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Ion channels and ATP-driven pumps involved in ion transport across the tonoplast of sugarbeet vacuoles

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The electrical properties of the tonoplast of mature sugarbeet root vacuoles have been studied using the patch-clamp technique. In whole-vacuole recordings, the addition of 5 mM Mg-ATP to the external solution activated a proton-translocating ATPase which produced inward currents of up to 65 pA. Furthermore, we identified a voltage-dependent membrane conductance which activated at hyperpolarized (inside-negative) potentials and decreased at positive potentials. Outside-out membrane patches predominantly contained a channel which showed an increasing probability of opening at potentials more negative than about −20 mV. These channels can account for the macroscopic currents recorded in whole vacuoles. The permeability sequence of the channel for cations and anions was: $P_{K^+} = P_{Na^+} > P_{Ac^-} > P_{NO_3^-} > P_{Mal^{2-}} > P_{Cl^-}$. The unit conductance of this channel was about 70 pS in symmetrical 50 mM KCl and 180 pS in symmetrical 200 mM KCl solutions. Another channel type of smaller conductance (15 pS in 50 mM KCl) was also present, but its properties have not yet been studied. The permeability sequence of the nonselective channel corresponds to that found by tracer measurements in vacuole suspensions, implying that the channel studied may present the molecular pathway for the movement of ions across the tonoplast.

Introduction

Physiological processes in plant cells are mediated by a complex interaction between the cytoplasm and the organelles. The vacuole, a large intracellular compartment (90% of the cell volume), participates in the maintenance of cytoplasmic homeostasis, cell turgor, accumulation of ions and metabolites [1–5]. Considerable experimental evidence already exists in different plant cells indicating that these transport mechanisms are driven by a proton gradient across the tonoplast (for reviews, see Refs. 3, 6–8). The fact that a positive membrane potential is created across the tonoplast of isolated mesophyll barley vacuoles by an electrogenic ATPase has been unequivocally established by direct measurement of the pump current by Hedrich et al. [9], using the patch-clamp technique. The presence of ionic channels has been demonstrated in vacuoles isolated from *Catharanthus roseus* cells cultured in suspension [10] and in barley leaf vacuoles [9]. In the latter, the channel described is permeable to both K^+ and malate.

The present study investigates the molecular mechanisms involved in the movement of solutes across the tonoplast of sugarbeet vacuoles. The

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Mes, 4 morpholineethanesulphonic acid; Tris, (hydroxymethyl)aminomethane.

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results obtained demonstrate that similarly to tonoplasts from photosynthetic cells, a channel with fairly low selectivity and a H^+-translocating ATPase may play a dominant role in regulating ion movements between cytoplasm and the vacuole in plant cells.

Materials and Methods

Sugarbeet roots were obtained from CERES (91660 Mereville, France) and from the Botanischer Garten of the University of Göttingen, F.R.G. A piece of about $1 \times 1 \times 2$ cm was cut from the root using a clean knife. Holding the piece with forceps a slice was cut off with a fresh razor blade and the surface rinsed with a few drops of the bathing medium and the drops collected directly into the recording chamber. When left undisturbed for a few minutes, vacuoles with diameters ranging from a few μm to about 50 μm were found attached to the glass bottom of the chamber. Little debris was present if the razor blade used for cutting was very sharp.

The bathing media were buffered with 10 mM Hepes adjusted to pH 7.2–7.5 with KOH or NaOH. The pipette (electrode)-filling solution was adjusted to pH 5.5–6.0. The osmotic pressure of the medium was adjusted with sorbitol or mannitol to 600 mosmol in the pipette and 650 in the bath. The ionic composition of solutions in different experiments are specified in the figure legends. All solutions were freshly made and filtered (Millipore, 0.2 μm) just before use.

Patch pipettes were pulled by a two-stage puller (Narishige, PP-83, or List Electronics, L/M-3P-A) using Kimax capillary tubing (Kimble Products, U.S.A.). Both untreated pipettes and fire-polished (microforge L/M CPZ-101, List Electronics) and coated pipettes (Sylgard, Rhone-Poulenc, France) as described by Corey and Stevens [11] were used. Fire-polishing was not essential for obtaining giga-seals. The resistances of the pipettes ranged between 1 and 50 MΩ depending on the ionic composition of the media and the size of the pipette tip.

