RESEARCH PAPER

Homeostatic control of slow vacuolar channels by luminal cations and evaluation of the channel-mediated tonoplast Ca\(^{2+}\) fluxes in situ

V. Pérez\(^1\), T. Wherrett\(^2\), S. Shabala\(^2\), J. Muñiz\(^1\), O. Dobrovinskaya\(^1\) and I. Pottosin\(^1\)*

\(^1\) Centro Universitario de Investigaciones Biomédicas. Universidad de Colima, 28045 Colima, Col., México

\(^2\) School of Agricultural Science, University of Tasmania, Tas7001, Australia

Received 12 June 2008; Revised 5 August 2008; Accepted 6 August 2008

Abstract

Ca\(^{2+}\), Mg\(^{2+}\), and K\(^+\) activities in red beet (Beta vulgaris L.) vacuoles were evaluated using conventional ion-selective microelectrodes and, in the case of Ca\(^{2+}\), by non-invasive ion flux measurements (MIFE) as well. The mean vacuolar Ca\(^{2+}\) activity was \(\sim0.2\) mM. Modulation of the slow vacuolar (SV) channel voltage dependence by Ca\(^{2+}\) in the absence and presence of other cations at their physiological concentrations was studied by patch-clamp in excised tonoplast patches. Lowering pH at the vacuolar side from 7.5 to 5.5 (at zero vacuolar Ca\(^{2+}\)) did not affect the channel voltage dependence, but abolished sensitivity to luminal Ca\(^{2+}\) within a physiological range of concentrations (0.1–1.0 mM). Aggregation of the physiological vacuolar Na\(^+\) (60 mM) and Mg\(^{2+}\) (8 mM) concentrations also results in the SV channel becoming almost insensitive to vacuolar Ca\(^{2+}\) variation in a range from nanomoles to 0.1 mM. At physiological cation concentrations at the vacuolar side, cytosolic Ca\(^{2+}\) activates the SV channel in a voltage-independent manner with \(K_d=0.7–1.5\ \mu\text{M}\). Comparison of the vacuolar Ca\(^{2+}\) fluxes measured by both the MIFE technique and from estimating the SV channel activity in attached patches, suggests that, at resting membrane potentials, even at elevated (20 \mu\text{M}) cytosolic Ca\(^{2+}\), only 0.5% of SV channels are open. This mediates a Ca\(^{2+}\) release of only a few pA per vacuole (\(\sim0.1\) pA per single SV channel). Overall, our data suggest that the release of Ca\(^{2+}\) through SV channels makes little contribution to a global cytosolic Ca\(^{2+}\) signal.

Key words: Calcium channel, calcium signalling, patch-clamp, SV channel, tonoplast, vacuole.

Introduction

Slow vacuolar (SV) channels are ubiquitous in all tissues of higher plants with several thousand per vacuole (Hedrich et al., 1988; Schulz-Lessdorf and Hedrich, 1995; Pottosin et al., 1997). The SV channel is a non-selective cation channel with significant Ca\(^{2+}\) permeability (Ward and Schroeder, 1994; Gradmann et al., 1997; Allen et al., 1998; Pottosin et al., 2001), encoded by the TPC1 (two-pore calcium channel) singleton gene (Peiter et al., 2005). The SV channel is undoubtedly the best explored and the most abundant vacuolar channel, as shown by electrophysiological and proteomics studies of the tonoplast (Schulz-Lessdorf and Hedrich, 1995; Carter et al., 2004; Pottosin and Schönknecht, 2007).

Because the vacuole is the largest Ca\(^{2+}\) store in plant cells, there is an ongoing debate as to the level of participation of the SV channel in Ca\(^{2+}\) signalling. For instance, based on the finding that the SV channel is activated by increased cytosolic Ca\(^{2+}\), Ward and Schroeder (1994) proposed that these channels take part in the so-called CICR (Ca\(^{2+}\)-induced Ca\(^{2+}\) release), a potentiation of the initial Ca\(^{2+}\) signal. However, experiments with tpc1-knockout Arabidopsis plants did not reveal any clear changes in the phenotype, thus questioning the participation of the SV channel in Ca\(^{2+}\) responses to a variety of stimuli (Peiter et al., 2005; Ranf et al., 2008).

Slow vacuolar channels are regulated by a variety of physiological factors (see Pottosin and Schönknecht,
2007, for a recent review). Most pronounced are the activation by cytosolic Ca\(^{2+}\) (Hedrich and Neher, 1987; Ward and Schroeder, 1994; Schulz-Lessdorf and Hedrich, 1995), and negative modulation by vacuolar Ca\(^{2+}\) (Pottosin et al., 1997, 2004). In the first attempt to quantify the joint impact of luminal and cytosolic Ca\(^{2+}\) on SV channel activity, Pottosin and co-workers (1997) demonstrated that the inhibitory effect of vacuolar Ca\(^{2+}\) prevailed the channel stimulation by cytosolic Ca\(^{2+}\). Hence, at realistic electrochemical Ca\(^{2+}\) gradients across the tonoplast that favour Ca\(^{2+}\) release from the vacuole, only a tiny fraction of SV channels is active.

