J. Membrane Biol. 183, 137–145 (2001)
DOI: 10.1007/s00232-001-0061-x

The Journal of
Membrane
Biology
© Springer-Verlag New York Inc. 2001

Anion Modulation of the Slowly Activating Vacuolar Channel

H. Miedema,[1] **O. Pantoja**

Departamento de Biología Molecular de Plantas, Instituto de Biotecnología, Universidad Nacional Autónoma de México, A.P. 510-3, Colonia Miraval, Cuernavaca, Morelos 62250, Mexico

Received: 4 December 2000/Revised: 25 May 2001

Abstract. This study addresses the permeability and modulation by anions of the slowly-activating vacuolar (SV) channel in the tonoplast of *Mesembryanthemum crystallinum.* Although we conclude that the *M. crystallinum* SV channel is impermeable to anions, the anion species at the cytosolic side of the membrane clearly affects the channel. We discuss two types of modulation, both consistent with a single binding site for anions at the cytosolic face of the channel. The first effect is related to the conducting properties of SV. When the site is occupied, the bound anion exerts an effect on the Ca^{2+} and Na^+ fluxes through the channel. Because the Stokes-diameter of both ion species is different, both fluxes are not influenced to the same extent. This differential effect on the permeation of both ion species is reflected in a permeability ratio Ca^{2+} over Na^+ (P_{Ca}/P_{Na}) that depends on the identity of the anion species. The second type of modulation deals with the activation of the channel by anions. Previous studies revealed that a low level of cytosolic Cl^- inactivates SV (Pantoja, Dainty & Blumwald, 1992a). We critically evaluated reported effects of anions on the whole-vacuole SV current. Given that cytosolic Ca^{2+} and Cl^- both modulate SV, we conclude that both levels of regulation show some kind of interaction. High cytosolic Ca^{2+} can overrule the inactivation caused by low cytosolic Cl^-.

Key words: Anion modulation — Malate — *Mesembryanthemum* — SV Channel

Correspondence to: O. Pantoja
omar@ibt.unam.xm

[1] *Present address:* Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA, UK

Introduction

The slowly-activating vacuolar or SV channel is the most abundant ion channel in the tonoplast and identified in all plant species studied so far. Analysis over the last decade of the regulation of SV shows a complexity that continues to increase. SV is modulated by voltage (Hedrich & Neher, 1987), cytosolic Ca^{2+} (Hedrich & Neher, 1987), luminal Ca^{2+} (Allen & Sanders, 1996 but see Cerana, Giromini & Colombo, 1999), calmodulin (Bethke & Jones, 1994), cytosolic pH (Schulz-Lessdorf & Hedrich, 1995), cytosolic Mg^{2+} (Pei, Ward & Schroeder, 1999), phosphorylation (Bethke & Jones, 1997), cytosolic Cl^- (Pantoja et al., 1992a), redox state (Carpaneto, Cantù & Gambale, 1999) and calcineurin (Allen & Sanders, 1995). Despite the vast amount of knowledge of the regulation of SV, its physiological role is still a matter of debate, for instance in the context of calcium-induced-calcium-release from the vacuole (Ward & Schroeder, 1994; Pottosin et al., 1997; Pei et al., 1999; Sanders, Brownlee & Harper, 1999). Another point of controversy is the question whether or not SV is permeable to anions. Initially, SV was identified as a rather nonselective channel, permitting the passage of cations as well as anions (Hedrich & Neher, 1987). Malate (Mal^{2-}) is one of the major osmotica in guard cells and it was hypothesized that SV plays a role in malate fluxes across the guard cell tonoplast (Hedrich, Flügge & Fernandez, 1986; Coyaud et al., 1987). In plants showing crassulacean acid metabolism (CAM), malic acid is a central carbohydrate intermediate (Taiz & Zeiger, 1998). Iwasaki et al. (1992) described an ion channel in the tonoplast of the CAM species *Graptopetalum paraguayense* that is selective for Mal^{2-}. Based on its voltage and Ca^{2+} sensitivity, the authors identified this channel as SV. The conclusion of Iwasaki et al. (1992) is the more interesting because since then the view on the ion selec-

tivity of SV has been changed drastically. According to the prevailing current opinion, SV is permeable to monovalent cations and Ca^{2+} but not to anions (Ward, Pei & Schroeder, 1995; Allen & Sanders, 1995, 1996). However, the anion permeability of SV 'remains contentious' (Allen & Sanders, 1997) and a study of Schulz-Lessdorf and Hedrich (1995) does indicate an anion permeability of SV. With the exception of just a few studies (Amodeo, Escobar & Zeiger, 1994; Ward & Schroeder, 1994), the discussion of the ion selectivity of SV is almost exclusively based on whole-vacuole measurements. This is rather peculiar considering the fact that the tonoplast contains not only various types of cation channels but also channels selective for anions (Pantoja, Gelli & Blumwald, 1992b; Plant, Gelli & Blumwald, 1994; Cerana, Giromini & Colombo, 1995). Although a proper choice of the ionic conditions allows the discrimination between the various types of channels (Allen & Sanders, 1996; Gambale et al., 1996), it seems more appropriate to study SV at the single-channel level. Our aim was to investigate the anion permeability of SV, in particular the putative permeability to Mal^{2-} as claimed by Iwasaki et al. (1992), in excised patches. We focused on the SV current across the tonoplast of *Mesembryanthemum crystallinum* L. (common ice plant), a plant species that also shows CAM.

In addition to the ion selectivity, we addressed the modulation of the *M. crystallinum* SV channel by anions. The existence of a binding site for anions has been implicated by previous findings of Pantoja et al. (1992a). When cytosolic Cl^- was substituted by another anion species, e.g., gluconate ($Gluc^-$), the authors observed a strong reduction of the whole-vacuole SV-current magnitude. A similar inhibition of SV by anions has since been reported in other plant species, using different anion species, e.g. Mal^{2-} (Cerana et al., 1995). The exact mechanism of this current inhibition is still unknown but there are two possible explanations. First, SV is regulated by cytosolic Cl^- and the removal of Cl^- inactivates the channel. Alternatively, $Gluc^-$ and Mal^{2-} directly block the SV channel. The present study sheds some new light on this phenomenon. In addition, we evaluated the effect of anion substitutions on the whole-vacuole SV current as reported in the literature and carefully analyzed the ionic conditions used. The data suggest a synergy between the modulation of SV by cytosolic Ca^{2+} and Cl^-.