Recordings were made from isolated sugarbeet vacuoles using the whole-vacuole (analogous to whole-cell [12]) and outside-out patch configurations. Whole vacuoles were obtained by first sealing the pipette onto the tonoplast. Access to the interior of the vacuole was gained by holding the pipette potential at -200 mV until the membrane patch underlying the pipette tip was ruptured. The vacuolar content rapidly equilibrated with the pipette solution [13], thus the ion concentrations were well defined on both sides of the membrane. Withdrawal of the pipette from the whole-vacuole configuration resulted in excision of an outside-out patch. In both configurations the vacuolar (inner) face is exposed to the pipette solution and the cytoplasmic face to the bathing medium. Current and potential measurements were made using a patch-clamp amplifier (RK300, Biologic or EPC7, List Electronics).

The membrane potentials described in this paper were corrected for the liquid junction potential as measured with a 3 M KCl reference electrode. The values obtained were in agreement with those calculated from the Henderson assumption [14]. Data were stored on tape using a video cassette recorder (Sony SL-C9F) after sampling with an analog/digital converter (Sony 701ES) modified as described by Benzanilla [15]. Data were analyzed using a digital oscilloscope (Nicolet 3091) after filtering at cut-off frequencies of 300 or 1000 Hz.

Results

Presence of an ATP-dependent pump

In the whole-vacuole configuration, when the membrane potential was clamped to 0 mV, addition of 5 mM Mg-ATP to the bath solution resulted in an inward (into the vacuole) current, of up to 65 pA, in 70% of the preparations (Fig. 1). When ATP was removed from the extravacuolar solution the current declined to the original level and again increased by a second addition of ATP. After addition of ATP the resting potential of the vacuole shifted to $+55$ mV. These results are comparable with those reported for barley vacuoles [9].

Voltage-dependent whole-vacuole currents

In order to determine the 'passive' ionic conductance of the tonoplast, whole-vacuole experiments were performed in symmetric solutions of 50 mM KCl. The membrane potential was held at

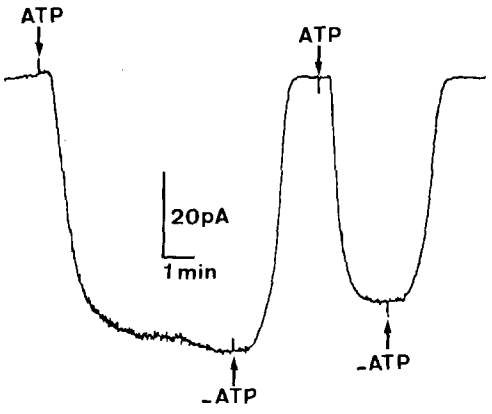

Fig. 1. Voltage-clamp recording of pump currents from a whole vacuole. The membrane potential was clamped at 0 mV; addition of 5 mM Mg-ATP to the extravacuolar solution generated inward currents of up to 65 pA. The current decreased to zero again when ATP was removed (−ATP). Pipette solution: 100 mM KCl/2 mM $MgCl_2$/5 mM Tris-Mes/1 mM EGTA (pH 5.5); bath solution: 100 mM KCl/2 mM $MgCl_2$/5 mM Tris-Mes/1 mM $CaCl_2$ (pH 7.5).

