

Sensitivity of the Plant Vacuolar Malate Channel to pH, Ca²⁺ and Anion-Channel Blockers

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Received: 27 April 2001/Revised: 9 October 2001

Abstract. The organic anion malate is accumulated in the central vacuole of most plant cells. Malate has several important roles in plant vacuoles, such as the maintenance of charge balance and pH regulation, as an osmolyte involved in the generation of cell turgor, and as a storage form of CO₂. Transport of malate across the vacuolar membrane is important for the regulation of cytoplasmic pH and the control of cellular metabolism, particularly in plants showing crassulacean acid metabolism (CAM), in which large fluxes of malate occur during the day/night cycle. By applying the patch-clamp technique, in the whole-vacuole configuration, to isolated vacuoles from leaf mesophyll cells of the CAM plant *Kalanchoë daigremontiana*, we studied the regulation of the vacuolar malate channel by pH and Ca²⁺, as well as its sensitivity to anion-channel blockers. Malate currents were found to be insensitive to Ca²⁺ on the cytoplasmic side of the membrane over a range from approximately 10⁻⁸ M to 10⁻⁴ M. In contrast, decreasing cytoplasmic pH below 7.5 had a significant modulatory effect on channel activity, reducing malate currents by 40%, whereas increasing cytoplasmic pH above 7.5 resulted in no change in current. Several known Cl⁻-channel blockers inhibited the vacuolar malate currents: niflumic acid and indanoyloxyacetic acid (IAA-94) proved to be the most effective inhibitors, exerting half-maximal effects at concentrations of approximately 20 μM, suggesting that the plant vacuolar malate channel may share certain similarities with other classes of known anion channels.

Key words: Malate channel — Tonoplast — CAM-*Kalanchoë* — Niflumic acid

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Introduction

Malate is a common organic anion accumulated in the vacuole of plant cells, where it often serves as one of the major charge-balancing ions and is involved in the maintenance of cell turgor. In addition, malate plays an important role in plants showing crassulacean acid metabolism (CAM) and C₄ photosynthesis by serving as a temporary storage molecule for CO₂. In CAM plants, large amounts of malate are accumulated in the vacuole of mesophyll cells during the night as a consequence of synthesis of malate via the enzyme phosphoenolpyruvate carboxylase (PEPC). During the subsequent light period, malate is released into the cytoplasm, where it is decarboxylated by NADP- and/or NAD-malic enzyme into pyruvate and CO₂, the latter then being assimilated in the chloroplasts via the Calvin Cycle (Smith & Bryce, 1992).

Until recently, the molecular pathway for transport of malate into the vacuole was unknown. However, with the application of the patch-clamp technique several reports (Iwasaki et al., 1992; Pantoja, Gelli & Blumwald, 1992; Cerana, Giromini & Colombo, 1995; Cheffings et al., 1997) have described the presence of a voltage-dependent, malate-selective ion channel in the plant vacuolar membrane (tonoplast). This channel is active only at physiological (cytoplasmic-side negative) potentials and could account for the accumulation of malate into the vacuole, driven by the negative tonoplast potential established by the activity of the two tonoplast H⁺-pumps that transport H⁺ out of the cytoplasm into the vacuole, the H⁺-ATPase (Barkla & Pantoja, 1996) and the H⁺-PPase (Rea & Poole, 1993). However, relatively little is known about the regulation of this important anion channel in plant cell biology. Partial characterization of the malate-selective channels from vacuoles of the CAM plants *Graptopetalum paraguayense* (Iwasaki et al., 1992) and *Kalanchoë daigremontiana* (Cheffings et al.,

1997), from sugar beet cell-suspension cultures (Pantoja et al., 1992) and stomatal guard cells (Pei et al., 1996) has been reported, either at the single-channel level or with whole vacuoles. In all cases, the activity associated with the malate channel was only observed at low concentrations of cytoplasmic Ca^{2+} ($\text{Ca}_{\text{cyt}}^{2+}$). In the particular case of guard cells, significant malate currents were only observed in the presence of a calmodulin-domain-containing protein kinase (CDPK; Pei et al., 1996).

In the present study, the regulation of the vacuolar malate channel in the CAM plant *Kalanchoë daigremontiana* was investigated with respect to its dependence on cytoplasmic Ca^{2+} and pH, as well as aspects of its pharmacology. Variations in $\text{Ca}_{\text{cyt}}^{2+}$ between approximately 10^{-8} M and 10^{-4} M had no effect on the vacuolar malate currents. However, cytoplasmic pH (pH_{cyt}) values below 7.5 inhibited the malate currents by about 40% at pH_{cyt} 6.5. The vacuolar malate channel appears to share some features with Cl^- channels from animal cells, as indicated by a comparable affinity towards the known Cl^- -channel blockers indanoyloxyacetic acid (IAA-94) and niflumic acid (NA).

Materials and Methods

PLANT MATERIAL

Plants of *Kalanchoë daigremontiana* Hamet et Perrier de la Bathie were propagated vegetatively and grown in John Innes No. 3 compost under glasshouse conditions. Plants were watered weekly with half-strength Hoagland solution No. 1 (Hoagland & Arnon, 1950) supplemented with full-strength Long Ashton micronutrients (Hewitt & Smith, 1975). Supplementary lighting (Osram 400 W SON-T sodium vapour lamps) was used to maintain a 14-hr day-length. Two weeks before use, plants were transferred to a growth room with illumination supplied by fluorescent tubes and tungsten lamps (Philips TLD 50 W/83 HF and Philips RS 60 W, respectively) for 14 hr at a photon flux density of $200 \mu\text{mol m}^{-2}\text{sec}^{-1}$ at mid-plant height. Room temperature was maintained at 25°C during the light period and at 14°C during the dark period.

ISOLATION OF VACUOLES

Vacuoles were obtained by osmotic shock of protoplasts from leaf mesophyll cells. Mesophyll cell protoplasts were isolated by the enzymatic digestion of the leaf blades. The upper and lower epidermis were removed and the blade pieces cut in small segments (approx. 1 mm^3) prior to their incubation in digestion medium containing: 0.5% (w/v) Cellulase RS (Yakult Honsha Co., Tokyo, Japan), 0.2% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical Co., Tokyo, Japan), 0.05% (w/v) BSA, 0.5 mM CaCl_2 , 0.4 M mannitol and 25 mM MES/KOH buffer, pH 5.5. After 1 hr at 25°C , leaf segments were filtered through two layers of Miracloth (Calbiochem, La Jolla, CA) and the mesophyll cell protoplasts were left to sink by gravity for about 5 min on ice. The supernatant was removed and the protoplasts were washed three to four times with a washing solution containing 0.4 M mannitol, 5 mM MgCl_2 , 0.5 mM CaCl_2 , 1.0 mM DTT and 25 mM MES/KOH buffer, pH 5.5. Protoplasts were lysed by mixing one volume of protoplasts with one volume of lysis medium (in mM: 2 EGTA, 2 EDTA, 20 KCl, 5 Tris,

pH 8.0) at 4°C for 1 to 2 min. Released vacuoles were then stabilized by the addition of four volumes of washing solution; they were then washed once with the washing solution and kept on ice. Vacuoles were used within 2 hr of isolation.