Material and Methods

Plant Material

Mesembryanthemum crystallinum L. was grown as described by Barkla et al. (1999). A detailed protocol for the isolation of leaf mesophyll protoplasts can be found in Miedema, Balderas and Pantoja (2000). Briefly, the epidermis at the abaxial side was stripped off and the leaf segments were incubated for 45 min at 30°C in an enzyme solution containing 0.05% cellulase RS (Onozuka, Japan), 0.01% pectolyase Y-23 (Seishin, Japan) and 1% BSA (Sigma, Mexico). Vacuoles were obtained by osmotic shock, using a 100 mM EGTA solution, adjusted with Tris to pH 7, resulting in a final osmolarity of 200 mM. To 0.3 ml of protoplast solution, 0.9 ml of EGTA solution was added and protoplast lysis was followed under the microscope. The addition of 10 ml bath solution stopped lysis. The vacuoles used for the patch-clamp experiments typically had a diameter of 100 μm. All experiments were performed at room temperature (19–21°C).

Patch-clamp

Patch-clamp recording and data analysis were performed using an Axopatch 200B amplifier, a 1200 Digidata interface and pClamp 6.0.4. software (Axon Instruments, Foster City, CA). Whole-vacuole and single-channel data were sampled at 2 and 5 kHz, respectively, and the data were filtered at a −3 dB frequency of 1 kHz using the electronics on the Axopatch amplifier. Pipettes were pulled from glass capillaries (Sigma, Mexico, # P-1174), using a two-stage pipette puller (Narishige, Tokyo, # PP-83).

For the experiments under Na_2Malate conditions, the pipette solution contained (in mM): 10 or 160 Na_2Malate and 10 MES, pH 5.5 and the bath solution: 10, 100 or 160 Na_2Malate and 10 HEPES, pH 7.5. For the experiments using NaCl, Na_2Malate was replaced by NaCl and the pipette solution contained 20 NaCl and the bath 250 NaCl. Unless stated otherwise, all solutions contained $CaCl_2$ resulting in a free Ca^{2+} activity of 0.3 mM and a Cl^- activity of 5–7 mM. Malate was added as malic acid and the pH of all solutions was adjusted with NaOH. In order not to complicate the interpretation of reversal potential (E_{rev}) measurements, we excluded Mg^{2+} from the solutions because SV shows a permeability to Mg^{2+} (Allen & Sanders, 1996; Cerana et al., 1999). Sorbitol was added to all solutions to a final osmolality of 500 mM. Ion activities were calculated according to Robinson and Stokes (1965) and Geochem-PC 2.0 (Parker, Norvell & Chaney, 1995). In the case both calculations gave slightly different values (Na^+), we used the values given by Robinson and Stokes. In order to account for the interaction between Ca^{2+} and $Gluc^-$, the pK value of Ca-Gluconate$^+$ was added to the Geochem database. Geochem requires pK values at zero ionic strength (IS). A pK value of 1.87 at $IS = 0$ results in a pK of 1.21 at $IS = 0.1$ and this is the value given by Martell and Smith (1977). The reference electrode was connected to the bath solution via a 2.5 M KCl/2% agar bridge. We followed the current and voltage convention for endomembranes proposed by Bertl et al. (1992). This implies that in the whole-vacuole and outside-out patch configurations a flux of cations into the vacuole is assigned as an outward, positive current. Likewise, the membrane potential (V_m) equals the negative of the applied pipette potential (V_p). Throughout the text, pipette and reversal potentials have been corrected for liquid junction potentials (LJP), according to $V_m = -V_p = LJP - V$, where LJP is defined as the potential of the bath with respect to the potential of the pipette solution and V is the potential read from the amplifier's display. LJPs were measured according to the protocol outlined by Ward and Schroeder (1994). Measured values of LJP were −12, 12 and 8 mV in (pipette/bath) 250/20, 20/250 and 20/100 mM NaCl, respectively and 12, −12 and −10 mV in 160/10, 10/160 and 10/100 mM Na_2Malate, respectively. Because selectivity measurements are solely based on the determination of reversal potentials, possible voltage offsets introduced by LJPs deserve extra attention. We therefore compared measured LJPs with values predicted by theory. The software of Barry (1994) was used to calculate LJPs. Using a molar (equivalent) conductivity for Mal^{2-} of 58.8 $10^{-4}m^2Smol^{-1}$ (The Handbook of Chemistry and

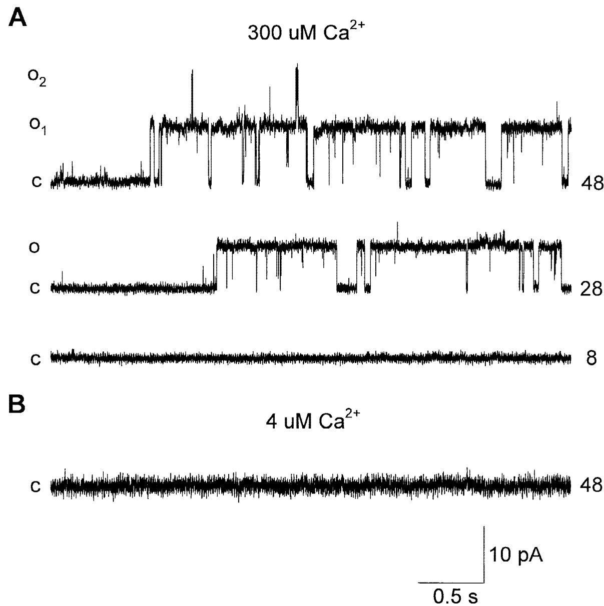

Fig. 1. (*A*) Recordings on an outside-out patch in 10/100 Na_2Malate, i.e., with 10 mM Na_2Malate in the pipette and 100 mM Na_2Malate in the bath solution. The activity of free Ca^{2+} in the bath solution, i.e., at the cytosolic side of the membrane, was 0.3 mM. The observed voltage dependence, activation at positive membrane potentials (i.e., vacuole negative) is in accordance with the strong rectification of SV. (*B*) Recording on the same outside-out patch as in *A* but with a cytosolic free Ca^{2+} concentration of 4 μM. With 4 μM cytosolic Ca^{2+}, SV inactivated at potentials that, at 0.3 mM cytosolic Ca^{2+}, activated it. Membrane potentials (in mV) in *A* and *B* are given next to the traces. Closed- and open-current levels are indicated by *c* and *o,* respectively.