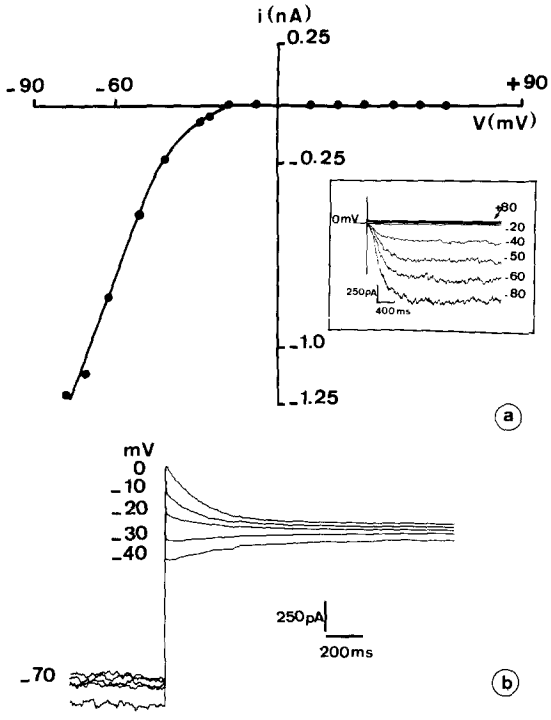

Fig. 2. Whole-vacuole currents recorded under voltage-clamp condition. (a) Current-voltage relationship for the steady-state currents of whole vacuoles. Inset: the vacuole was clamped at 0 mV and stepped to various potentials (+80 to −80 mV) during a 3 s voltage pulses. Pipette solution: 50 mM KCl/2 mM $MgCl_2$/10 mM Hepes (pH 6.0); bath solution: 50 mM KCl/2 mM $MgCl_2$/10 mM Hepes/3.5 mM KOH (pH 7.2). (b) The reversal of the tail currents were obtained by holding the potential at −70 mV and during 3 s voltage pulse to −40, −30, −20, −10 and 0 mV. Pipette solution: 50 mM KCl/2 mM $MgCl_2$/10 mM Hepes (pH 6.0); bath solution: 10 mM KCl/2 mM $MgCl_2$/10 mM Hepes/0.5 mM KOH (pH 7.2).

zero mV and during 3 s pulses stepped to various positive and negative levels. Pulses to negative potentials produced inward currents reaching steady-state within 2 s. The current amplitude rose with increasing negative potential. Voltage steps to potentials more positive than −20 mV revealed only very small currents (Fig. 2a, inset), indicating a markedly decreased open probability of the channels at positive potentials, as will be demonstrated in Figs. 3 and 4 in more detail. Fig. 2a illustrates the current-voltage relationship of the mean steady-state currents. In order to characterize the ions carrying these currents, vacuoles were exposed to asymmetric KCl solutions and the reversal potential of the current was recorded. With the vacuole potential held at −70 mV to obtain a steady-state current, the reversal potential was measured by applying 3-s pulses to various negative and positive values (Fig. 2b). With 50 mM KCl inside and 10 mM outside the vacuole currents ('tail'-currents) reversed between −25 and −30 mV (Fig. 2b). This indicated that the currents were carried by both K^+ and Cl^-. If the membrane was selectively permeable either to K^+ or Cl^- one would expect a reversal potential of −38 or +30 mV, respectively.

Single-channel recordings

The selectivity of the vacuolar channels was studied in more detail using isolated membrane patches. Pulling back the electrode from the whole-vacuole configuration excised outside-out membrane patches. This configuration allowed the resolution of current through single ion-channels. It should be noted that all membrane patches obtained in this study contained more than two channels. While single-channel events were recorded at low positive potentials, increasingly negative potentials caused the appearance of several channels (Fig. 3a).