ELECTRICAL MEASUREMENTS

Ionic currents across the vacuolar membrane (tonoplast) were measured by conventional patch-clamp techniques (Hamill et al., 1981). The seal between the patch pipette and the vacuole was obtained by bringing pipette and vacuole into contact with a hydraulic micromanipulator (WR-88, Narishige Scientific Instruments, Tokyo, Japan), while applying slight positive pressure to the pipette. Following contact, the positive pressure was removed, resulting occasionally in the immediate formation of a tight seal. However, sometimes it was necessary to apply slight suction to the pipette in order to form the seal between the vacuole and the pipette, thus obtaining the vacuole-attached configuration. The formation of the electrical seal was monitored on an oscilloscope (2225, Tektronix, OR) by recording the current in response to voltage pulses of 1 mV in amplitude and 10 msec in duration with a period of 70 msec. Pipette resistance was between 6 and 10 M Ω and seal resistances were of the order of 10 to 50 G Ω . Patch pipettes were fabricated from glass capillaries (P-1174, Sigma, Poole, U.K.) pulled in two stages with a pipette puller (L/M-3P-A, List-electronic, Darmstadt, Germany), fire-polished and coated with Sylgard (Dow Corning, Seneffe, Belgium) in an L/M CPZ-101 pipette forge (List-electronic, Darmstadt, Germany). The whole-vacuole configuration (Hedrich, Flügge & Fernandez, 1986) was obtained from the vacuole-attached mode by breaking the small area of membrane within the tip of the patch pipette by applying either a 5-msec voltage pulse of 1 V, or by applying a pulse of suction to the pipette. The whole-vacuole configuration was used to quantify the ionic currents across the intact tonoplast. Single-channel currents were recorded from isolated outside-out patches, obtained from the whole-vacuole configuration by pulling the patch pipette away from the vacuole (Hamill et al., 1981).

Whole-vacuole and single-channel currents were recorded at $20 \pm 2^\circ\text{C}$ with a patch-clamp amplifier (EPC-7, List). Whole-vacuole recordings were obtained with the pCLAMP program (version 5.1, Axon Instruments, Foster City, CA) and data were directly stored in a 50 MHz 486 microcomputer working on-line after filtering at 200 to 500 Hz with an eight-pole Bessel filter (Frequency Devices, MA). Vacuolar currents were elicited by applying 10-mV pulses of 10 sec duration from a holding potential of 0 mV. The voltage pulses were applied up to ± 100 mV every 15 sec. Current-voltage (I - V) relations were derived from steady-state currents measured at the end of 10-sec voltage pulses. Leakage currents were small compared to the steady-state currents and were not subtracted when plotting the I - V relations. Single-channel recordings were obtained by continuously polarizing isolated outside-out patches of tonoplast to potentials between ± 80 mV. The signal from the single-channel currents was digitized at 44 kHz by a pulse code modulator (PCM-701ES, Sony, Japan) and stored on videotape (NV-L25B, Panasonic, Osaka, Japan). For subsequent analysis, single-channel currents were digitized and processed with the PAT V 6.1 program (J. Dempster, University of Strathclyde, Glasgow, U.K.). Single-channel I - V relationships were obtained by plotting the single-channel current against the potential in the patch-pipette electrode.

SOLUTIONS

The composition of the pipette filling solution (vacuolar interior) was in mM: 2 MgCl_2 , 2 CaCl_2 , 5 KOH/MES, pH 5.5, 100 K_2 -

malate, adjusted to an osmolality of 440 mosmol kg⁻¹ with D-mannitol. The bath solution (cytoplasmic side) was composed of (in mM) 1 CaCl₂, 2 MgCl₂, 3 EGTA, 5 BTP/HCl, pH 7.5, 100 K₂-malate, adjusted to 440 mosmol kg⁻¹ with D-mannitol. The thermodynamic activity of free Ca²⁺ in the bath and pipette solutions was computed as 6.6 nM and 185 μM, respectively, with the program GEOCHEM (Parker, Zelazny & Kinraide, 1987).

Indanoyloxyacetic acid (IAA-94) and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) were a kind gift of Dr. A.J. Pope (SmithKline Beecham, Herts, U.K.); 2-[3-(trifluoromethyl)anilino]nicotinic acid (niflumic acid) and 4,4'-diisothiocyanatostillbene-2,2'-disulfonic acid (DIDS) were from Sigma (Poole, Dorset, U.K.).

Results

Vacuoles from leaf mesophyll cells of *K. daigremontiana* exposed to solutions of K₂-malate under patch-clamp conditions showed large inward currents at physiological (cytoplasmic-side negative) tonoplast potentials (Fig. 1A). (Following the sign convention established by Bertl et al. (1992), the reference phase is taken to be the extracytoplasmic side of the membrane, i.e., the vacuolar lumen, and the quoted potentials refer to the cytoplasmic side). These currents characteristically showed a fast, essentially instantaneous, activation at the onset of the voltage pulse, followed by a time-dependent component that required more than 8 sec to reach a steady level, and a fast deactivation at the end of the voltage pulse as indicated by the absence of tail currents (Fig. 1A). That the large inward currents corresponded to the movement of malate was further supported by the results shown in Fig. 1B. When the K₂-malate solution bathing the cytoplasmic side was replaced with KCl, negative tonoplast potentials induced only small instantaneous currents, without affecting the currents induced by positive potentials (Fig. 1B). The corresponding current-voltage (*I-V*) relationships demonstrated that the malate currents rectified strongly in the inward direction, corresponding to movement of malate²⁻ anions from the cytoplasmic side of the membrane into the vacuolar lumen (Fig. 1C). The similarity of the currents observed at positive potentials in malate and chloride solutions suggested that this might represent a 'leakage' current or the activity of relatively nonselective channels.