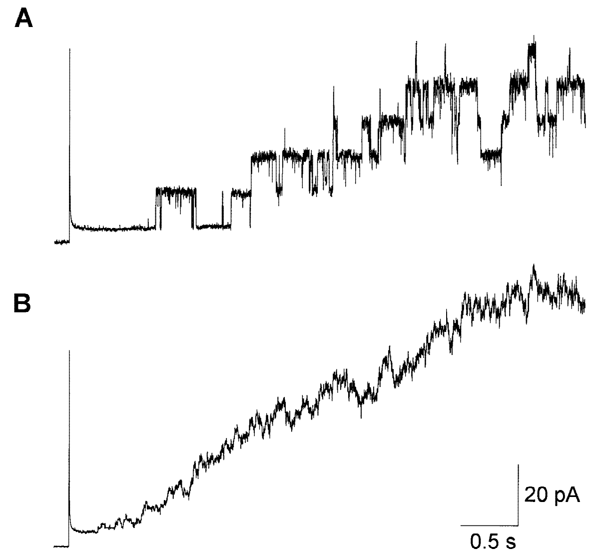

Fig. 2. Activation with time of SV in an outside-out patch with 10 mM Na_2Malate in the pipette and 100 mM Na_2Malate in the bath solution (10/100). Starting from a holding potential of –10 mV, SV was activated by pulsing to 90 mV. (*A*) Single current trace with at least five channels in the patch. (*B*) Average of nine traces as shown in *A*.

Physics, 1999–2000), we calculated a (normalized) mobility coefficient for Mal^{2-} of 0.4 (*see* Atkins, 1998). For the ionic conditions used, the measurements and the calculations of *LJP* were in excellent agreement.

The Ca^{2+} over Na^+ permeability ratio (P_{Ca}/P_{Na}) was calculated according to (*see* also Johannes & Sanders, 1995):

$$\frac{P_{Ca}}{P_{Na}} = -\frac{(Na_{cyt} - Na_{vac}\,exp(-V_mF/RT))(1 - exp(-2V_mF/RT))}{4(Ca_{cyt} - Ca_{vac}\,exp(-2V_mF/RT))(1 - exp(-V_mF/RT))} \quad (1)$$

where *R, T* and *F* have their usual meaning and $RT/F = 25.3$ mV (20°C).

Values of E_{rev}, P_{Ca}/P_{Na} and the single-channel conductance (*g*) are given ± standard error of the mean (SEM).

Results

Figure 1 shows current recordings of the *M. crystallinum* SV channel when studied in the excised outside-out patch configuration. This figure illustrates the sensitivity of SV to voltage and the cytosolic level of free Ca^{2+}. Both modes of regulation were already addressed in the seminal paper of Hedrich and Neher (1987) on SV in the tonoplast of *Vicia faba* guard cells. The threshold of SV for cytosolic Ca^{2+} depends on the ionic conditions, notably on the presence of Mg^{2+} (Pei et al., 1999). While Hedrich and Neher (1987) reported a threshold of 1 μM, SV in *M. crystallinum* already inactivated at 4 μM cytosolic Ca^{2+} (Fig. 1*B*). Thresholds for channel activation in the μM range have also been reported by Ward and Schroeder (1994) and Dobrovinskaya, Muñiz and Pottosin (1999). Another hallmark of SV are the slow activation kinetics and an example of an excised patch recording is shown in Fig. 2. Similar activation kinetics were observed at the whole-vacuole level (Fig. 3*A*) with half-times of activation of approximately 1–2 sec (at ≈90 mV). From double-pulse experiments as shown in Fig. 3*B* we calculated E_{rev}. Table 1 summarizes values of E_{rev} obtained from outside-out patch and whole-vacuole recordings. Because the measurements were performed under identical ionic conditions, Table 1 allows a comparison of E_{rev} in both configurations. Although the values of E_{rev} were very similar, E_{rev} in whole-vacuole was consistently 2–6 mV closer to E_{Na} than E_{rev} in excised patches. This observation reflects the fact that the vacuolar membrane contains various types of ion channels. The slow activation kinetics in Fig. 3 indicated that the whole-vacuole current was dominated by the SV conductance. Nevertheless, apparently other types of channels with a higher selectivity for Na^+ than SV contributed to the whole-vacuole current, thereby shifting E_{rev} in the direction of E_{Na}. We therefore decided to study the ion selectivity of SV in more detail at the single channel level.

The first question we addressed was whether the SV channel shows a permeability to Mal^{2-}. We tested this hypothesis by substituting Mal^{2-} in the bath solution by

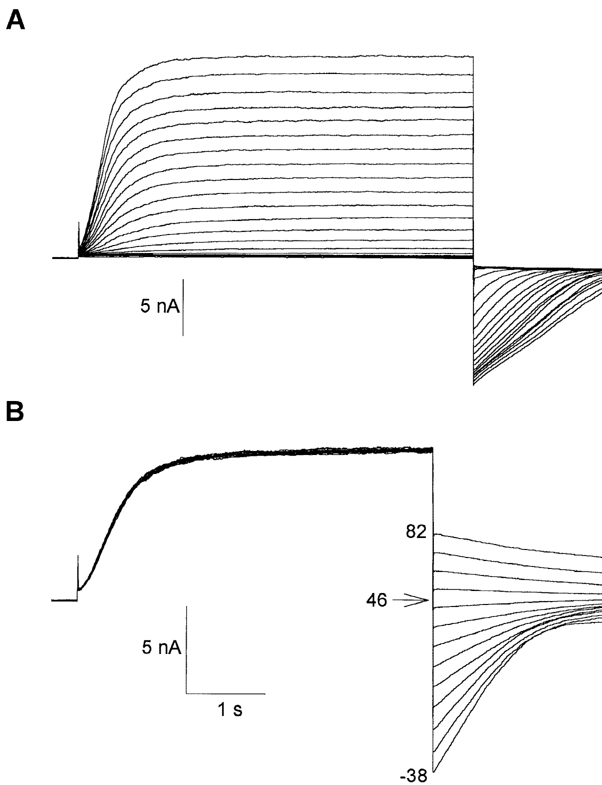

Fig. 3. Whole-vacuole recordings with 160 mM Na_2Malate in the pipette and 10 mM Na_2Malate in the bath solution (160/10). (*A*) Outward current was elicited by pulsing from a holding potential of 12 mV to V_m's ranging from 22 to 212 mV, in 10-mV steps, followed by a deactivating pulse to −48 mV. (*B*) Double-pulse experiment to determine the ion selectivity of the whole-vacuole current. The SV current was activated by pulsing from a holding potential of 12 mV to 132 mV, followed by a voltage pulse ranging from −38 to 82 mV, in 10 mV steps. E_{rev} was obtained by interpolation after plotting the amplitude of the tail currents, measured directly after applying the second voltage pulse, against V_m. As indicated, in this particular experiment, E_{rev} was 46 mV.