Two kinds of channels were seen in our record-

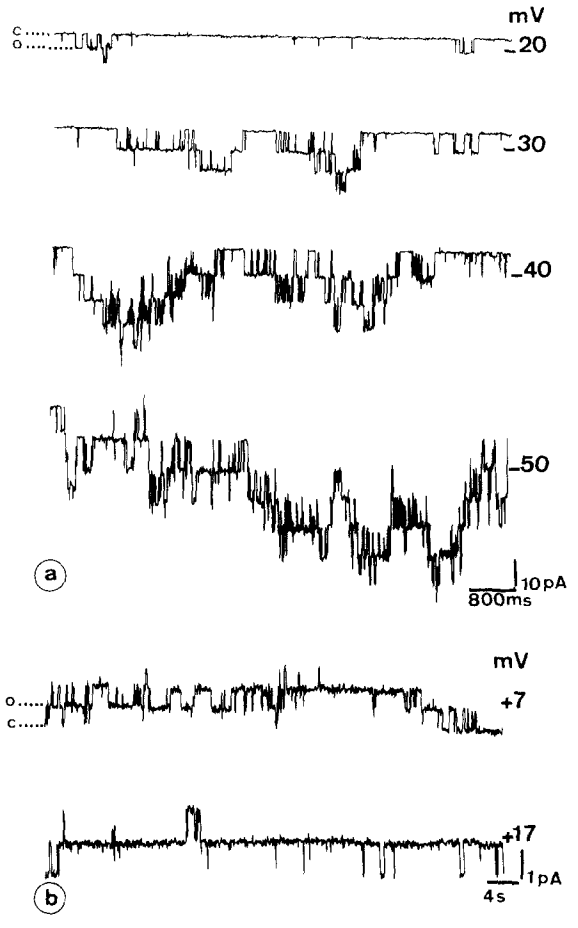

Fig. 3. Single-channel recordings from outside-out tonoplast patches. (a) Symmetrical KCl. Pipette solution: 200 mM KCl/2 mM $MgCl_2$/10 mM Hepes (pH 6.0); Bath solution: 200 mM KCl/2 mM $MgCl_2$/10 mM Hepes/3.5 mM KOH (pH 7.2). Current records are filtered at 300 Hz. The membrane patch displays an inward current which fluctuates between several levels caused by simultaneous openings of several channels in the patch. (b) Asymmetric K_2-malate. Pipette solution: 50 mM D-malic acid/88 mM KOH/2 mM $MgCl_2$/10 mM Hepes (pH 6.0); bath solution: 5 mM D-malic acid/14.2 mM KOH/2 mM $MgCl_2$/10 mM Hepes (pH 7.2). The membrane patch displays outward currents increasing with voltage; o, open level; c, closed level. Current records are filtered at 300 Hz.

ings which may be described as the 'nonselective' and the 'small' channel. The small channel had a conductance of about 15 pS in symmetrical 50 mM KCl solutions and appeared only infrequently. Under the experimental conditions shown the 'small' channel did not contribute significantly to the whole-vacuole current and was not considered further in this report.

At potentials more positive than −20 mV the behaviour of the nonselective channel consisted of short openings in between prolonged quiescent periods. However, using asymmetric ion concentrations on either side of the membrane, openings were also obtained for positive potentials (Fig. 3b). These results indicate that the nature of the ions and their concentrations may influence the threshold potential of activation of this channel. The current-voltage relation for the open channel was determined in the voltage range between −60 and +60 mV by holding the patch at a negative potential to open channels and then, while they were open, stepping the potential to more positive voltage levels. Even for very high positive potentials the channel did not close instantaneously, staying open long enough to allow current measurements (Fig. 4b). The closing of the channels following voltage steps to positive poten-

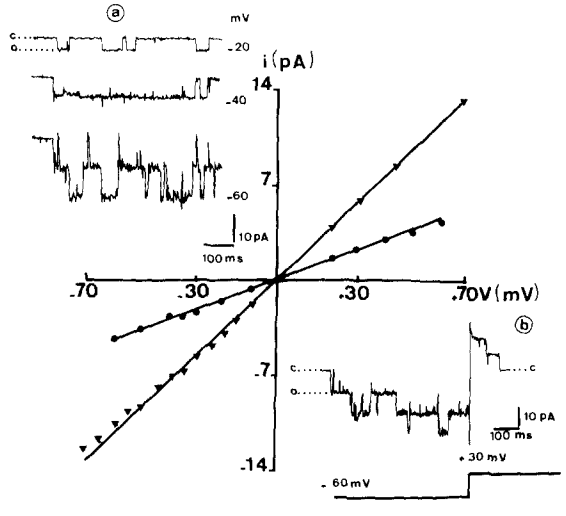

Fig. 4. Current-voltage relationship of single-channel currents recorded from outside-out membrane patches in solutions containing high and low KCl. Low KCl (●). Pipette solution: 50 mM KCl/2 mM $MgCl_2$/10 mM Hepes (pH 6.0); bath solution: 50 mM KCl/2 mM $MgCl_2$/10 mM Hepes/3.5 mM KOH (pH 7.2). High KCl (▼). Pipette solution: 200 mM KCl/2 mM $MgCl_2$/10 mM Hepes (pH 6.0); bath solution: 200 mM KCl/2 mM $MgCl_2$/10 mM Hepes/3.5 mM KOH (pH 7.2). (a) and (b) Single-channel recordings at negative and positive voltages in symmetrical 200 mM KCl, filtrated at 1000 Hz. (a) Current records at negative potentials, (b) current records at positive voltages; the membrane was clamped at −60 mV to open the channels and then stepped to +30 mV to record the open-channel amplitude from the closing channels.

tials is consistent with the rapid decay of the tail currents (Fig. 2b), indicating that the whole-vacuole current is carried by these nonselective channels. The observed channel properties closely resemble those identified as the 'nonselective' channel in barley vacuoles [9].

In the voltage range of −60 to +60 mV the current-voltage relation for this channel was linear for symmetrical ionic conditions. The single-channel conductance is about 70 pS in symmetric 50 mM KCl and about 180 pS in symmetrical 200 mM KCl solutions (Fig. 4). This disproportionality cannot be explained on the basis of differences in activity coefficients, since the activities of these solutions should have been 41 and 148 mM, respectively [16]. The channel conductance may therefore saturate, as it has been found for other ion channels [17].

Channel selectivity

The whole-vacuole and single channel recordings revealed that the channel is permeable to both cations and anions. The relative permeabilities were studied using outside-out patches in solutions containing different salts.