During development, leaves of *K. daigremontiana* switch modes of carbon assimilation from a C₃ metabolism initially in young leaves to CAM in mature leaves (Amagasa, 1982). Associated with this developmental switch to CAM is an increase in the amount of nocturnal malate accumulation in the vacuole of leaf mesophyll cells. As a consequence of this accumulation of malate, water is also stored in the vacuole, which results in the observed succulence of mature leaves. To determine whether there is a developmentally related upregulation of the tonoplast malate channel that could occur as a prerequisite for

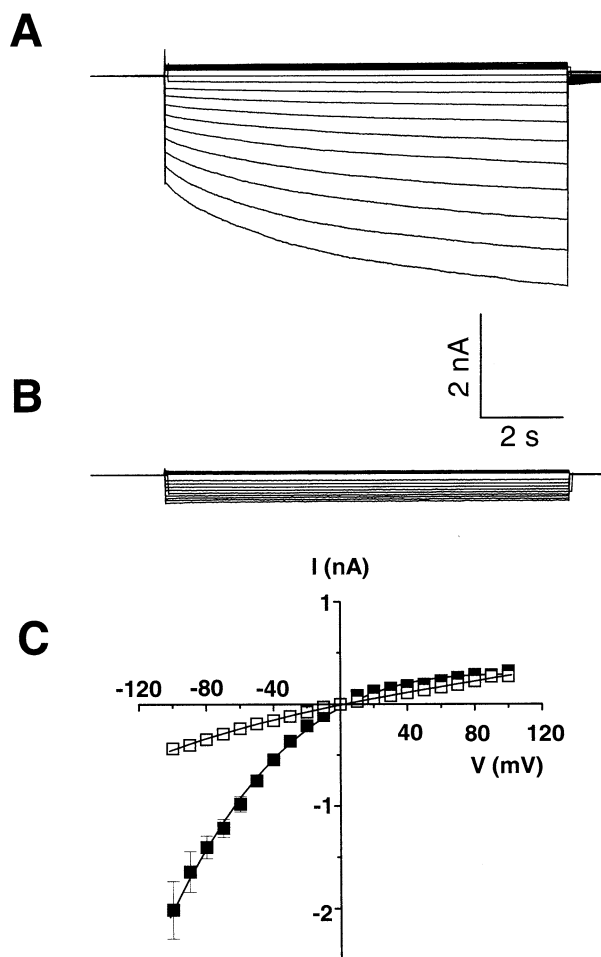


Fig. 1. Malate currents from isolated vacuoles of leaf mesophyll cells of *Kalanchoe daigremontiana*. Vacuoles were exposed to solutions (mM) of 100 K₂-malate, pH 7.5, 2 MgCl₂, 1 CaCl₂ and 3 EGTA (free Ca²⁺ = 6.6 nM) on the cytoplasmic (bath) side of the membrane, and 100 mM K₂-malate, pH 5.5, 2 MgCl₂, 2 CaCl₂ inside the vacuole (pipette: free Ca²⁺ = 185 μM). (A) Negative tonoplast voltage pulses elicited inward currents with two components, an instantaneous component (at the onset of the voltage pulse) and a time-dependent component (end of voltage pulse), corresponding to the movement of the malate²⁻ anion from the cytoplasmic side of the membrane (bath) into the vacuolar lumen (pipette). The malate current reached levels of up to -3 nA at -100 mV. Positive tonoplast voltage pulses elicited only smaller instantaneous outward currents and were independent of the cation used as counterion for malate (*data not shown*). The voltage pulse protocol was as follows: from a holding potential of 0 mV, voltage pulses were applied in steps of 10 mV between ±100 mV for 10 sec. (B) With a cytoplasmic solution of (mM) 100 KCl, pH 7.5, 2 MgCl₂, 1 CaCl₂ and 3 EGTA (free Ca²⁺ = 10⁻⁷ M), with the pipette solution as above, negative and positive voltage pulses only activated small instantaneous currents. (C) Current-voltage (*I-V*) relationship of the vacuolar currents recorded with either 100 mM K₂-malate (■) or 100 mM KCl (□) bath solutions, measured at the end of the 10-sec pulse. Results are the mean ± SEM from more than 20 vacuoles. If the SEM bar is not shown, SEM were smaller than the symbols.

transport of this anion into the vacuole, we made a comparative study of vacuolar currents employing

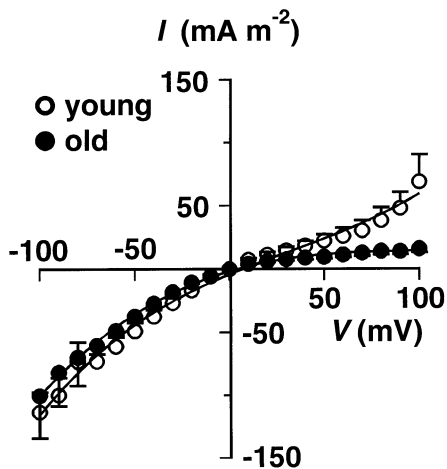


Fig. 2. I - V plot of the vacuolar malate currents from young (\circ) and mature (\bullet) leaves, expressed per m^2 of tonoplast. Currents were measured from vacuoles exposed to the same solutions as in Fig. 1A. Results are the mean \pm SEM of 20 vacuoles from mature leaves and of 8 vacuoles from young leaves.

vacuoles from mesophyll cells from young and mature leaves of *K. daigremontiana*. Under identical experimental conditions, the malate currents driven by negative tonoplast potentials were of smaller magnitude in vacuoles from young leaves than from mature leaves (*data not shown*). However, expressing these currents on an area basis, to compensate for the smaller size of the vacuoles from young leaves, revealed no difference in the magnitude of the currents from the two types of vacuoles (Fig. 2). However, the currents driven by positive tonoplast potentials were significantly larger in vacuoles from young leaves than those from mature leaves, particularly at potentials more positive than 50 mV (Fig. 2). It is now well established that the activity of ion channels in both plant and animal cells is regulated by cytoplasmic factors such as Ca^{2+} and pH (Hille, 1992). These two factors are also important in the physiology of the plant cell during the transduction of signals, in which they may act as second messengers (Blatt, 2000). In order to gain more information on the role of the tonoplast malate channel in the physiology of CAM plants, we studied the possible regulation of the malate channels by cytoplasmic Ca^{2+} and cytoplasmic pH.

CYTOPLASMIC pH

The magnitude of the tonoplast malate currents was directly affected by cytoplasmic pH (pH_{cyt}). Acidification of the cytoplasmic side of the vacuoles by 0.5 pH units reduced the magnitude of the malate currents at all values of tonoplast potential, without changing the magnitude of the outward currents (Fig. 3). Further acidification of the cytoplasm induced an additional reduction in the malate currents, which at