However, while the range of cytosolic Ca\(^{2+}\) variation is well established, all the information regarding the free vacuolar Ca\(^{2+}\) in higher plants comes essentially from a single study (Felle, 1988). In the absence of Ca\(^{2+}\), other vacuolar cations increase the threshold for the SV channel voltage activation, specifically in the case of Mg\(^{2+}\), Na\(^{+}\), and Cs\(^{+}\), or non-specifically via screening of the negative surface charge (K\(^{+}\), choline, NMDG) (Pottosin et al., 2004, 2005; Iwashikina and Hedrich, 2005; Ranf et al., 2008). Furthermore, the effects of different vacuolar cations were not additive. For instance, at zero vacuolar Ca\(^{2+}\), increases in the vacuolar K\(^{+}\) caused a positive shift of SV channel voltage dependence, whereas at 0.5 mM vacuolar Ca\(^{2+}\), the same increase in vacuolar K\(^{+}\) caused a negative shift (Pottosin et al., 2005). Also, cytosolic Mg\(^{2+}\), especially at low cytosolic Ca\(^{2+}\), acted as a positive regulator of the SV channel (Pei et al., 1999; Carpaneto et al., 2001). Consequently, the free concentrations of physiologically abundant vacuolar cations such as Na\(^{+}\), K\(^{+}\), Ca\(^{2+}\), and Mg\(^{2+}\), remain to be determined and their joint effect on the SV channel voltage dependence, evaluated.

In light of this, the vacuolar levels of these cations were measured and these values were used to quantify the SV channel activity under a physiological range of cytosolic Ca\(^{2+}\) and Mg\(^{2+}\) activities. This was then compared with the SV channel activity measured in situ from attached patches and with Ca\(^{2+}\) fluxes measured on isolated vacuoles using the MIFE technique. Our results suggest that SV channel mediation of Ca\(^{2+}\) release from the vacuole is insufficient to support global Ca\(^{2+}\) responses in higher plant cells.

### Materials and methods

#### Preparation

Vacuoles were isolated mechanically from *Beta vulgaris* L. taproot slices as described previously (Pottosin et al., 2001). Briefly, sections of a taproot were soaked for at least 20 min in a solution essentially identical to the bath solution used in patch-clamp experiments, but made slightly (by 30–40 mOsm) hypertonic. Sections were cut with preparation needles under stereoscopic control, and vacuoles released were collected with a micropipette and transported to the experimental chamber.

#### Intravacuolar cation activities measurements

Ion-selective microelectrodes for the determination of intravacuolar activities of Ca\(^{2+}\), Mg\(^{2+}\), and K\(^{+}\) were fabricated from 1.5 mm non-filamented borosilicate glass capillaries (GC 150–10, CDR Clinical Technology, Middle Cove, Australia) pulled to the smallest diameter (<1 μm) that still allowed backfilling. Blank capillaries were oven-dried at 225 °C overnight. They were then covered with a steel lid for 10–15 min, before 40–50 μl tributylchlorosilane (90796, Fluka Chemicals) was injected under the lid, and the lid removed 10 min later. Electrode blanks were baked for a further 40 min to ensure complete drying, and allowed to cool before storage in a closed container for up to 6 weeks.

Micropipettes were backfilled with the appropriate solution (Table 1) using a syringe with a thin metal needle. Filling with the ionophore (liquid ion exchanger, LIX) was performed immediately after backfilling by dipping the microelectrode into a wider tipped (30–50 μm) pulled capillary, filled by its submersion in the LIX stock solution. The LIX column obtained in the microelectrode tip was 100–200 μm long. Because the ion-selective microelectrode readings are affected by the ionic strength of the solutions, the activities of major osmotics (K\(^{+}\), Na\(^{+}\)) were determined first, and calibration solutions were prepared accordingly.

Potassium electrodes were calibrated in a background of 60 mM NaCl and Ca\(^{2+}\)-electrode calibration solutions included 115 mM KCl and 60 mM NaCl. The changes in Ca\(^{2+}\) activity between 150 μM and 350 μM, mimicking the variation in measured vacuolar Ca\(^{2+}\), did not affect the electric potential values reported by Mg\(^{2+}\)-electrodes, so no additional corrections for Ca\(^{2+}\) were made.

Microelectrode calibration was performed before and after impalement. Electrodes with slopes less than 50 mV (for K\(^{+}\)) or 25 mV per decade (for Ca\(^{2+}\), Mg\(^{2+}\)), or a correlation coefficient less than 0.999, were discarded. Typical resistance of the microelectrodes was 1–3 GΩ. Measured microelectrode voltages were corrected for potential values across the tonoplast and the liquid junction potentials (in mV: 1.3 for the Ca\(^{2+}\)-microelectrode, 1.4 for the K\(^{+}\)-microelectrode, and 6.6 for the Mg\(^{2+}\)-microelectrode). Results of trans-tonoplast electric potential difference, measured in a separate set of experiments with a glass microelectrode filled with 3 M KCl to minimize the liquid junction potentials, were used to correct potentials values reported by the ion-selective microelectrodes. The bath solution was 100 mM KCl, 1 mM HEPES (pH 7.4/ KOH), adjusted with sorbitol to be slightly more hyperosmotic than the measured osmolality values for squeezed beet juice (500–650 mOsm).

Due to the low selectivity of the Na\(^{+}\)-LIX against K\(^{+}\) and difficulties with front-filling H\(^{+}\)-LIX electrodes impalements, Na\(^{+}\) and H\(^{+}\) free activities could not be measured with ion-selective microelectrodes. Instead, centrifuged squeezed juice (mainly vacuolar sap) from beet taproots was used to measure the Na\(^{+}\) concentration using a flame photometer, and H\(^{+}\) activity, with conventional pH-electrodes.

| Ion | Liquid ion exchanger (LIX) | Backfilling solution |
|-----|---------------------------|----------------------|
| Ca\(^{2+}\) | Calcium ionophore I — cocktail A (Sigma I-1772) | 500 mM CaCl\(_2\) |
| Mg\(^{2+}\) | Magnesium ionophore I — cocktail A (Fluka 63048) | 500 mM MgCl\(_2\) |
| K\(^{+}\) | Potassium ionophore I — cocktail A (Fluka 60031) | 500 mM KCl |

### Table 1. Liquid ionophores and respective backfilling solutions used for ion selective microelectrodes
**Ca**²⁺ flux measurements with the MIFE technique**

**Ca**²⁺-fluxes from Beta vacuoles were quantified using the Micro-electrode Ion Flux Estimation (MIFE) technique (see Newman, 2001, for details and theoretical background). Microelectrodes fabricated for MIFE were similar to those used for vacuole impalements, except the tip diameter was 2–3 μm and drying time after silanization was 30 min.