Table 1. Comparison of E_{rev} measured in the single-channel (SC) and whole-vacuole (WV) configuration

Pipette/bath mM[1]	E_{rev} in SC mV	E_{rev} in WV mV	E_{Na} mV
160/10	41 ± 0.8 (4)	47 ± 1.0(9)	57
10/100	−24 ± 0.8 (31)	−26 ± 1.5(2)	−49
10/160	−32 ± 2.1 (13)	−36 ± 0.5(3)	−60

Numbers in parentheses represent the number of independent experiments.
[1] All measurements were performed with Na_2Malate in the pipette and bath solution.

the large anion gluconate ($Gluc^-$) that is generally considered to be impermeable (but *see* Schulz-Lessdorf & Hedrich, 1995). If SV is indeed permeable to Mal^{2-}, it would be expected that the perfusion with the gluconate

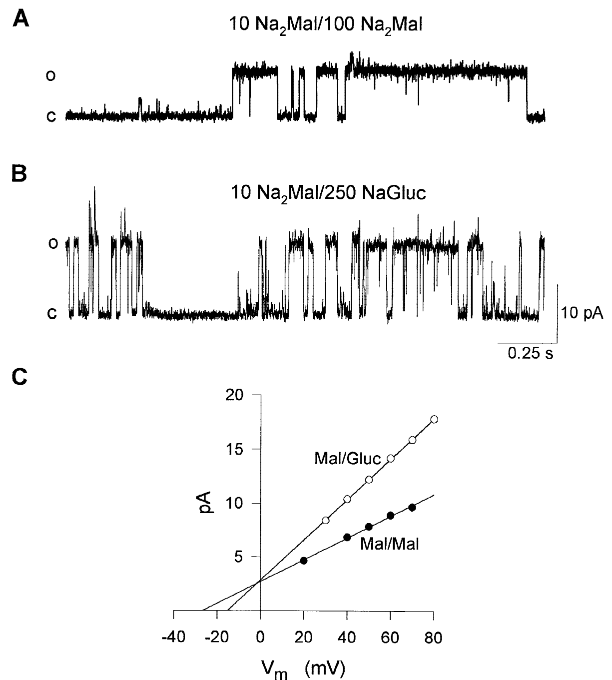

Fig. 4. The effect of the substitution of cytosolic Mal^{2-} by $Gluc^-$ on E_{rev} and *g*. The pipette solution contained 10 mM Na_2Malate. The outside-out patch was exposed first to 100 mM Na_2Malate (*A*) and then to 250 mM Na-Gluconate (*B*). V_m in *A* and *B* was 50 mV. Closed- and open-current levels are indicated by *c* and *o,* respectively. (*C*) *IV*-plots obtained from the same patch as the recordings shown in *A* and *B.* Na-Gluconate induced a difference in both E_{rev} and *g*. In this particular case, E_{rev} shifted from −27 mV to −15 mV while *g* increased from 101 pS in Na_2Malate to 187 pS in Na-Gluconate.

solution would induce a shift of E_{rev} in the direction of E_{Mal} (in order to be able to calculate E_{Mal}, the gluconate solution contained 10 μM Mal^{2-} and upon perfusion of the bath E_{Mal} shifted from 24 mV to −65 mV). Figure 4*A* shows current recordings from such a substitution experiment and Fig. 4*B,* the IV plots and the changes of E_{rev} and *g* in this particular experiment. Table 2 lists the results of eight independent experiments. With 10 mM Na_2Malate in the pipette, all recordings started in 100 mM Na_2Malate after which the bath solution was perfused with 250 mM Na-Gluconate. These substitution experiments revealed a change of E_{rev} from, on average, −24 mV to −19 mV. It should be noted that when the shift was calculated from each individual experiment, we obtained the same result, i.e., a mean shift of E_{rev} of 5 mV. Because the substitution of Mal^{2-} by $Gluc^-$ induced a shift of E_{rev} away from E_{Mal} we concluded that SV is impermeable to Mal^{2-}.

One may argue that because E_{rev} did shift in the direction of E_{Gluc}, these experiments could indicate that SV allows $Gluc^-$ to permeate. Although we cannot completely rule out this possibility, we postulate that the reason for the shift of E_{rev} can be found when searching

Table 2. Effect of the substitution of 100 mM Na_2Malate in the bath by 250 mM Na-Gluconate (n = 8)

Pipette/bath mM	Na_{pip} mM	Na_{bath} mM	Na_{bath}/Ca_{bath}	E_{rev} mV	E_{Ca} mV	E_{Na} mV	E_{Mal} mV	P_{Ca}/P_{Na}	g pS
10 Mal/100 Mal[1]	18	131	437	−24 ± 1.2	0	−50	24	44 ± 3.5	107 ± 2.3
10 Mal/250 Gluc[2]	18	190	950	−19 ± 1.7	6	−59	−65	111 ± 15.9	180 ± 3.8

Na_{pip} and Na_{bath} refer to the activity of Na^+ in the pipette (vacuolar lumen) and bath solution (cytosol), respectively. Na_{bath}/Ca_{bath} is the ratio of the Na^+ and Ca^{2+} activities in the bath solution.
[1] The activity of free Ca^{2+} in the 10 mM Na_2Malate pipette solution and the 100 mM Na_2Malate bath solution was 0.3 mM.
[2] In the 250 mM Na-Gluconate solution free Ca^{2+} was 0.2 mM.

in quite another direction. In order to calculate levels of free Ca^{2+}, initially, we treated $Gluc^-$ the same as Cl^-. However, with a pK of 1.21, $Gluc^-$ chelates Ca^{2+} stronger than Cl^- (pK = −0.99). Consequently, after the perfusion with $Gluc^-$, the level of free Ca^{2+} decreased from 0.3 to 0.2 mM and, as a result, E_{Ca} changed from 0 to 6 mV (Table 2). The change in E_{rev} thus coincided with the change in E_{Ca} and therefore we considered it more plausible that the shift in E_{rev} simply reflected the shift in E_{Ca}. Because the change of E_{Ca} could account for the shift of E_{rev}, the overall conclusion was that neither Mal^{2-} nor $Gluc^-$ permeates the SV channel.

