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Characterization of Vacuolar Malate and K^+ Channels under Physiological Conditions[1]

Omar Pantoja[2], Angie Gelli, and Eduardo Blumwald*

Centre for Plant Biotechnology, Department of Botany, University of Toronto, Toronto, Ontario M5S 3B2, Canada

ABSTRACT

Patch-clamp techniques were employed to study the electrical properties of vacuoles from sugar beet (*Beta vulgaris*) cell suspensions at physiological concentrations of cytoplasmic Ca^{2+}. Vacuoles exposed to K^+ malate revealed the activation of instantaneous and time-dependent outward currents by positive membrane potentials. Negative potentials induced only instantaneous inward currents. The time-dependent outward currents were 10 times more selective for malate than for K^+ and were completely blocked by zinc. Vacuoles exposed to KCl developed instantaneous currents when polarized to positive or negative membrane potentials. The time-dependent outward channels could serve as the route for the movement of malate into the vacuole, whereas K^+ could move through the time-independent inward and outward channels.

Accumulation of ions and metabolites in the vacuole and their subsequent release are fundamental processes for the control of metabolism and osmoregulation in plant cells (3). The presence in the tonoplast of an H^+-ATPase (17), an H^+-PPiase (16), and H^+/ion (1) and H^+/metabolite transporters (6) has been shown. Ion channels have also been found in the tonoplast; however, the conditions in which the activity of these channels have been detected may be found only in special circumstances. Large negative membrane potentials and high levels of Ca^{2+} (10^{-3} M) in the cytoplasmic side of the tonoplast were required for the activation of these channels (4, 5, 9, 14). So far, only the work by Hedrich and Neher (11) has demonstrated the activity of channels under physiological conditions. We present in this report results that indicate that at cytoplasmic Ca^{2+} concentrations of 10^{-7} M two different channels are present in the tonoplast of sugar beet cells that may serve as the pathway for the movement of K^+ and malate into the vacuole.

MATERIALS AND METHODS

Plant Material

Cell suspension cultures of sugar beet (*Beta vulgaris*) were grown as previously described (2).

Isolation of Vacuoles

Vacuoles with a diameter between 25 and 30 μm were isolated by osmotic shock of protoplasts as described previously (15).

Electrical Recording

Patch-clamp techniques were used to record ionic currents from whole vacuoles and isolated outside-out patches (8). The vacuole-attached configuration was initially obtained by applying gentle suction to the pipette resulting in seals with resistances of 6 to 10 GΩ. Access to the interior of the vacuole was gained by applying a voltage pulse of 1 V for 30 ms, thus obtaining the whole-vacuole configuration. Liquid junction potentials were minimized by filling both the recording and the reference electrode pipettes with the same solution. Recording and reference pipettes were connected, respectively, to the amplifier and to ground through Ag-AgCl electrodes. Pipettes were pulled from borosilicate glass capillaries (Rochester Scientific Co., Rochester, NY) in two stages with a vertical pipette puller (Narishige Co., Tokyo, Japan). Pipettes with tip resistances of 3 to 5 MΩ were coated with a layer of silicone (Sigmacote, Sigma) and heat polished for 3 to 4 min.

Whole-vacuole currents were obtained by holding the membrane potential at 0 mV and applying alternate pulses of ± 10 mV (or ± 20 mV as indicated) every 10 s with increments of ± 10 mV up to ± 100 mV or as described in the legends to the figures. Vacuolar currents were obtained with the pCLAMP program (version 5.0, Axon Instruments) and data stored in a PCII-386 computer operating online. Outside-out patches were obtained from the whole vacuole configuration by withdrawing the pipette from the vacuole. Single-channel currents were measured by continuously polarizing the isolated patch to potentials between ± 100 mV. The signal from the patch-clamp system was digitized at 44 kHz by a pulse code modulator and stored on videotape (DAS 900, Dagan Corporation, Minneapolis, MN). Single-channel data were digitized and processed with the PAT V 6.1 program developed by J. Dempster (University of Strathclyde, Glasgow, UK) in a PCII-386 computer. Voltage clamp measurements of whole-vacuole and single-channel currents were done at 20 to 23°C with a Dagan 3900 integrating patch clamp amplifier (Dagan), and the currents were filtered at 200 Hz with a four-pole Bessel filter contained in the patch-clamp amplifier.