Calibration of Ca²⁺-selective MIFE-electrodes was performed in a background of 100 mM K⁺. MIFE set-up and experimental procedure was essentially as described previously (Wherrett et al., 2005). The MIFE electrode was positioned at a distance of 10 μm from the vacuole surface and moved back and forth 50 μm horizontally to avoid the influence of ion leakage fluxes from the underlying glass chamber. Calcium fluxes were calculated by subtracting the background flux determined in the immediate proximity to the vacuole from the measured integral flux, taking into account the diameter of each analysed vacuole. Ca²⁺-fluxes were estimated against a background of 100 mM KCl, 1 mM HEPES (pH 7.4 with KOH), and either 0, 20, 50, 70, 100, 150, or 250 μM Ca²⁺, without Ca²⁺-buffering (background Ca²⁺-contamination was ~2 μM, as reported by Ca²⁺-selective electrodes). The statistical significance of Ca²⁺-flux changes under treatment with Mg²⁺ and Zn²⁺ was evaluated by a one way analysis of variance using SPSS, and mean comparison using Duncan’s multiple range test.

**Patch-clamp protocols and analyses**

Patch pipettes fabrication, patch-clamp set-up, and record acquisition and analyses were as described previously (Pottosin et al., 2001). The osmolality of all solutions was adjusted by sorbitol to isotonic levels with respect to the vacuolar sap (range 520–680 mOsm), verified using a cryoscopic osmometer (OSMOMAT 030, Gonotec, Germany).

The effects of the major vacuolar cations were examined in a set of experiments on excised patches with 100 mM KCl, 2 mM CaCl₂, 10 mM HEPES, pH 7.5, (with KOH) at the cytosolic side, and either 100 mM, 125 or 187 mM KCl, 0 or 62 mM NaCl, 0 or 8 mM MgCl₂, with a variable free Ca²⁺ (0, 0.01, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0 or 10.0 mM), 10 mM MES/KOH (pH 5.5 or 6.4), at the vacuolar side. For measurements in vacuole-attached configuration, patch pipettes were filled with the same cytosolic solution as above, while the bath contained 100 mM KCl, and 0, 0.05, 0.1, 0.2, 0.5, 1.0, 2 or 10 mM Ca²⁺, 10 mM HEPES-KOH (pH 7.5). Voltages were corrected for the trans-tonoplast potential from values measured with a 3 M KCl-filled microelectrode in bath solutions containing the same free Ca²⁺-concentration.

SV currents in vacuole-attached patches and small right-oriented (cytosolic side out) vacuolar vesicles were also analysed at ionic conditions mimicking the physiological cation concentrations. The vacuolar solution used was in mM: 125 K⁺, 62 Na⁺, 8 Mg²⁺, 0.25 Ca²⁺, pH 5.5 (MES/KOH), and the cytosolic solution contained: 100 mM K⁺, 10 mM HEPES/KOH (pH 7.5) and 0.1, 1.0, 7.0 or 20 μM free Ca²⁺, supplemented with either 0.5 or 1.5 mM free Mg²⁺. Solutions with a certain free Ca²⁺ and Mg²⁺ concentrations were prepared using a combination of chelating agents (2 mM of EGTA, HEDTA, and/or NTA). The free divalent cation concentration and concentrations of added chelating agents including ATP, taking into the account the ambient pH, temperature and ionic strength (WinMAXC v.2.50, Chris Patton, Stanford University).

SV channel currents were evoked by a series of positive voltage steps from a holding potential of −100 mV (cytosol minus vacuole) or −20 mV (in experiments with low cytosolic Ca²⁺), up to +200 mV in 20 mV increments. Unitary current–voltage (I/V) relationships at different ionic conditions and patch configurations were obtained by the application of 15 ms voltage-ramps between +200 and −200 mV as previously described (Pottosin et al., 2001). The SV activation curve (number or relative number of open channels versus trans-tonoplast electric potential difference) was obtained as described in detail by Pottosin et al. (2005). To describe the shift of the voltage dependence by vacuolar Ca²⁺, a threshold voltage value (Vₜ₈), i.e. the voltage at which 1% of maximal SV channel activity was reached (P/Pₘₐₓ=0.01), was estimated at each given condition. Non-linear regression fitting of the data was performed using the GraFit v.6 data analysis and graphics program (Erithacus Software, Ltd).

**Results**

**Vacular Ca**²⁺ **fluxes and cation concentrations**

One of the primary aims of this study was to evaluate net Ca²⁺ release from sugar beet vacuoles. As one possible route of Ca²⁺ escape from vacuoles are Ca²⁺-activated SV channels, by applying the MIFE technique Ca²⁺ flux measurements were performed at different levels of cytosolic (bath) Ca²⁺ (Fig. 1A). However, the minimal Ca²⁺ free concentration achieved was ~2 μM (reflecting contamination of the bath solution by the vacuolar isolation procedure) due to the inability of the MIFE technique to estimate net Ca²⁺ fluxes in the presence of Ca²⁺ buffers. This concentration is referred to as the ‘trace bath Ca²⁺’ when referring to MIFE results. Under such minimal conditions, the net Ca²⁺ release from vacuoles was close to the detection limit of the method (~1 nmol m⁻² s⁻¹). This flux tripled and reached its maximum at ~20 μM free Ca²⁺ in the bath, while further increases in the free cytosolic Ca²⁺ caused a gradual decrease of Ca²⁺ flux, until it reversed polarity at ~200 μM cytosolic Ca²⁺. This non-monotonic behaviour is consistent with passive Ca²⁺ transport, mediated by some (presumably SV) Ca²⁺-permeable channel, activated by cytosolic Ca²⁺. Moreover, the observed Ca²⁺ flux was significantly (P < 0.05) enhanced by 1.5 mM Mg²⁺ and strongly suppressed by 0.1 mM Zn²⁺ (Fig. 1B), known activator and blocker, respectively, of SV channels (Hedrich and Kurkdjian, 1988; Gambale et al., 1993; Pei et al., 1999; Carpaneto et al., 2001).