Despite the fact that SV is thus impermeable to Mal^{2-} and $Gluc^-$, the identity of the anion species at the cytosolic side of the membrane clearly exerts an effect on the SV channel behavior. First, upon the substitution of Mal^{2-} by $Gluc^-$, the gating kinetics of the channel slightly changed with a more frequent gating in $Gluc^-$ than in Mal^{2-} (Fig. 4*A*). Secondly, the 5 mV shift of E_{rev} in the positive direction indicated that P_{Ca}/P_{Na} more than doubled from 44 to 111. The increase of P_{Ca}/P_{Na} was accompanied by an increase of g from 107 to 180 pS (Table 2). To test the hypothesis that the anion species affects the SV channel, we performed measurements under essentially the same cationic conditions (182–198 mM Na^+ and 0.3 mM Ca^{2+}) but in the presence of either Cl^- or Mal^{2-} in the bath solution (Table 3). As can be seen, on average, the values of g and P_{Ca}/P_{Na} were lowest in NaCl and highest with Na-Gluconate in the bath solution. Note the correlation in Table 3 between g and P_{Ca}/P_{Na}; an increase of g was accompanied by an increase of P_{Ca}/P_{Na}.

Discussion

One of the main conclusions of the present study is that the *M. crystallinum* SV channel is impermeable to Mal^{2-}. This conclusion contrasts with the findings of Iwasaki et al. (1992) on the tonoplast of *Graptopetalum paraguayense.* The authors described a channel that was more permeable to Mal^{2-} than to K^+. Because the *G. paraguayense* channel showed a similar sensitivity to voltage and cytosolic Ca^{2+} as SV, they concluded that the currents were mediated by SV. If the channel studied in *G. paraguayense* was indeed SV, a second difference with the *M. crystallinum* SV channel is the much lower single-channel conductance of 27 pS (in 15/50 mM K_2Malate) versus 108 pS (±31, n = 31) for *M. crystallinum* (in 10/100 mM Na_2Malate).

We focused on the putative permeability of Mal^{2-} and, by implication, $Gluc^-$. We did not, for instance, substitute Cl^- by $Gluc^-$ because these experiments have already been undertaken, either at the whole-vacuole level (Allen & Sanders, 1996; Schulz-Lessdorf & Hedrich, 1995) or at the single-channel level, although glutamate and sulfate were used as large impermeable anions instead of $Gluc^-$ (Amodeo et al., 1994). Despite the limited number of anion species tested, in the more general sense, our conclusion is in accordance with earlier findings: SV is permeable to monovalent cations and Ca^{2+} but not to anions (reviewed in Allen and Sanders, 1996). In this context, our study contributes to settle a long-lasting debate on the ion selectivity of SV. While Allen and Sanders (1996) studied SV at the whole-vacuole level, our conclusion is based on excised-patch measurements. That these two studies arrived at the same conclusion may suggest that in order to study SV, both types of measurements must render identical results. The fact, however, that we did find a discrepancy in E_{rev} between the two types of recordings (Table 1) emphasizes that E_{rev}'s obtained from whole-vacuole SV currents should be interpreted with caution.

The argument whether SV permeates anions is mainly based on substitution experiments as the one shown in Fig. 4. In our experiments E_{rev} shifted after replacing Mal^{2-} by $Gluc^-$ but we nevertheless concluded that SV is impermeable to Mal^{2-}. Our conclusion was based on the size of the shift, only a mere 5 mV, and even more important, on the direction of the shift, away from E_{Mal} and in the direction of E_{Ca} (Table 2). Allen and Sanders (1996) and Schulz-Lessdorf and Hedrich (1995) both performed similar substitution experiments on SV in *Vicia faba* guard cells. In the experiments of Allen and Sanders, $Gluc^-$ failed to shift E_{rev} and they argued that SV is thus impermeable to Cl^-. In contrast, Schulz-Lessdorf and Hedrich (1995) did observe a shift in E_{rev}

Table 3. Effect of anion species in the bath solution on P_{Ca}/P_{Na} and g in excised patch measurements.

Bath solution mM	Na_{pip} mM	Na_{bath} mM	Na_{bath}/Ca_{bath}	P_{Ca}/P_{Na}	g pS	n
250 NaCl[1]	17	182	607	31 ± 3.1	95 ± 3.5	7
160 Na_2Malate[2]	19	198	660	48 ± 7.6	113 ± 19.3	13
250 Na-Gluconate[2]	18	190	950	111 ± 15.9	180 ± 3.8	8

Na_{pip} and Na_{bath} refer to the activity of Na^+ in the pipette (vacuolar lumen) and bath (cytosol) solution, respectively. Na_{bath}/Ca_{bath} is the ratio of the Na^+ and Ca^{2+} activities in the bath solution. In all pipette and bath solutions the activity of free Ca^{2+} was 0.3 mM, except in the 250 mM Na-Gluconate bath solution where it was 0.2 mM.
[1] Pipette solution contained 20 mM NaCl.
[2] Pipette solution contained 10 mM Na_2Malate.

upon the replacement of KCl with K-Gluconate. The reason for the different outcome of these two studies remains hard to understand, especially because they were conducted on the same cell type of the same plant species. Possibly, part of the explanation may be found in the manner in which permeability ratios were calculated. Although E_{rev} shifted away from E_{Gluc}, Schulz-Lessdorf and Hedrich (1995) concluded that SV permeates $Gluc^-$, though to a very limited extent. The authors fitted whole-vacuole currents to the Goldman-Hodgkin-Katz (GHK) current equation assuming a permeability for five ion species, including $Gluc^-$. Such a fitting procedure differs from resolving the GHK equation for two or three permeable ion species in order to obtain an analytical solution, a procedure not possible in the case of five ion species. The question to what extent the final result depends on the type of calculation remains, however, speculative.