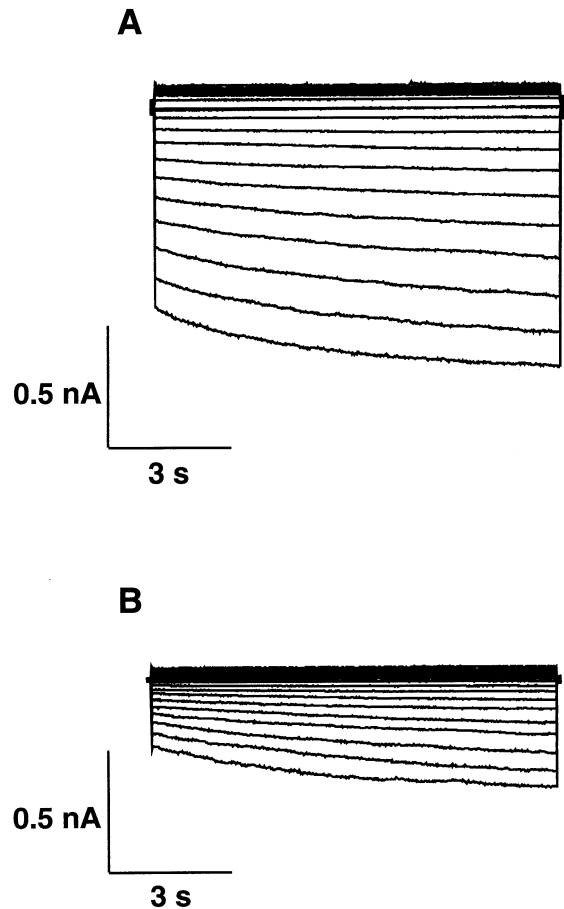


Fig. 3. Inhibition of the malate vacuolar currents by acidic pH_{cyt} . Original record showing inhibition of the malate vacuolar currents by acidification of the cytoplasmic side of the tonoplast from pH 7.5 (A) to pH 7.0 (B). A decrease of 0.5 pH units from pH 7.5 to pH 7.0 reduced the thermodynamic activity of malate $^{2-}$ of 29 mM by less than 0.5%.

pH_{cyt} 6.5 were reduced by almost 50% compared with the currents at pH_{cyt} 7.5. In contrast, increasing pH_{cyt} above 7.5 to 8.5 did not affect the vacuolar malate currents (Fig. 4A). From analysis of the Hill plot of the effects of pH_{cyt} between 6.0 to 8.5 on the malate currents (Fig. 4B), a Hill coefficient of 0.81 and an apparent K_d of 8.3 μM could be calculated. These results suggest that inhibition of the vacuolar currents by acidic pH_{cyt} could be explained by the binding of one H^+ per channel to a functional group with an apparent pK_a of 5.1. Contrary to the dependence of the malate vacuolar currents on pH_{cyt} , changes in the pH on the inside of the vacuole (vacuolar lumen) had no effect on these currents (*data not shown*).

CYTOPLASMIC Ca^{2+}

Regulation of channel activity in plants by cytoplasmic Ca^{2+} ($\text{Ca}_{\text{cyt}}^{2+}$) has been reported for channels present in the plasma membrane (Schroeder & Hagiwara, 1989; Hedrich, Busch & Raschke, 1990;

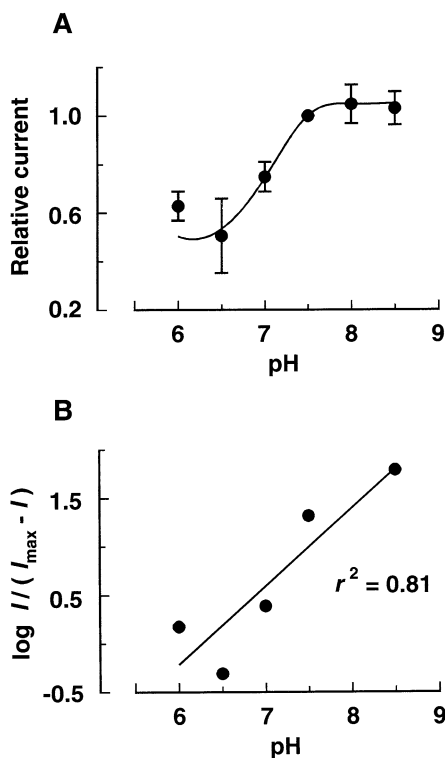


Fig. 4. Summary of the effects of pH_{cyt} on the vacuolar malate currents. (A) Variations in the pH on the cytoplasmic side of the tonoplast between 8.5 and 7.5 had a negligible effect on the malate currents. However, acidification of the cytoplasmic side by 0.5 pH units below physiological values reduced the magnitude of the vacuolar currents by 30%, and by almost 50% with a change of 1.0 to 1.5 pH units. Within this pH range, malate $^{2-}$ activity decreased from 29 mM at pH 7.5 to 27 mM at pH 6.0. (B) Hill plot of the effects of cytoplasmic pH on malate currents. Linear regression analysis gave a Hill coefficient of 0.81 and an apparent K_d of 8.3 μM ; the straight line is the best fit to these values. Results are the mean \pm SEM of at least 4 vacuoles.

Schroeder & Keller, 1992; Grabov & Blatt, 1997), as well as in the tonoplast (Hedrich & Neher, 1987). As demonstrated in Fig. 5, variations in free $\text{Ca}_{\text{cyt}}^{2+}$ between 6.6 nM and 84 μM did not change the response of the vacuolar malate currents between +100 and -100 mV. It must be noted that vacuoles from *Kalanchoë*, in contrast to other plant vacuoles studied to date, did not show the activity of the SV currents that are activated at high concentrations of free $\text{Ca}_{\text{cyt}}^{2+}$ (Hedrich & Neher, 1987; Barkla & Pantoja, 1996).

ANION-CHANNEL INHIBITORS

The use of chemical probes has been a rewarding approach for the study of the properties of ion channels and the biochemical characterization of channel proteins (Landry et al., 1987; Cabantchik & Greger, 1992). With the aim of finding a probe that might be useful in future work towards the isolation

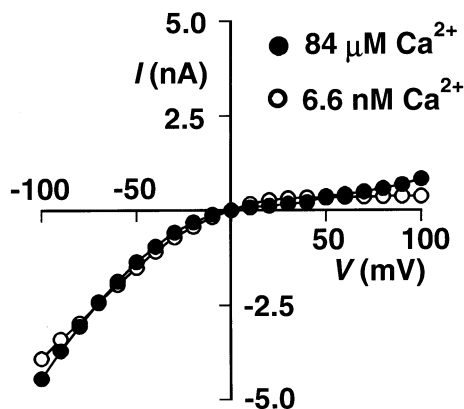


Fig. 5. Effects of free $\text{Ca}_{\text{cyt}}^{2+}$ on the vacuolar malate currents. The current-voltage (I - V) relations show that the magnitude of the vacuolar currents remained constant notwithstanding the variations in the cytoplasmic (bath) Ca^{2+} between 84 μM (●) and 6.6 nM (○). Results are the mean \pm SEM of at least 10 vacuoles.

and purification of the malate-channel protein, we studied the effects of several known anion-channel blockers on the tonoplast malate currents from *K. daigremontiana*.