The SV channel is controlled by different mono and divalent cations present at cytosolic and vacuolar sides of the tonoplast. Therefore, to link the vacular Ca²⁺ flux to the SV channel activity, a further attempt was made to evaluate the activities of physiologically relevant vacuolar cations. Because single-barrelled electrodes were used to determine the activities of Ca²⁺, Mg²⁺, and K⁺ in fresh isolated sugar beet vacuoles (see Materials and methods for details), electrode readings were corrected for trans-tonoplast electric potential difference with measurements from a separate set of experiments using 3 M KCl-filled microelectrodes (Fig. 1C). However, due to the poor selectivity of all available Na⁺ resins (Chen et al., 2005),
vacuolar Na⁺ concentrations were measured using conventional flame photometry. The results of these measurements are summarized in Table 2. Notably, the Ca²⁺ activity was an order of magnitude lower (Table 2) than previously reported for distinct higher plant preparations (Felle, 1988). Moreover, the same Ca²⁺ activity value was independently estimated from the reversal condition (respectively bath Ca²⁺ concentration) of the Ca²⁺ flux reported by MIFE, assuming equilibrium conditions for Ca²⁺ exchange across the tonoplast and correcting the data for the resting tonoplast potential.

Modulation of the SV channel voltage dependence by vacuolar cations

Our previous study has demonstrated a very strong dependence of the SV channel activation threshold on vacuolar Ca²⁺ (Pottosin et al., 2004). However, the above study was performed at a non-physiological vacuolar pH (7.5), while the actual pH of the vacuolar sap is around 5.9±0.3 (n=11 separate preparations). Accordingly, in this work the Ca²⁺ modulation of the SV channel voltage dependence has been analysed at more physiologically relevant vacuolar pH values (6.4 and 5.5, respectively). The summary of the activation curves at different vacuolar Ca²⁺ levels and pH 5.5 is presented in Fig. 2A. To make a single-parameter comparison of the Ca²⁺ dependence at different vacuolar pH values, the membrane potential values at which 1% of available SV channels are open (V₁%; see Pottosin et al., 2005, for a justification) have been estimated. This technical activation threshold was plotted as a function of the vacuolar free Ca²⁺ (Fig. 2B). At vacuolar pH 7.5 within a range from 10⁻⁵ M to 10⁻¹ M Ca²⁺, the SV channel behaves as a ‘super-Ca²⁺’ electrode. The curve V₁% versus the vacuolar free Ca²⁺ has a slope of ~40 mV per 10-fold increase in Ca²⁺ concentration (as compared to 29.5 mV for an ideal Ca²⁺ electrode). This is because the SV channel is able to bind more than one Ca²⁺ ion from the vacuolar side (Pottosin et al., 2004).

Table 2. Activities of physiological cations in red beet (Beta vulgaris L.) vacuoles

| Ion | Activity (mM) | Method                  |
|-----|--------------|-------------------------|
| K⁺  | 116±2 (n=8)  | K⁺-selective microelectrodes |
| Na⁺ | 62±5 (n=5)   | Flame photometry         |
| Mg²⁺| 8.3±0.7 (n=7)| Mg²⁺-selective microelectrodes |
| Ca²⁺| 0.20±0.02 (n=7) | Ca²⁺-selective microelectrodes 0.21 |

* Concentration instead of activity was determined in this case.
channel voltage dependence and $V_{1\%}$ at zero vacuolar Ca$^{2+}$ (Fig. 2).