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[2] Present address: Department of Plant Sciences University of Oxford, South Parks Rd, Oxford OX1 3RB, England.

Solutions

Vacuoles were suspended (cytoplasmic side) in a solution containing either 100 mM KCl or 100 mM K_2mal[3] along with 2 mM $MgCl_2$, 5 mM Tris/Mes, pH 7.5, 1 mM $CaCl_2$, 3 mM EGTA, or 2.1 mM bis-(α-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid to give a final concentration of free Ca^{2+} of 10^{-7} M. The pipette filling solution (vacuolar interior) consisted of either K_2mal or KCl at the concentrations indicated in the legends to the figures. The vacuolar solution also contained 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM Tris/Mes, pH 6.0. Both the bathing and the pipette solutions were adjusted to 450 mOsmol with D-mannitol.

Calculation of the mal^{2-}/K^+ Permeability Ratio

A modification by Lewis (13) of the Goldman-Hodgkin-Katz equation was used to calculate the permeability ratio between mal^{2-} and K^+ as follows:

$$V = \frac{RT}{F} \ln \frac{[K]_o + 4\frac{P'_{mal}}{P_K}[mal]_i}{[K]_i + 4\frac{P_{mal}}{P_K}[mal]_o\, e^{FV/RT}}$$

with P' defined as:

$$P'_{mal} = \frac{P_{mal}}{1 + e^{FV/RT}}.$$

RESULTS

Under voltage-clamp conditions and at physiological levels of Ca^{2+}_{cyt}, vacuoles of sugar beet showed two different responses depending on the K salt used in the bathing solution (cytoplasm). With Ca^{2+}_{cyt} at 10^{-7} M and symmetrical 100 mM KCl, negative and positive voltage pulses stimulated instantaneous inward and outward currents, respectively. These currents remained constant during the 5-s pulses (Fig. 1B). Similar results were obtained when KCl in the pipette (KCl_{vac}) was replaced by potassium malate (K_2mal_{vac}) (data not shown). Substitution of KCl_{cyt} by K_2mal_{cyt} induced a different response. In addition to the instantaneous currents, time-dependent outward currents were also stimulated by positive voltage pulses, whereas negative pulses stimulated only instantaneous inward currents (Fig. 1C). Similar currents were also recorded with K succinate in the cytoplasmic side of the tonoplast and with KCl_{vac} (data not shown). Current-voltage plots showed that the currents recorded in the presence of KCl_{cyt} as well as the instantaneous currents obtained with K_2mal_{cyt} were of similar magnitude at all levels of potential and show a slight inward rectification, similar to the results obtained by Hedrich and Neher (11) (Fig. 1D). Figure 1D also demonstrates that the time-dependent outward currents obtained with K_2mal_{cyt} rectified, as indicated by the nonlinear current-voltage relationship.