Addition of indanoyloxyacetic acid (IAA-94) or niflumic acid (NA) to the cytoplasmic side of the vacuoles caused a reduction in the magnitude of the inward currents between 0 and -100 mV, with no effect on the outward currents (Fig. 6). The inhibition of the malate currents by these two anion-channel blockers was similar. At concentrations of 1 μM , both compounds reduced the malate vacuolar currents by approximately 15%. Increasing their concentration to 100 μM further reduced the currents, to 80% of the control levels in the presence of IAA-94, and to 70% of the control with NA (Figs. 7A and 7C, respectively). Analysis of the inhibitory effects of the two blockers using the Hill equation gave a Hill coefficient of 0.69 and an apparent IC_{50} of 19 μM for IAA-94; and a Hill coefficient of 0.55 with an apparent IC_{50} of 23 μM for NA (Figs. 7B and 7D, respectively). Attempts were made to study the effects of three additional anion-channel blockers; furosemide, DIDS (4,4'-diisothiocyanatostilbene-2,2'-sulfonic acid) and NPPB (5-nitro-2-(3-phenylpropylamino)benzoic acid). However, a study of the effects of NPPB was prevented due to the insolubility of this compound in ethanol. Although it was possible to dissolve NPPB in DMSO, control experiments showed that this solvent inhibited the vacuolar malate currents, even at DMSO concentrations as low as 0.001% (v/v) (*data not shown*). Initial experiments with furosemide and DIDS demonstrated that at concentrations as high as 100 μM these inhibitors reduced the malate currents by only 30% (*data not shown*). Due to their low affinity, no further studies were carried out with these two compounds.

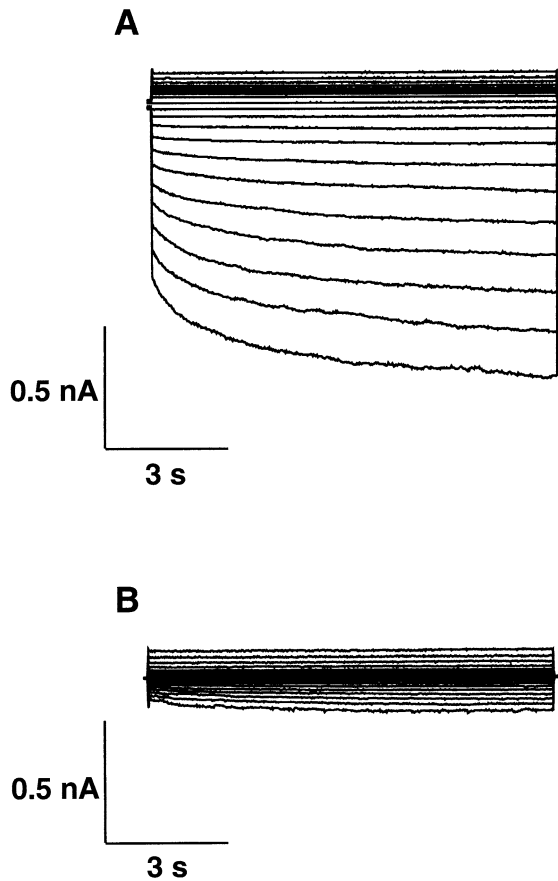


Fig. 6. Inhibition of the vacuolar malate currents by IAA-94. Original records showing the levels of the vacuolar currents before (*A*) and after (*B*) the addition of 100 μM IAA-94 to the cytoplasmic side of the tonoplast. Records show that the inward currents corresponding to the movement of malate²⁻ into the vacuole were inhibited almost completely in the presence of IAA-94.

SINGLE-CHANNEL RECORDINGS

Studies of the tonoplast malate currents from *K. daigremontiana* at the single-channel level have been hindered by what appears to be a “patchy” distribution of these channels in the tonoplast. This has been made apparent by the common occurrence of either the following cases: In one case, the absence of channels from the patch was suggested by a failure to record the opening and closing of single channels, even when the patch was polarized to potentials between +150 and -150 mV for periods of several minutes. In the other case, the presence of too many channels in a patch to allow resolution at the single-channel level was indicated by the recording of a noisy signal, even when the seal resistance was maintained at $>5 \text{ G}\Omega$. However, it was possible to record single-channel activity from patches obtained from vacuoles exposed to the anion-channel blockers. Figure 8*A* shows the activity of channels from an isolated patch of tonoplast exposed to 200 μM NA. The corresponding amplitude histograms demon-

strated that, in the presence of the inhibitor, the channels spent most of the time in the “blocked” state, as indicated by the lower amplitude of the Gaussian curve fitted to the 7.45 pA current level, compared to the amplitude of the Gaussian fit to the closed state (0 pA). The blocking effect of NA was reversible, as demonstrated by an increase in the open time of the single channels upon the washing out of the blocker, and is graphically shown in Fig. 8*B* by the larger magnitude of the Gaussian at the 7.45 pA level.

MALATE-CURRENT INACTIVATION

During this study, while attempting to determine the selectivity of the vacuolar malate currents by the analysis of tail-current recordings, it was observed that the magnitude of the inward instantaneous component was diminished whenever a negative voltage pulse was preceded by positive polarization of the tonoplast. This suggested the possible inactivation of the malate channels by voltage. In order to define this effect, we studied how currents elicited by a standard test pulse to -100 mV were affected by the voltage of the positive prepulse. Figure 9*A* shows a representative record of such an experiment. As the prepulse voltage was made more positive, the magnitude of the instantaneous component of the malate current decreased, without significantly affecting the final, steady-state level attained at the end of the 12-sec test pulse to -100 mV. Plotting the magnitude of the instantaneous current against the voltage of the prepulse shows the steady-state inactivation (*h*) of the instantaneous component of the malate currents (Fig. 9*B*), where the smooth curve was drawn according to the equation:

$$h_{\infty} = [1 + \exp(E - E_{h1/2})/k_h]^{-1}$$

where *E* is the prepulse potential, $E_{h1/2}$ the midpoint potential, and k_h is a measure of the steepness of the curve (Lizardi et al., 1992). In the experiment shown in Fig. 9*B*, the fitted parameters were $E_{h1/2} = 69.3 \text{ mV}$ and $k_h = 13.9 \text{ mV}$. At a potential of 50 mV, 20% of the channels were inactivated. From eight different vacuoles, the best-fit parameters were: $E_{h1/2} = 65.6 \pm 3.6 \text{ mV}$ and $k_h = 17.7 \pm 1.3 \text{ mV}$. We also studied the effects of duration of the prepulse on the instantaneous component of the malate currents, using a prepulse of 50 mV for durations of between 0.5 sec and 10 sec. However, the magnitude of these currents remained unchanged, indicating the absence of a time-dependent inactivation component (*data not shown*).