Sugar beet is a salt-tolerant species that uses Na$^+$ for maintaining cell turgor. Accordingly, the impact of vacuolar sap Na$^+$ on the voltage dependence of the SV channel at zero and 0.5 mM vacuolar Ca$^{2+}$ was verified (Fig. 3). At zero Ca$^{2+}$, addition of 62 mM Na$^+$ caused an increase in the voltage threshold of SV channel activation. This exceeded the effect of the addition of equimolar concentration of K$^+$ (Fig. 3A). However, in the presence of 0.5 mM Ca$^{2+}$ at the luminal side, addition of Na$^+$ resulted in the opposite effect, i.e. a decrease in the activation threshold (Fig. 3B). Alternatively, the results presented in Fig. 3 could be read in a different manner. Indeed, the activation curves are the same at zero and 0.5 mM Ca$^{2+}$ in the presence of Na$^+$, indicating that in the presence of 62 mM Na$^+$, a vacuolar Ca$^{2+}$ increase from zero to 0.5 mM does not have a significant impact on the SV channel voltage dependence. In other words, the presence of physiologically relevant Na$^+$ concentrations in cell vacuoles desensitizes the SV channel to vacuolar Ca$^{2+}$ variation between zero and 0.5 mM.
Finally, the modulation of the SV channel voltage dependence by vacuolar Ca$^{2+}$ at pH 5.5 in the presence of physiological concentrations of K$^+$, Na$^+$, and Mg$^{2+}$ was analysed. Addition of 62 mM Na$^+$ and 8 mM Mg$^{2+}$ to the vacuolar solution shifted the activation threshold at zero vacuolar Ca$^{2+}$ by $\sim 35$ mV to more positive potentials (Figs 4A, 2A). Also the threshold dependence on vacuolar Ca$^{2+}$ flattened: not only the Ca$^{2+}$ change from 0.1 mM to 1 mM produced little change in the threshold but, in addition, the Ca$^{2+}$ increase from zero to 0.1 mM caused a lesser $V_{1\%}$ shift compared to the case without Mg$^{2+}$ and Na$^+$ at the luminal side (Fig. 4B). For comparison, threshold potentials were evaluated in vacuole-attached patches, with patch-pipettes filled with the standard cytosolic high Ca$^{2+}$ solution and variable free Ca$^{2+}$ in the bath. Threshold $V_{1\%}$ values (in mV) were as follows: $-14.4\pm0.1$, $-11.3\pm8.7$, $-14.9\pm7.4$, and $+5.3\pm0.3$ ($n=4$–5 vacuoles) using bath free Ca$^{2+}$ of 0, 0.1, 0.5, and 1 mM, respectively. Increased threshold at 1 mM free Ca$^{2+}$ in the bath probably reflects an increase of vacuolar Ca$^{2+}$, due to Ca$^{2+}$ ions entering the vacuole from the bath via open Ca$^{2+}$-activated SV channels. Indirect evidence that such a process takes place comes from the membrane potential measurements (Fig. 1C). At [Ca$^{2+}$] $>1$ mM, the membrane potential became more sensitive to changes in bath Ca$^{2+}$, implying a larger contribution of the tonoplast Ca$^{2+}$-permeable channels. Providing at bath Ca$^{2+}$ < 1 mM the vacuolar sap composition is unperturbed due to the exchange with the bath, the $V_{1\%}$ mean values for vacuole-attached patches would then be in the $-15$ mV to $-11$ mV range. These values can be compared with those obtained on excised patches at different vacuolar Ca$^{2+}$ levels, with the concentrations of other cations at the luminal side mimicking the vacuolar content. For excised patches $V_{1\%}$ values between $-15$ mV and $-11$ mV were obtained at vacuolar Ca$^{2+}$ levels between 0.1 mM and 0.7 mM (Fig. 4B). This is in a good agreement with a mean vacuolar free Ca$^{2+}$ activity $\approx 0.2$ mM reported by ion-selective microelectrodes (Table 2). Thus, in intact isolated vacuoles, the voltage-dependent activity of the SV channel is most likely controlled by a joint action of major vacuolar cations (K$^+$, Na$^+$, Mg$^{2+}$, Ca$^{2+}$) and pH.

**The SV channel activity at low cytosolic Ca$^{2+}$**

All the results illustrated in Figs 2–4 were obtained at non-physiologically high cytosolic Ca$^{2+}$ concentrations (2 mM). Now, the SV channel voltage-dependent activity was analysed at lower cytosolic Ca$^{2+}$ (0.1–20 $\mu$M), in the presence of physiologically relevant concentrations of major cations and pH at the vacuolar side. The effects of increasing cytosolic Mg$^{2+}$ from 0.5 mM to 1.5 mM were also checked. Because at high positive potentials, huge SV channel-mediated currents from a typical vacuole tend to saturate the amplifier, small vacuolar vesicles with a visible diameter of 5–10 $\mu$m were isolated after achieving the whole vacuole configuration. The advantage of this compared to a standard outside-out configuration, is a larger current magnitude and a higher precision of measurement of the membrane capacitance (range 1–4 pF). This enabled the evaluation of the SV current per unit membrane area. A typical example of the effect of cytosolic Ca$^{2+}$ and Mg$^{2+}$ variation on SV currents is illustrated in Fig. 5A. Both cations increase the SV current measured at high positive potentials. Titration curves for Ca$^{2+}$ at +100 and +180 mV
yielded \( K_d \) values of 1.4\( \pm 0.7 \) \( \mu \)M and 0.7\( \pm 0.1 \) \( \mu \)M, respectively (Fig. 5B), implying that cytosolic Ca\(^{2+}\) binding is essentially voltage-independent. In addition, a detailed analysis of the voltage-dependence at 0.1 \( \mu \)M and 20 \( \mu \)M cytosolic Ca\(^{2+}\) revealed only a small shift in the activation curve at 0.5 mM cytosolic Mg\(^{2+}\), with no shift whatsoever at higher (1.5 mM) Mg\(^{2+}\) (Fig. 5C; Table 3). Furthermore, the slope (determined by the gating charge, \( z \)) of voltage dependence increased only slightly upon the increase of cytosolic Ca\(^{2+}\) from 0.1 \( \mu \)M to 20 \( \mu \)M (Table 3). Altogether, this suggests that the contribution of Ca\(^{2+}\) binding from the cytosolic side to the SV channel voltage gating is insignificant. Increasing the cytosolic Mg\(^{2+}\) concentration caused about a 2-fold stimulation of the SV channels. This effect was comparable at all membrane voltages and cytosolic Ca\(^{2+}\) concentrations (Fig. 5; Table 3). Therefore, for this range of cytosolic free cation concentrations (Ca\(^{2+}\): 0.1–20 \( \mu \)M, Mg\(^{2+}\): 0.5–1.5 mM), divalent cations increase the mean number of voltage-activated SV channels without a substantial alteration of their voltage dependence. One may presume, however, that this does not remain true for higher cytosolic Ca\(^{2+}\). The potential at which 1% of voltage-activated SV channels are open at 20 \( \mu \)M cytosolic Ca\(^{2+}\) is about +48 mV (Fig. 5C). For a comparison, at very high (2 mM) cytosolic Ca\(^{2+}\) and physiologic (0.2 mM) vacuolar Ca\(^{2+}\) (Fig. 4B), the \( V_{1\%} \) value was \( \sim -15 \) mV.