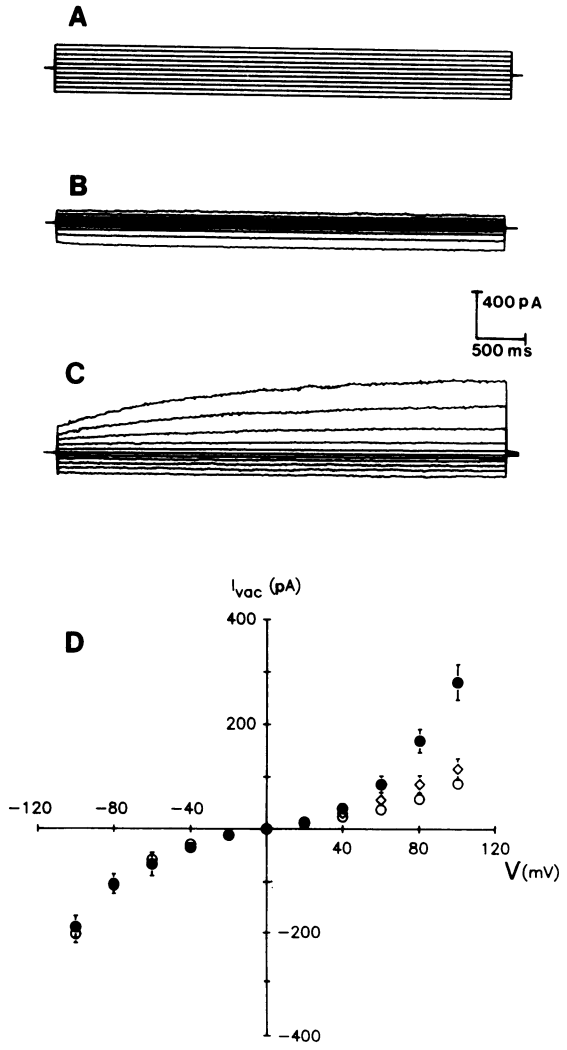

Figure 1. Voltage-clamp recordings from whole vacuoles of sugar beet cells. A, Vacuolar currents were activated by applying voltage pulses between +100 and −100 mV in steps of 20 mV from a holding potential of 0 mV. B, In vacuoles exposed to symmetrical 100-mM KCl solutions, positive and negative potentials stimulated currents that did not change in magnitude during the duration of the pulses. C, Under symmetrical 100 mM K_2mal, both instantaneous and time-dependent outward currents were stimulated by positive voltage pulses. Negative pulses induced only instantaneous currents. D, Current levels measured at the onset of the pulses (instantaneous) and after 4.8 s (steady state) were plotted against their corresponding voltages. Instantaneous (◇) and time-dependent (●) currents obtained with symmetrical K_2mal. O, Currents at 4.8 s obtained with symmetrical KCl. Points are the mean ± SE of at least seven vacuoles.

Selectivity of the putative channels mediating the inward and outward currents with K_2mal_{cyt} and KCl_{cyt} were determined by employing tail current experiments. The time-dependent outward currents of vacuoles exposed to a K_2mal gradient (10 mM vacuole, 100 mM cytoplasm) reversed at

[3] Abbreviations: K_2mal, potassium malate; mal^{2-}, malate ion; Ca^{2+}_{cyt}, KCl_{cyt}, K_2mal_{cyt}, cytoplasmic concentrations of Ca^{2+}, KCl, and K_2mal_{cyt}, respectively; KCl_{vac}, K_2mal_{vac}, vacuolar concentrations of KCl and mal^{2-}, respectively; $E_{mal^{2-}}$, E_{K^+}, equilibrium potentials for mal^{2-} and K^+, respectively; E_{rev}, reversal potential; I_{SC}, single-channel currents.