Discussion

The principal ionic currents in vacuoles from leaf mesophyll cells of the CAM plant *Kalanchoë daigre-*

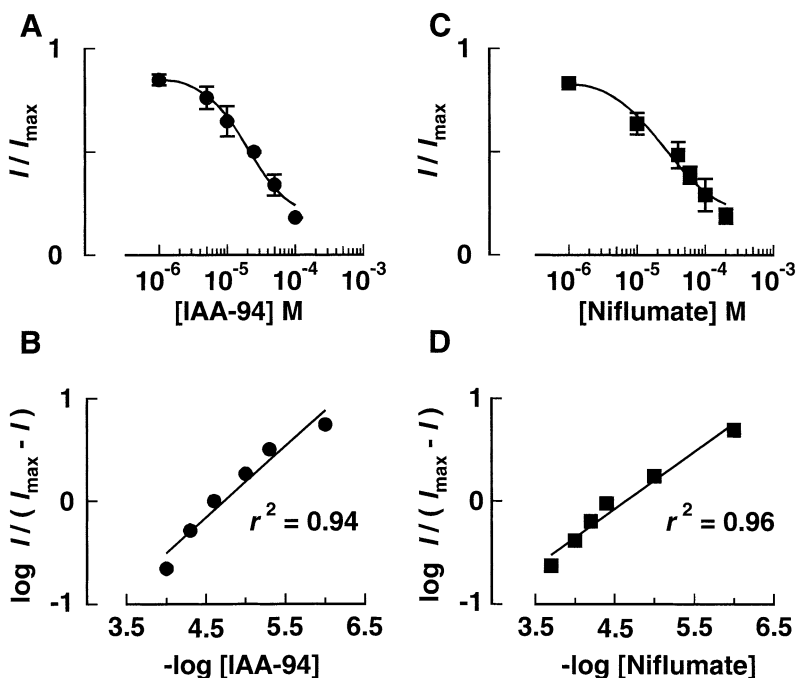


Fig. 7. Inhibition of the vacuolar malate currents by anion-channel blockers. The magnitude of the malate currents was reduced by increasing cytoplasmic concentrations of IAA-94 between 1 and 100 μM (A), and of NA between 1 and 200 μM (C). A Hill plot of these results gave a Hill coefficient of 0.69 and an apparent K_d of 19 μM for IAA-94 (B); the equivalent values for NA were 0.55 and 23 μM , respectively (D). Results are the mean \pm SEM of at least 8 vacuoles.

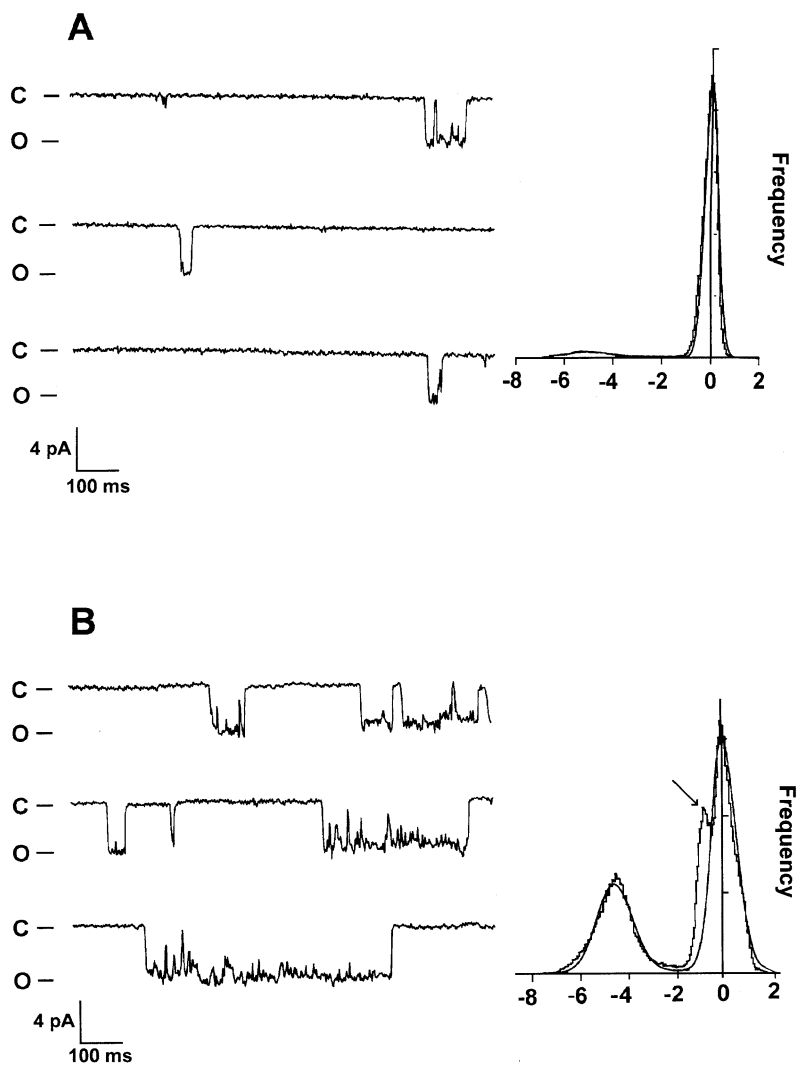


Fig. 8. Vacuolar malate currents at the single-channel level. (A) Original traces from an isolated outside-out patch of tonoplast clamped at -70 mV and exposed to the same conditions as in Fig. 1A, but in the presence of 200 μM NA on the cytoplasmic side. The amplitude histogram distribution shows that in the presence of NA the channel was closed for approximately 96% of the recording time. (B) The same patch following the removal of NA. An increase in opening frequency of the single channel was recorded on washing out the inhibitor. The amplitude histogram shows that in the absence of NA the opening of the channel increased to 33% of the recording time. The appearance of a substate is indicated by the presence of a second Gaussian close to that corresponding to the closed state (arrow).

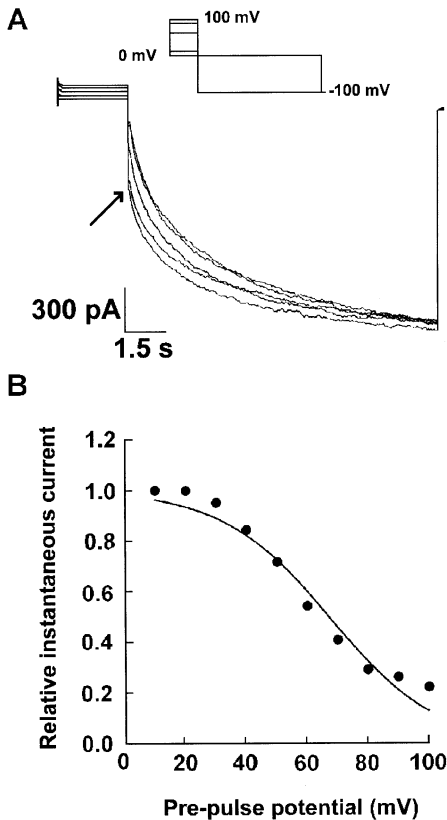


Fig. 9. Voltage-inactivation of the vacuolar malate currents. (A) An original record showing the effects of the positive prepulse on the magnitude of the instantaneous malate currents. Clamping the vacuole to more positive potentials in increments of 10 mV during the prepulse resulted in a progressive decrease in the instantaneous component of the malate current elicited by the subsequent test pulse to -100 mV (arrow). The magnitude of the prepulse potential had only a small effect on the final, steady-state current activated by the -100 mV test pulse. **Inset.** Pulse protocol: from a holding potential (V_h) of 0 mV, a prepulse to 10 mV with a duration of 0.5 sec was applied before the 12-sec test pulse to -100 mV. This procedure was repeated every 25 sec, increasing the value of the prepulse each time by 10 mV up to 100 mV. For clarity, only the traces corresponding to prepulses of 10, 20, 60, 90 and 100 mV are shown. (B) Relationship between the malate instantaneous currents elicited by the test pulse to -100 mV and the tonoplast potential during the preceding prepulse, normalized to a relative instantaneous current of 1.0 for a prepulse potential of 10 mV (data from A). Similar results were recorded with 9 vacuoles. The curve was drawn according to the equation: $h_\infty = [1 + \exp((E - E_{h1/2})/k_h)]^{-1}$.