The SV channel shows the characteristics of a multi-ion pore (Allen & Sanders, 1996; Gambale et al., 1996) and such pores often show concentration-dependent permeability ratios. Therefore, is it possible that the effects on P_{Ca}/P_{Na} and g in Tables 2 and 3 are due to changes in the ratio of the Na^+ and Ca^{2+} activities in the bath solution? This seems especially relevant for the $Mal^{2-}/Gluc^-$ substitution experiments in Table 2. Here, the levels of both Na^+ and Ca^{2+} slightly changed after the perfusion of the bath with the Na-Gluconate solution. Although we acknowledge that such an anomalous mole fraction effect may have had an influence on P_{Ca}/P_{Na} and g, we nevertheless consider the possibility that it was the major reason for the observed changes quite remote. The reason is that the bath solutions used in the studies in which the multi-ion pore mechanism of SV was postulated contained relatively high Ca^{2+} levels. For instance, in the study of Allen and Sanders (1996), the Ca^{2+} mole fraction (%Ca) in the bath solution ranged from 0.47 to 100 (with a constant 200 mM KCl in the pipette solution). We, on the other hand, always recorded in bath solutions with much higher Na^+/Ca^{2+} ratios. Even compared to the 250 mM Na-Gluconate solution with a Na^+/Ca^{2+} of 950 and a %Ca of 0.11, with Na^+/Ca^{2+} as high as 437 and %Ca as low as 0.23 (Table 2) the 100 mM Na_2Malate bath solution still contained an excess of Na^+.

Although we thus conclude that the *M. crystallinum* SV channel is impermeable to Mal^{2-} and $Gluc^-$ and discount the possibility that the multi-ion pore behavior interfered with our measurements, under otherwise almost identical ionic conditions the anion species clearly affected the values of P_{Ca}/P_{Na} and g (Table 3). What then is the mechanism by which these anions exert an effect on SV? Calculated permeability ratios of multi-ion pores derived from the GHK current equation only give a qualitative measure of the relative permeability and should therefore be interpreted with caution (Johannes & Sanders, 1995). It should be stressed, however, that we are dealing with quite another phenomenon. In the case of the *M. crystallinum* SV channel, the calculated permeability ratio of two *permeable* ion species (P_{Ca}/P_{Na}) is sensitive to the presence of an *impermeable* anion species. In this section we propose a simple mechanism that explains the effects of the anion species on the calculated values of P_{Ca}/P_{Na} and g in terms of an anion-binding site and the radii of the hydrated Na^+ and Ca^{2+} ions. Because of a multi-ion pore permeation mechanism, in terms of rate theory, P_{Ca}/P_{Na} of SV is determined by occupancy, well depths and peak heights (Hille, 1992). Based on the previous findings of Pantoja et al. (1992a) and Cerana et al. (1995), we postulate a binding site for anions at the cytosolic side of the channel. One can imagine that the binding of an anion to this putative site would cause steric hindrance for Na^+ and Ca^{2+}, thereby affecting the peak height for either entering or leaving the channel. The hindrance experienced by the two cations is not necessarily the same. Although the crystal or ion radii of Na^+ and Ca^{2+} are very similar, 0.95 and 0.99 Å, respectively, the Stokes-diameter (r_s), representing the radius of the hydrated ion, for both ions is quite different. The ionic mobility (μ) of both ion species is about the same (~6 10^{-8} $m^2sec^{-1}V^{-1}$). Because r_s is directly proportional to the ratio of the valency of the ion (z) and μ, r_s of Na^+ is about half that of Ca^{2+} (Atkins, 1998). Because of this difference in r_s, it is conceivable that, depending on the anion species bound to the channel, the flux of

Table 4. Effect of the anion species on the SV current

Reference	Cl^- (mM)	NO_3^- (mM)	$Gluc^-$ (mM)	Mal^{2-} (mM)	Ca^{2+} (μM)	Current reduction
Pantoja et al., 1992a[a]	80 → 3		78		36 → 23	Yes
Pantoja et al., 1992a[a]	80 → 3	78			36 → 36	Yes
Cerana et al., 1995[b]	146 → 0			28	293 → 85	Yes
Cerana et al., 1995[b]	146 → 0	149			293 → 296	No
Schulz-Lessdorf & Hedrich, 1995[a]	78 → 9		77		37 → 23	Yes
Allen & Sanders, 1996[a]	17 → 0		19		580[c] → 580[c]	No

'Current reduction' refers to the current amplitude after the substitution of Cl^- by one of the anion species listed, compared to the current amplitude in the presence of KCl. The activities of Cl^- and Ca^{2+} are given before and after the perfusion of the bath solution. In all studies except the one of Allen and Sanders (1996), the bath solution contained Mg^{2+} and the activity of free Mg^{2+} was at least 180 μM.

[a] pH 7.5.

[b] pH 7.2, the solution also contained 3 mM SO_4^{2-}.

[c] Activities given by the authors.

Ca^{2+} ions is more affected than the flux of Na^+. Suppose that because of the size of the $Gluc^-$ ion ($C_6H_{11}O_7^-$, MW = 195), the anion-binding site has a low affinity for this ion species. Then, according to this view, the calculated P_{Ca}/P_{Na} in the presence of $Gluc^-$ represents the 'control' permeability ratio of the ion channel, i.e., the ion selectivity calculated with the anion-binding site unoccupied. The high P_{Ca}/P_{Na} of 111 (Table 3) indicates that the selectivity filter of the channel has a much higher affinity for Ca^{2+} than for Na^+. In addition, the high conductance of 180 pS indicates that the Ca^{2+} and Na^+ ions experience very little hindrance, if any at all, when entering or leaving the channel pore. The $Gluc^-$ solution also contained 5 mM Cl^-. If we assume that in the presence of the $Gluc^-$ solution the binding site is unoccupied, it is implied that the site has a rather low affinity for Cl^-. Now, imagine that we strongly increase the cytosolic Cl^- concentration. As soon as the binding site is occupied by Cl^-, the permeation of Ca^{2+} and Na^+ is affected. Due to the difference in diameter between the hydrated Ca^{2+} and Na^+ ions, Ca^{2+} ions are more impeded than Na^+ ions. The fact that the flux of Na^+ is favored is reflected in the lower P_{Ca}/P_{Na} of 31 compared to P_{Ca}/P_{Na} of 111 in the presence of $Gluc^-$. But because Na^+ does experience some hindrance by the binding of Cl^-, the single channel conductance drops to almost half its value in $Gluc^-$, from 180 to 95 pS (Table 3).

Compared to the Cl^- and $Gluc^-$ ion, the Mal^{2-} ion ($C_4H_4O_5^{2-}$, MW = 132) is of an intermediate size. Imagine that the anion-binding site binds Mal^{2-} as well but with a lower affinity than Cl^-, because Mal^{2-} is considerably larger than Cl^- and, in addition, is divalent. Because of the size of the Mal^{2-} ion, the binding of Mal^{2-} causes steric hindrance for both Ca^{2+} and Na^+. However, because of the difference in size, Ca^{2+} will still experience slightly more hindrance than Na^+ and this may explain the intermediate P_{Ca}/P_{Na} of 48 in Mal^{2-} (Table 3). The observation that g was very much the same in NaCl and Na_2Malate (95 and 113 pS, respectively) does not per se negate the assumption that the cation fluxes are more affected in NaCl than in Na_2Malate. It may reflect the fact that P_{Ca}/P_{Na} is more sensitive to the ionic conditions than is g.

