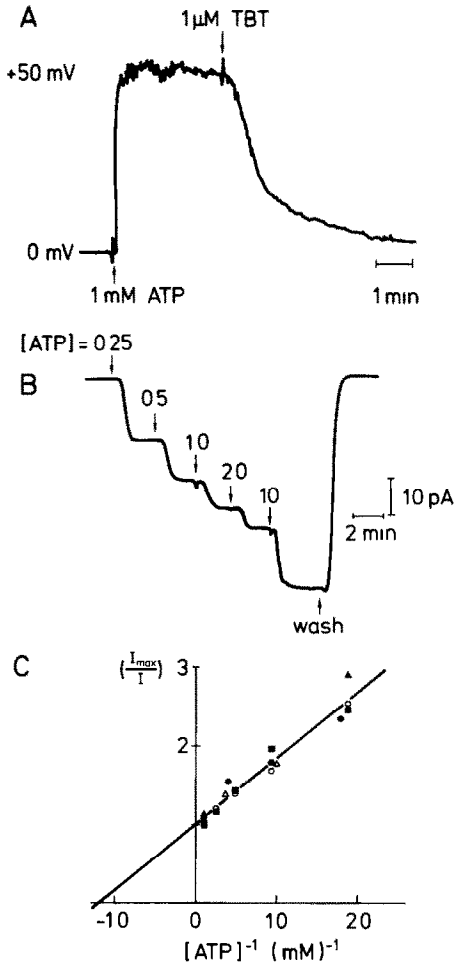

Fig.3. (A) Current-clamp recording of the membrane potential of an isolated barley vacuole. After the application of 1 mM ATP the membrane potential depolarized to 50 mV. 1 μM tributyltin (TBT) abolished the membrane potential. The solutions were: symmetric 250 mM NMG-glutamate, pH 7.3; 1 mM $CaCl_2$ and 2 mM $MgCl_2$. (B) Voltage-clamp recordings of ATP-induced pump currents. The membrane potential was held at 0 mV. Increasing the Mg-ATP concentration in steps (numbers above the arrows indicate Mg-ATP concentrations in mM) caused a stepwise increase of the total inward current from 0 up to 65 pA. (C) Lineweaver-Burk plot of the data from four different experiments obtained by a similar procedure to that in B. In order to compare vacuoles of different sizes, the ATP-induced currents are expressed as I/I_{max}. The apparent K_m for ATP is 0.8 mM and I_{max} 50–85 pA. Solutions in B and C were: 50 mM KCl, 50 mM Hepes/imidazole (pH 7.3), 1 mM $CaCl_2$ inside the bath and 50 mM KCl, 5 mM Hepes/ Mes (pH 5.5), 1 mM $CaCl_2$ in the pipette.

day-night cycles of carbon metabolism. These results also show that a likely pathway for K^+ and malate movements across the vacuolar membrane is the inwardly rectifying channels described here. Under physiological conditions the electrogenic pump probably cannot depolarize the vacuolar membrane as much as shown in fig.3A because of the presence of the inward rectifier, which was blocked in fig.3. If electrogenic pumping depolarizes the vacuolar membrane beyond the malate equilibrium potential, malate could accumulate in the vacuole through the inward rectifier (which can be active at positive potentials, as shown in fig.1B). A reduction of the pump activity similarly could lead to a loss of vacuolar malate.

The large conductance and poor selectivity of the inward rectifier are reminiscent of the gap-junction channels [19] that provide an ionic and metabolic continuum between adjacent animal cells; similarly, the inward rectifier channels connect the vacuolar and cytoplasmic spaces.

The plant cell vacuole is known to be the site of accumulation of many substances of nutritive and pharmaceutical value [15] (i.e. organic acids, sugars, etc.). Therefore, an understanding of the molecular mechanisms responsible for solute transport across the vacuolar membrane should bring significant practical benefit.

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