TABLE I

PERMEABILITY RATIOS (P_{K^+}/P_{anion}) OF THE 'NONSELECTIVE' CHANNEL CALCULATED FROM THE GOLDMAN-HODGKIN-KATZ EQUATIONS

The experiments were conducted using outside-out patches from tonoplasts of sugarbeet root vacuoles. Bath and pipette solutions were adjusted, respectively, to pH 7.2 and 6.0 with 10 mM Hepes-KOH. Junction potentials used to correct the potential values are expressed as $V_{pip} - V_{bath}$; reversal potential (E_{rev}); acetate$^-$ (Ac^-); malate^{2-} (Mal^{2-}).

Pipette (mM)	Bath (mM)	E_{rev} (mV)	P_{K^+}/P_{anion}	Junction potential (mV)
100 K^+ 104 NO_3^-	13.6 K^+ 16.6 NO_3^-	−20.0	3.2	0
100 K^+ 110 Ac^-	10.0 K^+ 8.0 Ac^-	−19.0	2.7	−13
94 K^+ 50 Mal^{2-}	13.5 K^+ 5.0 Mal^{2-}	−16.5	5.1	−13
50 K^+ 50 Cl^-	11.5 K^+ 8.0 Cl^-	−23.0	6.0	0

Starting with symmetric KCl and replacing KCl by NaCl in the bath the currents reversed at 0 mV, indicating a permeability ratio $P_{K^+}/P_{Na^+} = 1.0$. The current-voltage curve was linear for negative potentials, but had a marked outward rectification for potentials greater than 30 mV (not shown).

The anion selectivity of the channel was tested with different potassium salts in symmetrical solutions. The salts and their concentrations used are listed in Table I. Relative permeabilities were calculated by the Goldman-Hodgkin-Katz (G-H-K) equation using the activities rather than concentrations of the ions. A decreasing permeability relative to K^+ from acetate to chloride was shown.

$$P_{acetate} > P_{nitrate} > P_{malate} > P_{chloride}$$

In the case of malate, only the bi-ionized form was taken into consideration since the activity of the mono-ionized form was less than 10% of the bi-ionized form, pK_a 3.40 and 5.11 [18], in the pH range used.

Discussion

The ATP-dependent pump currents measured in the tonoplast of single isolated vacuoles present direct evidence for an electrogenic H^+-ATPase which has been postulated from pH dye experiments [19]. In the presence of 5 mM Mg-ATP this pump is able to produce an inward directed proton current of up to 65 pA per vacuole (2.3 $\mu A/cm^2$). This indicates that the H^+ current would be sufficient to generate a positive tonoplast potential as observed in vivo [20]. Moreover, the accumulation of various anions, such as malate, citrate and Cl^- has been shown to be increased by the presence of ATP in the extravacuolar solution [21,22]. The ATP-induced currents (Fig. 1) and the permeability sequence (Table I) corresponds to that found by tracer measurements in suspensions of vacuoles [21,22], implying that the H^+ pump and the nonselective channel studied may represent the molecular pathway for the movement of ions across the tonoplast.

Ion transport across the tonoplast of sugarbeet tap roots is involved in the annual accumulation and mobilization of sugars as well as the equilibration of changes in osmotic pressure during sugar

translocation [23,24]. Vacuoles from photosynthesis cells compared to storage tissue show diurnal changes of their anion content [25]. The maximal rate of uptake and release of malate or Cl^- has been shown to be in the range of 0.1 fmol/vacuole [21,22], corresponding to an ionic current of 10 pA. Under physiological conditions the membrane potential of the vacuole may be shifted either to +20 mV or −20 mV from its resting potential, for example, by modulating the pump activity. Taking the permeability ratios of Table I into account, the accumulation or release of anions would require the opening of approx. 30–60 channels per vacuole, to produce about an anion current of 10 pA. Based on the estimates of the overall channel density of a vacuole with a mean diameter of 30 μm, either from conductance measurements of whole vacuoles and single channels or from the active channels per patch (about 2) and a patch area of 1–10 μm [26], one would expect at least 600 active channels per vacuole.

The channel openings at −20 and +17 mV (Fig. 3) are sufficient to carry the required amount of ions to account for the 'rapid', diurnal concentration changes in leaf vacuoles as well as the expected 'low', annual fluxes in storage vacuoles. However, there may be also other cytoplasmic or vacuolar factors involved in the regulation of vacuolar channels, which will be the object of future studies.

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