montiana exposed to K_2 -malate solutions are time-dependent inward currents (Fig. 1A), which correspond to the movement of malate anions out of the cytoplasm into the vacuole when the tonoplast is polarized to physiological (cytoplasmic-side negative) potentials. Such currents have now been observed in several diverse species and different cell types, suggesting that malate currents may be a common feature of the tonoplast of plant cells (Iwasaki et al., 1992; Pantoja et al., 1992; Cerana et al., 1995; Pei et al., 1996; Cheffings et al., 1997). In CAM plants,

the vacuolar malate (VMAL) channels seem to dominate the electrical characteristics of the tonoplast (Fig. 1), at least in mature cells performing CAM photosynthesis (Fig. 2); relatively little current passes at the positive potentials at which SV and FV channels predominate in other species (Hedrich and Neher, 1987; Allen and Sanders, 1997). Thus, it appears that the VMAL channels provide the main pathway for vacuolar accumulation of malate that occurs as a consequence of nocturnal fixation of CO_2 in CAM plants (Smith et al., 1996; Cheffings et al. 1997). Because of the major role these channels may play in the control of carbon fluxes during CAM, we decided to study the regulation of the VMAL channel by cytoplasmic factors.

Several lines of evidence have suggested that CAM activity increases during leaf development in *K. daigremontiana*, such as an increase in the day-night changes in cell-sap malate concentrations as the leaves expand (Amagasa, 1982). An increase in the activity of the key enzyme phosphoenolpyruvate carboxylase (PEPC) has also been observed during leaf development in *K. daigremontiana* in parallel with an increase in the fluctuations of cell-sap malate during the day/night cycle (Ingram, 1993). White & Smith (1989) reported an increased activity of the vacuolar H^+ -ATPase with leaf age, which they related to the development of CAM. These authors also attributed the increased ATP-dependent H^+ transport rate observed in membrane vesicles in the presence of malate to an increased permeability of the tonoplast towards malate during leaf development. However, the results obtained in the present study using the patch-clamp technique with vacuoles isolated from young and old leaves suggest that the density of malate channels (or at least their combined activity) in the tonoplast of *K. daigremontiana* remains effectively constant per unit area of membrane with increasing leaf age (Fig. 2). Since the size of the large central vacuole of mesophyll cells of *K. daigremontiana* increases considerably during leaf development (Ingram, 1993), the tonoplast membrane will presumably come to represent a higher fraction of the total cell membrane in fully expanded cells. The observations of White & Smith (1989) regarding the apparent increase in malate permeability of the tonoplast vesicles might then be explained by a higher purity of the tonoplast fraction isolated from mature leaves of *K. daigremontiana* (CAM state), which would also account for the higher activity of the tonoplast H^+ -ATPase in the CAM state. Whether this explanation also applies to the increased transport activities associated with CAM induction in other species, such as the halophytic plant *Mesembryanthemum crystallinum* (Lüttge et al., 2000), remains to be established. It would also be of interest to establish whether the currents seen at positive potentials, which were a distinctive feature of vacuoles from young leaves (Fig. 2),

are specifically linked to C_3 metabolism, since they are significantly reduced in magnitude when the developing photosynthetic cells switch to CAM.

The regulation of ion channels by pH has been well characterized in animal cells (Hille, 1992), and more recently in plant cells (Blatt, 1992; Blatt & Armstrong, 1993; Grabov & Blatt, 1997). Furthermore, due to the pH dependence of the dissociation state of malate, it was important to study the effects of pH_{cyt} on the vacuolar malate channel. A decrease in pH_{cyt} reduced the magnitude of the malate currents (Fig. 3), an effect most likely due to a direct interaction between the channel and H^+ because, within this range of pH, malate exists primarily as the divalent anion malate²⁻. This inhibition of the malate channel by H^+ may be of physiological relevance as it occurs within the range of expected cytoplasmic pH values (7.0 to 7.5). One case in which acidification of the cytoplasm may be important is during the light period of CAM, when a net efflux of vacuolar malic acid occurs (Smith et al., 1996). Lowered cytoplasmic pH would result in inhibition of the malate channel, which would help to prevent a futile cycle of malate transport back into the vacuole. This would ensure sustained efflux of malate from the vacuole during the light period, as required for fixation of CO_2 in the Calvin Cycle (Smith & Bryce, 1992).

Analyzing the effects of acidic pH_{cyt} on the malate vacuolar currents can be useful for understanding the mechanism by which H^+ inhibits the malate currents. Figure 3 shows that the main effect of low pH_{cyt} was to reduce the magnitude of the malate currents, with no effect on either the response time (activation and deactivation) or voltage dependence. This would suggest that the inhibitory effect of H^+ is due to a direct block of the malate channel without any effect on its gating properties. According to the Hill plot obtained from the effects of pH_{cyt} on the malate currents (Fig. 4B), the blocking of the channel was on a one-to-one basis, as suggested by the Hill coefficient of 0.81, with a calculated dissociation constant pK_a of 5.1. It is possible that inhibition of the malate currents by H^+ , is due to the presence of acidic amino-acid residues surrounding or inside the channel pore, which, following protonation, create a conformational change (Heinemann et al., 1992) or cause a reduction in size of the pore (Yool & Schwarz, 1991; Heinemann et al., 1992), thereby obstructing the free movement of malate. The results from this study are similar to those obtained in animal cells for the blocking of Na^+ and K^+ channels (Cook, Ikeuchi & Fujimoto, 1984; Cornejo, Guggino & Guggino, 1989; Deutsch & Lee, 1989; Hille, 1992) and Ca^{2+} channels (Prod'hom, Pietrobon & Hess, 1987) by H^+ , where the protonation of an acidic amino-acid group has been proposed as the mechanism of inhibition. In plant cells, inhibition of channel activity by H^+ has been reported for the outward-rectifying K^+ channel

in the plasma membrane of stomatal guard cells (Blatt & Armstrong, 1993), a channel involved in abscisic acid-induced stomatal closing. Regulation of vacuolar malate channels and plasma membrane K^+ channels by H^+ strongly support the view of pH_{cyt} acting as a second messenger in plants (Felle, 1989).