In addition to an effect on the conducting properties, anions also influence SV in quite another way. Several studies revealed a strong current reduction after Cl^- in the bath solution was replaced by Mal^{2-} (Cerana et al., 1995), NO_3^- (Pantoja et al., 1992a) or $Gluc^-$ (Pantoja et al., 1992a; Schulz-Lessdorf & Hedrich, 1995) as the major anion species. A complication in the interpretation of steady-state whole-vacuole current magnitudes is the difficulty to distinguish effects on the open probability (P_o) from those on g. This may explain the different interpretation given for the observed current reduction in the presence of $Gluc^-$. Gluconate permeation impedes the permeation of K^+ (Schulz-Lessdorf & Hedrich, 1995) versus the inactivation of SV in the absence of cytosolic Cl^- (Pantoja et al., 1992a). The data presented here unambiguously show that it is not the mere presence of Mal^{2-} or $Gluc^-$ that blocks the SV channel and reduces g. On the contrary, in the presence of Cl^- and Mal^{2-}, g was about the same (Table 3). Moreover, g almost doubled after the replacement of Mal^{2-} by $Gluc^-$ (Table 2). This implies that the observed inhibition of whole-vacuole current was caused by an effect on P_o rather than on g. We analyzed the ionic conditions that prevailed in four studies that reported on the effect of the anion species on SV and the data are summarized in Table 4. Pantoja et al. (1992a) concluded that the reduction in cytosolic Cl^- was responsible for the current inhibition after Cl^- in the bath had been replaced by another anion species. Although this conclusion still seems justified, it requires some modification in that the modulation of SV by cytosolic Ca^{2+} has to be included. The reason is that Cerana et al., (1995) and Allen and Sanders (1996) found no current reduction upon the total removal of Cl^-. The data in Table 4 suggests that high cytosolic Ca^{2+} (~300 μM) can prevent the inhibition of SV induced by low

levels of cytosolic Cl^-. The modulation of SV by Ca^{2+} and Cl^- is an intriguing idea because the opposite seems not to be true: high cytosolic Cl^- is not able to lift the inactivation of SV at low cytosolic Ca^{2+}. These findings show strong parallels with the observation that cytosolic Mg^{2+} sensitizes SV to cytosolic Ca^{2+} (Pei et al., 1999). In the presence of Mg^{2+}, less Ca^{2+} is required to activate SV than in the absence of Mg^{2+}. Pei et al. (1999) explained this result by postulating two cytosolic binding sites for cations, one with a high affinity for Ca^{2+} but no affinity for Mg^{2+} and a second one with a low affinity for both Ca^{2+} and Mg^{2+}. Although, the sensitization to Ca^{2+} by Cl^- can be explained in similar terms, it is hard to envisage that a single binding site shows an affinity for both Cl^- and Ca^{2+}. We therefore postulate that in addition to the two sites described by Pei et al. (1999), SV possess a third binding site, specifically for anions, in particular Cl^-. As long as the cytosolic Ca^{2+} level is high, both the low- and high-affinity sites for Ca^{2+} are occupied. As a result, the channel is activated and the concentrations of Mg^{2+} and Cl^- are rather irrelevant. This is nicely illustrated by the study of Allen and Sanders (1996, *see* Table 4). Even with zero Mg^{2+} and zero Cl^-, the whole-vacuole SV current amplitude did not reduce. Only at strongly reduced cytosolic Ca^{2+} levels become the concentrations of Mg^{2+} and Cl^- relevant for the activation level of the SV channel.

In conclusion, this study addresses two effects of anions on SV, of which the first one is related to the conducting properties and the second to the activation kinetics of the channel. Although we describe two separate effects, both types of modulation can be explained by a single binding site for anions at the cytosolic face of the channel. One effect is related to the way impermeable anions affect the fluxes of the two permeable cation species Na^+ and Ca^{2+}. Depending on the anion species, the Na^+ and Ca^{2+} fluxes experience steric hindrance. Because the Stokes-diameters of Na^+ and Ca^{2+} are different, both fluxes are not affected to the same extent and as a result the calculated P_{Ca}/P_{Na} is sensitive to the anion species and anion concentration. The second effect concerns the activation of SV by cytosolic Cl^-. We argue that existing literature provides convincing evidence for a synergy between the modulation of SV by Ca^{2+} and Cl^-. High cytosolic Ca^{2+} can overcome the lack of SV activation at low cytosolic Cl^-. Although this effect shows strong parallels with the sensitization to Ca^{2+} by Mg^{2+}, considering the opposite valencies of the Cl^- and the Mg^{2+} ions, it seems unlikely that the same two binding sites are involved. More likely, the SV channel possesses three cytosolic binding sites, one for Ca^{2+}, one for Ca^{2+} and Mg^{2+} and one for Cl^-. The interaction between these sites in the overall activation of SV is the subject of future research.

The help and advice of Drs. B. Barkla (style improvement), P. Barry (on the mobility coefficient of Mal^{2-}), M. Tester (on the interaction between $Gluc^-$ and Ca^{2+}) and R. Vera-Estrella (for providing *Mesembryanthemum* plants) are highly acknowledged.

This study was supported by Grants awarded to O.P. by CONACyT (4745-N) and DGAPA (IN-202000).