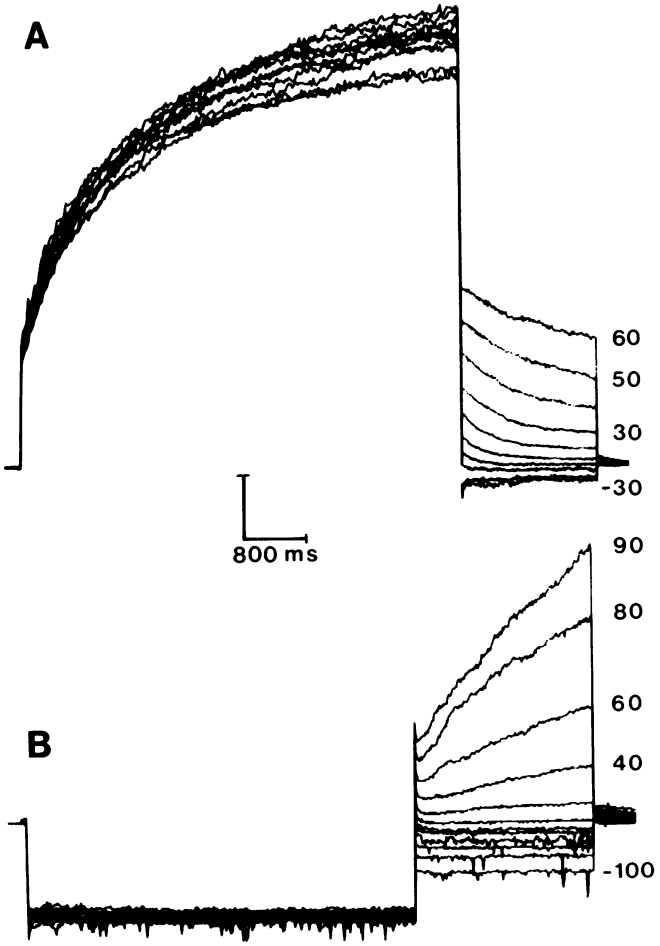

Figure 2. Reversal potential of the vacuolar currents. A, Voltage pulses of +120 mV activated outward currents for 7.5 s. Steady-state currents were deactivated by stepping down the voltage to +80 mV. This protocol was repeated 15 times with a subsequent decrease in the deactivating pulse of 10 mV. Tail currents reversed direction (E_{rev}) between −15 and −25 mV. B, Inward currents were activated by voltage pulses of −140 mV for 7.5 s. Stepping up the voltage pulse in increments of 10 mV resulted in the deactivation of the inward currents. The development of tail currents was not clearly observed, thus preventing the determination of E_{rev}. Vacuoles were exposed to 10 mM K_2mal_{vac} and 100 mM K_2mal_{cyt}. Similar results were obtained from at least four vacuoles. Vertical calibration bar is 50 pA for A and 20 pA for B.

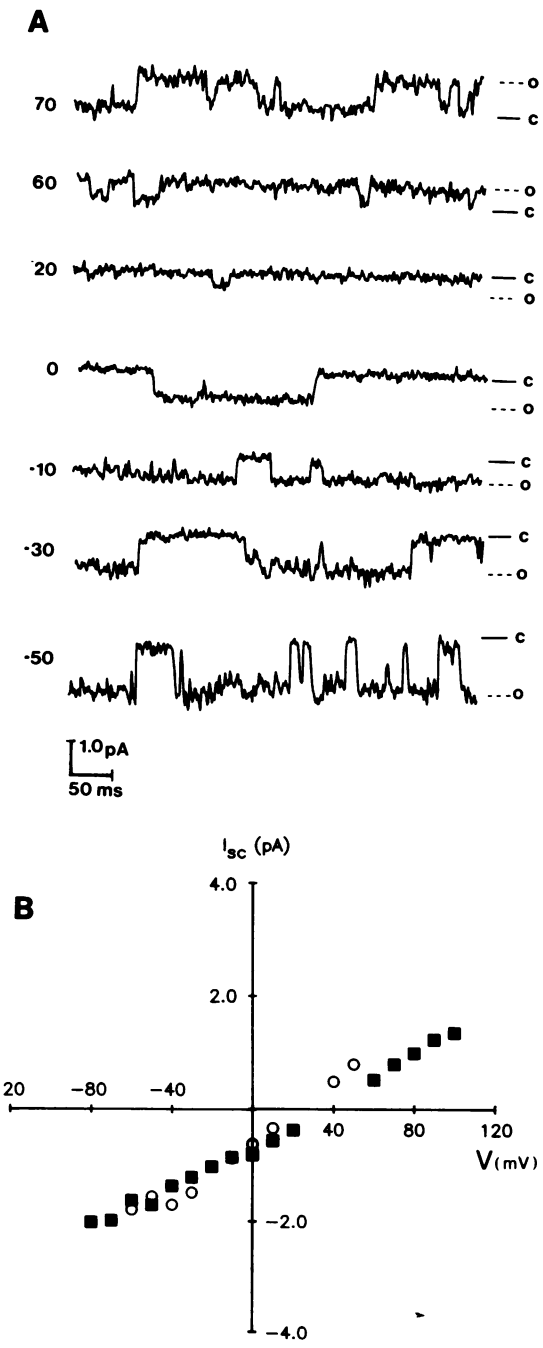

Figure 3. Single channel recordings. A, Original record of I_{SC} from an isolated outside-out patch exposed to 10 mM K_2mal_{vac} and 100 mM K_2mal_{cyt}. Currents were recorded between ±100 mV and the direction of the openings reversed between +20 and +60 mV. B, Single-channel current-voltage plots were constructed from records similar to those shown in A. Under a K_2mal (■) or a KCl (○) concentration gradient, as in A, the I_{SC} reversed direction between +20 and +30 mV. Data points are the mean ± SE of at least three patches.