Malate transport across the tonoplast has been studied previously using both isolated vacuoles (Marigo, Bouyssou & Laborie, 1988; Rentsch & Martinoia, 1991) and purified tonoplast vesicles (Oleski, Mahdavi & Bennett, 1987; Bettey & Smith, 1993; Lüttge et al., 2000). However, these studies have not been conclusive regarding the mechanism involved in the uptake of malate or in identifying the species of organic anion transported. Based on the stimulation of malate uptake into vacuoles of *Catharanthus roseus* by acidic pH_{cyt} , Marigo et al. (1988) concluded that Hmalate¹⁻ had the highest affinity for the carrier. Stimulation of malate (and citrate) uptake by acidic pH_{cyt} was also reported by Rentsch & Martinoia (1991) for barley mesophyll vacuoles; however, these authors concluded that the species recognized by the carrier was malate²⁻. A similar conclusion was reached by Oleski et al. (1987), who reported a stimulation of citrate uptake for tomato-fruit tonoplast by acidic pH_{cyt} , and concluded that citrate³⁻ was the form recognized by the carrier/channel. Oleski et al. (1987) also concluded that the citrate carrier/channel was involved in malate transport based on the strong inhibition of citrate uptake observed in the presence of malate. Further, the stimulation of malate/citrate vacuolar uptake reported for *C. roseus*, barley and tomato by acidic pH_{cyt} has been linked to a histidine residue as the active site (Dietz, Canut & Marigo, 1992). The inhibition of vacuolar malate currents by acidic pH_{cyt} observed in the present work (Fig. 3) is in contrast to these reports and suggests that in addition to the malate-selective channel reported here, another transporter may also be involved in the transport of malate across the tonoplast (Smith et al., 1996; Lüttge et al., 2000).

The importance of Ca_{cyt}^{2+} as a factor regulating several cellular processes has been well established (Berridge, 1993; Blatt, 2000). Variations in Ca_{cyt}^{2+} in the range between 10^{-4} M and 10^{-8} M had no effect on the vacuolar malate currents from *K. daigremontiana* (Fig. 5), indicating an independence of the currents from Ca_{cyt}^{2+} . In contrast to this result, the vacuolar malate channels from *Graptopetalum paraguayense* (Iwasaki et al., 1992) and sugar beet (Pantoja et al., 1992) were only active at physiological concentrations of free Ca_{cyt}^{2+} below 10^{-6} M; however, the present results are similar to those reported by Cerana et al. (1995) for vacuoles of *Arabidopsis* cell suspensions. An interesting result obtained with the vacuoles of *K. daigremontiana* is the apparent absence of the SV channels at high levels of Ca_{cyt}^{2+} , which have been observed in the majority of plant vacuoles studied so

far (Hedrich & Schroeder, 1989; Barkla & Pantoja, 1996; Allen & Sanders, 1997). This indicates that the main conductance in the vacuoles of *K. daigremontiana* is associated with the malate channels, emphasizing the important role that these channels play in the vacuolar accumulation of malate.

One property that differentiates the vacuolar malate channel from other channels characterized to date is the selectivity of this channel towards an organic anion rather than an inorganic one, which suggests that its molecular properties may be distinctive from those of the best known Na^+ , K^+ , and Cl^- channels. In order to gain information on the biochemical and molecular characteristics of the malate channel, we have made use of several known anion-channel blockers to compare the inhibitor sensitivity of this channel to the Cl^- channels of animal cells (Kozłowski, 1999). Niflumic acid (2-[3-(trifluoromethyl)anilino]nicotinic acid) and IAA-94 (indanoyloxyacetic acid) inhibited the vacuolar malate currents with similar IC_{50} values of approximately 20 μM (Fig. 7). These values are comparable to those reported for the inhibition of the Cl^- channel from the apical membrane of the trachea (Landry et al., 1989) and skeletal muscle (Weber-Schürholtz et al., 1993), and the Ca^{2+} -activated Cl^- channel of *Xenopus* oocytes (White & Aylwin, 1990). Moreover, the reversibility of the effects of NA on the malate channel was similar to results reported for the Ca^{2+} -activated Cl^- channel of *Xenopus* oocytes (White & Aylwin, 1990). Niflumic acid and IAA-94 have also been used to study the properties of a Cl^- channel from the plasma membrane of stomatal guard cells in plants, where half-inhibition of the peak Cl^- current was obtained with 20 μM NA and 7 μM IAA-94 (Marten et al., 1992). Moreover, niflumic acid inhibits (though with slightly lesser potency) a malate-permeable channel found in the plasma membrane of root apical cells in wheat plants (Zhang, Ryan & Tyerman, 2001). These results suggest that the vacuolar malate channel and the plasma membrane Cl^- channels from animal cells and guard cells may share some similarity at the molecular level, particularly at the binding site for the anion-channel blockers, as indicated by the almost identical affinities that these channels show for the inhibitors.

The high affinities of NA and IAA-94 for the vacuolar malate channel (Fig. 7) may provide a tool for the biochemical identification of the channel protein, although ion channels are notoriously low-abundance proteins. In the past, several laboratories have reported the purification of Cl^- -channel proteins by affinity chromatography employing IAA-23 (an IAA-94 analog) from kidney and trachea cells (Landry et al., 1989), as well as from skeletal muscle (Weber-Schürholtz et al., 1993).

Polarization of the vacuole to negative potentials caused a decrease in the magnitude of the instantane-

ous component elicited by the subsequent test pulse to -100 mV (Fig. 9A), indicating that this component of the malate currents inactivates in a voltage-dependent manner (Fig. 9B). Inactivation is a process well characterized for Na^+ and K^+ channels in animal cells, and in these systems is also dependent on voltage (Armstrong, 1981; Hille, 1992). Extensive research on Na^+ and K^+ channels from animal cells has shown that inactivation may be due to a ball and chain mechanism, in which a cytoplasmic segment of the channel protein plugs the channel pore following activation (opening) (Armstrong, 1981; Stühmer et al., 1989; Hoshi, Zagotta & Aldrich, 1990). In comparison with this mechanism, voltage-dependent inactivation of the malate currents at more positive potentials is manifested as a decrease in the size of the instantaneous component elicited at the onset of the voltage pulses, without any effect on the final, steady-state current. This indicates that the relative contribution of the instantaneous and time-dependent components to the final, steady-state current can vary reciprocally, consistent with the notion that they are two kinetic components of the same channel (Cheffings et al., 1997). The mode of inactivation of the vacuolar malate channel is therefore likely to be different to that proposed for Na^+ and K^+ channels. One possible model is found in the Cl^- family of Cl^- channels, in which voltage-dependent gating can be mediated by the permeating anion (Jentsch, Friedrich & Schriever, 1999). Further studies will be needed to characterize in more detail the gating mechanism of the vacuolar malate channel in plants.

This work was supported by the Biotechnology and Biological Sciences Research Council (UK).

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