Finally, the voltage-dependent activity of the SV channel was analysed in vacuole-attached patches at high (20 \( \mu \)M) and low (0.1 \( \mu \)M) Ca\(^{2+}\). As the membrane capacitance in these experiments was very low (<1 pF), its precise value could not be determined accurately. Thus, membrane capacitance, yielding the mean number of the open channels per pF. Data are means \( \pm \)SEM (n=4–8 vacuoles). Solid lines are the best fits to the Boltzmann equation with parameter values given in Table 3.

| Cytosolic free Ca\(^{2+}\) and Mg\(^{2+}\) concentrations | Maximum open SV channels number | Gating charge \( (z) \) | Midpoint potential \( (mV) \) |
|----------------------------------------------------------|-------------------------------|----------------|--------|
| 0.1 \( \mu \)M Ca\(^{2+}\)+0.5 mM Mg\(^{2+}\)             | 11\( \pm 2 \)pF               | 1.14\( \pm 0.20 \) | 146\( \pm 8 \) |
| 0.1 \( \mu \)M Ca\(^{2+}\)+1.5 mM Mg\(^{2+}\)             | 16\( \pm 2 \)pF               | 1.32\( \pm 0.18 \) | 134\( \pm 7 \) |
| 20 \( \mu \)M Ca\(^{2+}\)+0.5 mM Mg\(^{2+}\)              | 87\( \pm 8 \)pF               | 1.41\( \pm 0.06 \) | 133\( \pm 4 \) |
| 20 \( \mu \)M Ca\(^{2+}\)+1.5 mM Mg\(^{2+}\)              | 163\( \pm 5 \)pF              | 1.43\( \pm 0.05 \) | 133\( \pm 2 \) |
| Attached                                                  |                               |                |        |
| 0.1 \( \mu \)M Ca\(^{2+}\)+0.5 mM Mg\(^{2+}\)             | 4.2\( \pm 0.5 \)              | 1.18\( \pm 0.06 \) | 136\( \pm 6 \) |
| 20 \( \mu \)M Ca\(^{2+}\)+0.5 mM Mg\(^{2+}\)              | 44\( \pm 1 \)                 | 1.32\( \pm 0.15 \) | 96\( \pm 3 \) |

\( ^a \) Potential values for the attached patches are referred to the command voltage and should be corrected for the resting tonoplast electric potential difference, \( \sim +10 \) mV at these conditions.
the channel density could not be determined either. Instead, the mean number of open SV channels per patch was accessed as a function of membrane potential (Fig. 6; Table 3). The actual electric potential difference across a membrane patch in attached configuration is a sum of the resting trans-tonoplast potential and the command voltage. In a separate set of experiments, using small-tip patch-clamp pipettes filled with 3 M KCl the resting trans-tonoplast potential difference was determined. For bath solutions with 0.1 μM and 20 μM Ca²⁺, resting potentials were +12±3 mV (n=11 vacuoles) and +9±2 mV (n=9), respectively. Such cytosolic-side positive potentials were due to the absence of H⁺-pump activity. In the presence of 2 mM Mg-ATP, respective resting tonoplast potentials for 0.1 μM and 20 μM Ca²⁺ baths became −4±2 mV (n=11) and −12±4 mV (n=11), respectively.

Correcting the membrane voltage in attached patches for a resting potential difference, the parameters of the SV channel voltage dependence in attached and excised (outside-out) patches became identical at low (0.1 μM) cytosolic Ca²⁺ (Table 3). However, in contrast to outside-out patches, increasing the cytosolic Ca²⁺ from 0.1 μM to 20 μM caused a relatively large (40 mV; Table 3) shift of the voltage dependence to more negative potentials. Further on, the 1% voltage threshold (corrected for the resting potential) was about +20 mV for attached patches at 20 μM cytosolic Ca²⁺, compared to the −5 to −25 mV range obtained for vacuolar-attached patches facing very high (2 mM) cytosolic Ca²⁺ (Fig. 4B).

**Discussion**

*Other vacuolar cations desensitize the SV channel to changes in luminal Ca²⁺*

In this study, the free vacuolar concentrations of all major physiological cations were determined (Table 2). To our knowledge, this is the first report of free vacuolar Mg²⁺ concentrations in higher plants. In addition, using two independent approaches, it was found that the mean free vacuolar Ca²⁺ concentration in Beta taproots was ~0.2 mM, an order of magnitude lower than previously reported values for Zea roots and Riccia rhizoids (Felle, 1988). Therefore, a re-evaluation of the modulation of SV channel activity by vacuolar Ca²⁺ under more realistic vacuolar cation concentrations and pH is required.

Our main findings can be summarized as follows: in the presence of cations other than Ca²⁺ at their physiological concentrations and at acidic pH inside the vacuole, the SV channel voltage-dependent activity becomes insensitive to vacuolar Ca²⁺ variation within the physiological range (Fig 4B). Part of this effect is non-specific and related to the screening of the negative charge at the membrane surface, thus reducing vacuolar Ca²⁺ binding at high ionic strength (Pottosin *et al.*, 2005). However, some monovalent cations such as Na⁺ and Cs⁺ appear to exert specific effects on the SV channel voltage dependence when compared to K⁺ (Ivashikina and Hedrich, 2005; Ranf *et al.*, 2008). The Na⁺-induced shift in the SV channel voltage dependence found here is comparable to that reported by Ivashikina and Hedrich (2005) at similar (zero vacuolar Ca²⁺) conditions. However, in a background of more realistic vacuolar Ca²⁺ concentrations (0.5 mM in our case), Na⁺ caused an opposite shift (Fig. 3B). Therefore, accumulation of Na⁺ in the vacuole during salt stress could ameliorate the inhibitory effects of vacuolar Ca²⁺ on the SV channel.