References

Allen, G.J., Sanders, D. 1995. Calcineurin, a Type 2B protein phosphatase, modulates the Ca^{2+}-permeable slow vacuolar ion channel of stomatal guard cells. *Plant Cell* **7:**1473–1483

Allen, G.J., Sanders, D. 1996. Control of ionic currents in guard cell vacuoles by cytosolic and luminal calcium. *Plant J.* **10:**1055–1069

Allen, G.J., Sanders, D. 1997. Vacuolar ion channels in higher plants. *In:* The plant vacuole. R.A. Leigh and D. Sanders, Eds. pp. 217–252. Academic Press, London

Amodeo, G., Escobar, A., Zeiger, E. 1994. A cationic channel in the guard cell tonoplast of *Allium cepa*. *Plant Physiol.* **105:**999–1006

Atkins, P.W. Physical Chemistry. 1998. 6th Ed. Oxford University Press, Oxford, UK

Barkla, B.J., Vera-Estrella, R., Maldonado-Gama, M., Pantoja, O. 1999. ABA induction of vacuolar H^+-ATPase activity in *Mesembryanthemum crystallinum* L. is developmentally regulated. *Plant Physiol.* **120:**811–819

Barry, P.H. 1994. JPCalc, a software package for calculating liquid junction potentials corrections in patch-clamp, intracellular, epithelial and bilayer measurements and for correcting junction potential measurements. *J. Neuroscience Methods* **51:**107–116

Bertl, A., Blumwald, E., Coronado, R., Eisenberg, R., Findlay, G., Gradmann, D., Hille, B., Köhler, K., Kolb, H.A., MacRobbie, E., Meissner, G., Miller, C., Neher, E., Palade, P., Pantoja, O., Sanders, D., Schroeder, J., Slayman, C., Spanswick, R., Walker, A., Williams, E. 1992. Electrical measurements on endomembranes. *Science* **258:**873–874

Bethke, P.C., Jones, R.L. 1994. Ca^{2+}-Calmodulin modulates ion channel activity in storage protein vacuoles of barley aleurone cells. *Plant Cell* **6:**277–285

Bethke, P.C., Jones, R.L. 1997. Reversible protein phosphorylation regulates the activity of the slow-vacuolar ion channel. *Plant J.* **11:**1227–1235

Carpaneto, A., Cantù, A.M., Gambale, F. 1999. Redox agents regulate ion channel activity in vacuoles from higher plant cells. *FEBS Lett.* **442:**129–132

Cerana, R., Giromini, L., Colombo, R. 1995. Malate-regulated channels permeable to anions in vacuoles of *Arabidopsis thaliana. Aust. J. Plant Physiol.* **22:**115–121

Cerana, R., Giromini, L., Colombo, R. 1999. On the gating mechanism of the slowly activating vacuolar channels of *Arabidopsis thaliana* cultured cells. *J. Plant Physiol.* **154:**617–622

Coyaud, L., Kurkdjian, A., Kado, R., Hedrich, R. 1987. Ion channels and ATP-driven pumps involved in ion transport across the tonoplast of sugar beet vacuoles. *Biochem. Biophys. Acta* **902:**263–268

Dobrovinskaya, O.R., Muñiz, J., Pottosin, I.I. 1999. Inhibition of vacuolar ion channels by polyamines. *J. Membrane Biol.* **167:**127–140

Gambale, F., Bregante, M., Stragapede, F., Cantù, A.M. 1996. Ionic channels of the sugar beet tonoplast are regulated by a multi-ion single-file permeation mechanism. *J. Membrane Biol.* **154:**69–79

Hedrich, R., Neher, E. 1987. Cytoplasmic calcium regulates voltage dependent ion channels in plant vacuoles. *Nature* **329:**833–835

Hedrich, R., Flügge, U.I., Fernandez, J.M. 1986. Patch-clamp studies of ion transport in isolated plant vacuoles. *FEBS Lett.* **204:**228–232

Hille, B. 1992. Ionic Channels of Excitable Membranes. Sinauer Associates, Massachusetts

Iwasaki, I., Arata, H., Kijima, H., Nishimura, M. 1992. Two types of channels involved in the malate ion transport across the tonoplast of a Crassulacean acid metabolism plant. *Plant Physiol.* **98:**1494–1497

Johannes, E., Sanders, D. 1995. Lumenal calcium modulates unitary conductance and gating of a plant vacuolar calcium release channel. *J. Membrane Biol.* **146:**211–224

Martell, A.E., Smith, R.M. 1977. Critical Stability Constants. Plenum Press, NY

Miedema, H., Balderas, E., Pantoja, O. 2000. Current oscillations under voltage clamp conditions: an interplay of negative slope conductance and series resistance. *J. Membrane Biol.* **173:**31–37

Pantoja, O., Dainty, J., Blumwald, E. 1992a. Cytoplasmic chloride regulates cation channels in the vacuolar membrane of plant cells. *J. Membrane Biol.* **125:**219–229

Pantoja, O., Gelli, A., Blumwald, E. 1992b. Characterization of vacuolar malate and K^+ channels under physiological conditions. *Plant Physiol.* **100:**1137–1141

Parker, D.R., Norvell, W.A., Chaney, R. L. 1995. GEOCHEM-PC: A chemical speciation program for IBM and compatible personal computers. In: Chemical Equilibrium and Reaction Models, Eds. R.H. Loeppert, A.P. Schwab and S. Goldberg, pp. 253–269. SSSA Special publication number 42, Soil Science Society of America/American Society of Agronomy, Madison, WC

Pei, Z-M., Ward, J.M., Schroeder, J.I. 1999. Magnesium sensitizes slow vacuolar channels to physiological cytosolic calcium and inhibits fast vacuolar channels in *Fava* bean guard cell vacuoles. *Plant Physiol.* **121:**977–986

Plant, P.J., Gelli, A., Blumwald, E. 1994. Vacuolar chloride regulation of an anion selective tonoplast channel. *J. Membrane Biol.* **140:** 1–12

Pottosin, I.I., Tikhonova, L.I., Hedrich, R., Schönknecht, G. 1997. Slowly activating vacuolar channels can not mediate Ca^{2+}-induced Ca^{2+}-release. *Plant J.* **12:**1387–1398

Robinson, R.A., Stokes, R.H. 1965. Electrolyte Solutions. Butterworths, London

Sanders, D., Brownlee, C., Harper, J.F. 1999. Communicating with calcium. *Plant Cell* **11:**691–706

Schulz-Lessdorf, B., Hedrich R. 1995. Protons and calcium modulate SV-type channels in the vacuolar-lysosomal compartment-channel interaction with calmodulin inhibitors. *Planta* **197:**655–671

Taiz, L., Zeiger, E. 1998. Plant Physiology. 2nd Ed. Sinauer Associates, Inc., Sunderland, MA

Ward, J.M., Schroeder, J.I. 1994. Calcium-activated K^+ channels and calcium-induced calcium release by slow vacuolar ion channels in guard cell vacuoles implicated in the control of stomatal closure. *Plant Cell* **6:**669–683

Ward, J.M., Pei, Z-M., Schroeder, J.I. 1995. Roles of ion channels in initiation of signal transduction in higher plants. *Plant Cell* **7:**833–844