potentials (E_{rev}) between −15 and −25 mV (Fig. 2A). Using these values of E_{rev}, a permeability ratio, $P_{mal^{2-}}/P_{K^+}$, of 6 to 10 was calculated using a modification of the Goldman-Hodgkin-Katz equation. Permeability ratios for the inward currents were difficult to obtain because tail current experiments did not give a clear E_{rev}, as shown in Figure 2B. This figure also shows that deactivation of the inward currents to positive membrane potentials led to the activation of the time-dependent outward currents, suggesting that the gating of these two currents may be different. In the presence of a similar KCl gradient, tail current experiments also failed to give clear E_{rev} for both inward and outward currents (data not shown). However, E_{rev} between +20 and +30 mV were obtained from

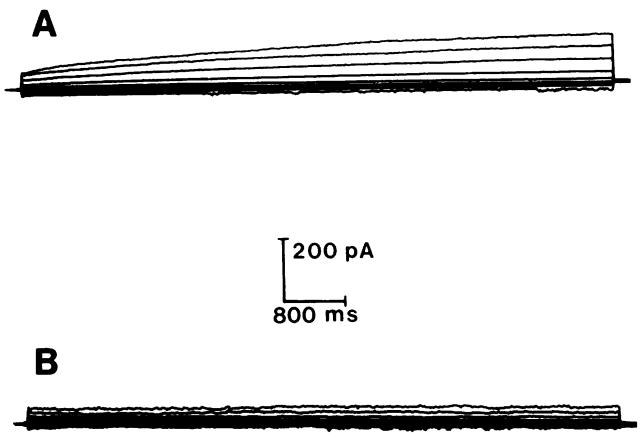

Figure 4. The effects of anion channel blockers on the vacuolar outward and inward currents. A, Time-dependent outward currents were activated by polarizing the tonoplast to positive voltages under symmetrical 100 mM K_2mal. Negative voltages stimulated only small instantaneous currents. B, The time-dependent outward currents observed in A were completely inhibited upon the addition of 2.5 mM Zn_2Cl to the cytoplasmic side of the vacuoles; however, the instantaneous inward and outward currents were unaffected. Similar effects of Zn^{2+} were observed in at least four vacuoles.

single channel recordings (Fig. 3) that were used to calculated a P_{K^+}/P_{Cl^-} ratio of 5 to 6. The time-dependent outward currents recorded in the presence of K_2mal_{cyt} (Fig. 4A) were completely inhibited by the addition to the cytoplasmic side of 2.5 mM Zn^{2+}, an anion channel blocker (12), without affecting the instantaneous currents (Fig. 4B).

To identify the unitary components of the vacuolar currents, single-channel recordings were performed in isolated outside-out patches. With 10 mM K_2mal_{vac} and 100 mM K_2mal_{cyt}, I_{SC} were recorded at membrane potentials between −80 and +80 mV. Under these conditions, the I_{SC} showed a linear relationship with voltage and reversed direction (E_{rev}) at +30 mV (Fig. 3, A and B). Substitution of K^+ for Na^+ in the cytoplasmic side of the patch did not alter the reversal potential of the single channel currents (data not shown). These results indicated that these channels were as permeable to K^+ as to Na^+ and 10 times more permeable to cations than to mal^{2-}. I_{SC} were also recorded from patches exposed to 10 mM KCl_{vac} and 100 mM KCl_{cyt}. These currents varied linearly with voltage and were five to six times more selective for K^+ than for Cl^- (as calculated from the E_{rev} of +20 to +30 mV).

DISCUSSION

The results obtained from experiments in which whole vacuoles were exposed to symmetrical 100 mM KCl at physiological levels of $Ca^{2+}{}_{cyt}$ indicate that the tonoplast of sugar beet cells possess channels that are activated instantaneously at all levels of membrane potential (Fig. 1B). These currents are comparable to the fast-activated vacuolar channels reported by Hedrich and Neher (11) at 10^{-7} M $Ca^{2+}{}_{cyt}$. The activation of instantaneous and time-dependent outward currents by positive potentials when KCl in the cytoplasm was substituted by K_2mal may indicate the presence of two different channels (Fig. 1C). The similarity in the magnitudes of the currents recorded with KCl and the instantaneous currents obtained in the presence of K_2mal suggest that these two currents are through the same channel, whereas the outward rectification shown by the time-dependent currents is probably through a different type of channel (Fig. 1C).