The effect of vacuolar Mg²⁺ was qualitatively similar to that of Ca²⁺, but smaller in magnitude (Pottosin *et al.*, 2004). However, bearing in mind the much higher vacuolar Mg²⁺ concentration compared to that of Ca²⁺ (Table 3), the actual effects of these two ions on the SV channel voltage dependence may be comparable.

In a previous study (Pottosin *et al.*, 2004), a detailed kinetic model of stabilization of the closed states of the SV channel by vacuolar Ca²⁺ and Mg²⁺ was generated. In particular, it was found that up to three Ca²⁺ or Mg²⁺ ions could be bound in one of the closed states: C2. For simplicity, it was proposed that divalent cation binding at this state was non-co-operative and occurred with the same $K_d$ values. In the present work it is demonstrated

![Fig. 6. Slow-vacuolar channel activity in vacuole-attached patches. Bath and pipette solutions are identical, 100 mM KCl (pH 7.4) and free Ca²⁺ and Mg²⁺ concentrations are as indicated at the top of the curves. A macroscopic SV current was divided by the single channel current at each potential, yielding the mean number of open channels per patch (NPO). For comparison, assuming the same channel density as in outside-out patches (Fig. 5), the mean open SV channel number per typical vacule of 50 μm diameter is given at the second y-axis on the right. Data are means ± SEM (n=4). Solid lines are the best fits to the Boltzmann equation with parameter values given in Table 3. Voltages were command potentials for this case; to convert them to the actual tonoplast electric potential difference, the resting potential value (about +10 mV at these conditions, as verified by 3 M KCl-filled microelectrode impalements) should be totalled.](image-url)
that, at acidic pH, the dependence of the activation threshold on vacuolar Ca\(^{2+}\) (reflecting mainly the stabilization of C2-state by Ca\(^{2+}\)) becomes complex, with a distinct plateau in a physiological Ca\(^{2+}\) range (Fig. 2B).

Curiously, protons by themselves do not affect SV channel voltage dependence at zero vacuolar Ca\(^{2+}\). This may be due to the fact that vacuolar protons bind with equal pKs in closed and open states, so not affecting the distribution between states at any voltage. However, H\(^+\) binding obviously affects the vacuolar Ca\(^{2+}\) binding. The effect of acidic pH presented in Fig. 2B may be explained for example, if the protonation of the SV channel protein results in a much higher \(K_d\) for binding of the second and third Ca\(^{2+}\) ions. Therefore, a plateau between 0.1 mM and 1 mM Ca\(^{2+}\) may reflect the saturation of the first Ca\(^{2+}\) ion binding in all states. [At these Ca\(^{2+}\) concentrations, in accordance with \(K_d\) values for a single Ca\(^{2+}\) ion binding from Pottosin et al. (2004), all states (open and two closed: C1 and C2) will contain a bound Ca\(^{2+}\) ion. Hence, further stabilization of closed states and respective voltage threshold shift is only possible with binding of extra Ca\(^{2+}\) ions to the C2-state.] It should be noted though, that the effects of different cations are not additive. Rather, it seems that they compete with Ca\(^{2+}\) binding, thus diminishing the modulation effect of Ca\(^{2+}\) on the SV channel.

Our working hypothesis therefore, is that the existence of diverse modulatory effects exerted by multiple cations in the vacuolar lumen assists in ‘buffering’ the SV channel activity. Consequently, the variation of vacuolar concentration of a single component, even one as powerful as Ca\(^{2+}\), would not result in a large shift in the SV channel voltage dependence.

The SV channel activity at resting trans-tonoplast potentials

In the absence of externally applied voltage and at resting cytosolic Ca\(^{2+}\) (100 nM), on average 0.01 SV channels per patch will be open, as judged by the activation curve shown for vacuole-attached patches (Fig. 6). This is equivalent to 0.023% of the number of channels open at 20 \(\mu\)M cytosolic Ca\(^{2+}\) and high membrane potentials. Assuming the same channel density as in outside-out patches (see Fig. 5C, 1 pF approximately corresponds to a 100 \(\mu\)m2 membrane area), for a typical vacuole of 50 \(\mu\)m diameter or 7855 \(\mu\)m\(^2\) membrane surface area, as an average only 1.5 SV channels per vacuole will be open at any time. At 20 \(\mu\)M cytosolic Ca\(^{2+}\), about 40 SV channels per vacuole will be open at resting potentials (Fig. 6), and this can be taken as an upper estimate (see the dose dependence in Fig. 5B).

The properties of Ca\(^{2+}\) fluxes reported by the MIFE technique, their stimulation by Mg\(^{2+}\) and a strong inhibition by Zn\(^{2+}\), suggests that they are mediated by SV channels. Moreover, the Ca\(^{2+}\) release from isolated beet vacuoles, similar to the SV current, was stimulated by cytosolic Ca\(^{2+}\), reaching a maximum of 2.8±0.6 nmol m\(^{-2}\) s\(^{-1}\) (Fig. 1) at 20 \(\mu\)M cytosolic Ca\(^{2+}\). Assuming a 50 \(\mu\)m vacuole diameter, this equates to a current of 4.3±0.9 pA per vacuole. Comparison of the Ca\(^{2+}\) flux magnitude and the number of open SV channels at 20 \(\mu\)M cytosolic Ca\(^{2+}\), suggests that each SV channel mediates a net Ca\(^{2+}\) current of ~100 FA at the resting trans-tonoplast potential. This value fits remarkably well the theoretical predictions for Beta SV channel: 100–120 FA of the Ca\(^{2+}\) current per channel at zero voltage, weakly sensitive to \(pCa\) for a physiological range of Ca\(^{2+}\) gradients (Gradmann et al., 1997).