Time-dependent outward currents were observed only when mal^{2-} and $succinate^{2-}$ were the counterion for K^+ in the cytoplasmic side (Fig. 1C), indicating that the time-dependent currents correspond to mal^{2-} or $succinate^{2-}$ moving into the vacuole. Outward currents correspond to the movement of either positive charges moving from the inside of the vacuole to the outside, or negative charges moving in the opposite direction.

Further evidence supporting the idea that the time-dependent outward currents correspond to the movement of mal^{2-} into the vacuole was obtained from tail current experiments (Fig. 2). Reversal of the tail currents (between −15 and −25 mV) was closer to the equilibrium potential for mal^{2-} ($E_{mal^{2-}}$ = −23 mV) than for K^+ (E_{K^+} = +46 mV), indicating that these channels are between 6 and 10 times more permeable to mal^{2-} than to K^+. Development of the time-dependent outward currents in the presence of a reduced concentration of K^+ in the vacuole further indicates that these currents correspond to the movement of mal^{2-} into the vacuole.

The complete inhibition of the time-dependent outward currents by the anion channel blocker Zn^{2+} (Fig. 4) lends support to the view that these currents are carried by mal^{2-}. Zinc is known to inhibit anion channels in animal cells (12), and inhibition of vacuolar currents by Zn^{2+} has also been reported by Hedrich and Kurdjian (10).

Recordings from isolated patches demonstrate that the unitary components of the time-dependent vacuolar currents are channels (Fig. 3A). These recordings partially corroborated the results obtained from the whole-vacuole experiments. Reversal of the I_{SC} in Figure 3B occurred at +30 mV and thus corresponded neither to the $E_{mal^{2-}}$ (−23 mV) nor to E_{K^+} (+46 mV). The permeability ratio $P_{K^+}/P_{mal^{2-}}$ calculated from E_{rev} indicates that these channels are 10 times more selective for K^+ than for mal^{2-}. These results differ from those obtained with the whole vacuole, where a higher selectivity for mal^{2-} was calculated. However, it is possible that during the single-channel recordings only the channels conducting the instantaneous currents were active. This is supported by the results obtained from tonoplast patches exposed to KCl, where channels six times more selective for the cation (K^+) than for the anion (Cl^-) were recorded (Fig. 3B). Under these conditions, only instantaneous currents were observed in the whole vacuole (Fig. 1B). Similar results have been reported by Hedrich and Neher (11), who attributed I_{SC} with a P_{K^+}/P_{Cl^-} ratio of about 6 to the fast-activated vacuolar currents. The activity of only one channel from the single-channel recordings suggests that the channels conducting the time-dependent current may have been inactive. This inactivation could be the result of some mechanical stress imposed on the tonoplast patch during its isolation.

From our results, we can propose a role for the time-dependent outward channels recorded in the presence of

K_2mal_{cyt}. Accumulation of mal^{2-} would occur only when the vacuoles were polarized to potentials beyond $E_{mal^{2-}}$, which, according to data of Gerhardt and Heldt (7), would be at potentials more positive than +30 mV. Our results clearly show that at physiological Ca^{2+}_{cyt} (10^{-7} M), the time-dependent outward channels are active beyond $E_{mal^{2-}}$, thus providing a pathway for the movement of mal^{2-} into the vacuole. A similar role has been assigned to the inward-rectifying cation channels (10), although the activity of these channels has only been observed at unphysiological levels of Ca^{2+}_{cyt} (10^{-3} M) and at more negative potentials than $E_{mal^{2-}}$ (9). In addition to the more physiological conditions in which the time-dependent outward channels are active, their higher selectivity for mal^{2-} than for K^+ suggests that these channels are a more feasible pathway for the movement of mal^{2-} into the vacuole than the inward-rectifying channels. Malate inside the vacuole could act as a counterion for K^+ and, concomitantly, also be involved in mechanisms of cellular osmoregulation.

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