In reality, SV-mediated Ca\(^{2+}\) release could be even smaller. Indeed, the resting potential of the isolated vacuoles in our experiments were depolarized (~+10 mV for cytosolic bath with 0.1–20 \(\mu\)M Ca\(^{2+}\) plus 0.66 mM Mg\(^{2+}\)) in the absence of substrates for vacuolar H\(^+\) pumps. The addition of 2 mM Mg-ATP into the bath solutions resulted in more negative membrane potential values, ~4 to ~12 mV. This is comparable to directly measured values for the tonoplast in intact barley roots and leaves, ~10 to ~15 mV and ~0 mV, respectively (Walker et al., 1996; Cuin et al., 2003). An even lower negative value of ~30 mV was reported for Arabidopsis leaves (Miller et al., 2001). Based on the parameters values for the SV voltage dependence (Table 3), a negative shift of membrane potential by 16–21 mV results in a 2–3 times lower SV channel activity. However, a more negative potential implies an increase in a driving force (resting potential minus \(E_Ca\)) for vacuolar Ca\(^{2+}\) release. For 20/200 \(\mu\)M Ca\(^{2+}\) gradient the relative increase in the Ca\(^{2+}\) driving force is significant, from ~20 mV in the absence of ATP to ~41 mV in the presence of 2 mM Mg-ATP (negative sign implies the preferential Ca\(^{2+}\) influx to the cytosol from the vacuole). Bearing in mind that Ca\(^{2+}\) flux under asymmetric conditions is not linear and displays an inward rectification (increase of chord conductance when the potential difference between cytosol and vacuole is made more negative) (Gradmann et al., 1997), one may expect that at resting tonoplast potential Ca\(^{2+}\) flux through an open SV channel will increase more than the increase in driving force, i.e., ~2 times. Therefore, at high (e.g. 20 \(\mu\)M) cytosolic Ca\(^{2+}\) the decrease of the SV channel open probability will be almost compensated by the increase of Ca\(^{2+}\) flux through a single channel. At low cytosolic Ca\(^{2+}\) the relative change of Ca\(^{2+}\) driving force due to a negative shift of resting tonoplast potential will be less significant. Therefore, total Ca\(^{2+}\) release from the vacuole will be reduced in this case, due to a decrease in the mean number of open SV channels.

The cytosolic Ca\(^{2+}\) level of 20 \(\mu\)M considered above may lead to another overestimate of the SV channel activity. Under physiological ionic conditions, half-activation of
the SV channel occurs at 0.7–1.4 μM cytosolic Ca2+ (Fig. 5B), close to \( K_d = 1.5 \) μM defined by Hedrich and Neher (1987). These values are near the peak global free Ca2+ levels reached during plant responses to several abiotic stresses (Ranf et al., 2008). Thus, the overestimation will be approximately 2-fold. For isolated vacuoles, the SV-mediated Ca2+ release will come closer to that measured by the MIFE technique in the absence of added Ca2+: 1 nmol m\(^{-2}\) s\(^{-1}\) or 1.5 pA per vacuole.

Considering therefore both the more negative trans-tonoplast potential and lower cytosolic Ca2+, the whole vacuole population of SV channels would produce a global Ca2+ release into the cytosol of ≤1 pA. Assuming that for a typical cell the vacuole and the cytosol occupy 80% and 20% of the volume, respectively, and that a vacuole has a diameter of 50 μm, cytosolic volume will be ≈16 pl. Therefore, net cytosol-directed Ca2+ current of 1 pA will tend to change the total cytosolic Ca2+ concentration at 35 μM min\(^{-1}\) rate. However, cytosolic Ca2+ is strongly buffered. For plant cells, the estimated buffering capacity \( B_{\text{max}} \) is 0.2–0.5 mM (Trewavas, 1999) whereas the effective dissociation constant \( K_d \) for a cytosolic Ca2+ buffer in different cell types ranges between 0.15 μM and 0.6 μM (Martinez-Serrano et al., 1992; Kuratomi et al., 2003). Assuming a simple Michaelis–Menten behaviour, the relation between total and free cytosolic Ca2+ may be expressed as 1 + \( B_{\text{max}}/(C_{\text{afree}} + K_d) \). Therefore, when free cytosolic Ca2+ approaches the \( K_d \) of about 1 out of 500 Ca2+ ions as an average could be found in the free form. Thus, the SV channel mediated increase of free cytosolic Ca2+ would be ≈70 nM min\(^{-1}\), which is by several orders of magnitude slower than the Ca2+ responses to most abiotic stresses, and still slower than elicitor-induced Ca2+ signals (Ranf et al., 2008). Yet, as discussed below, in intact cells the SV channel reactivity could be locally increased due to the formation of a high Ca2+-microdomain resulting from channel gathering, as well as tight contact with Ca2+-permeable channels located at other membranes.

Thus, SV channels make little, if any contribution to global cytosolic Ca2+ responses, as convincingly demonstrated in a recent study by Ranf et al. (2008). This result is expected and perfectly reasonable in view of the fact that the vacuole is an inexhaustible Ca2+ store. Hence, the analogy with the CICR in animal cells may be not valid, because the latter phenomenon is assigned to a different Ca2+ store, such as the endoplasmic reticulum (ER) that can be readily and reversibly depleted. In contrast to the small-sized ER store, depleting a large vacuole, which can occupy up to 90% of the plant cell volume, will inevitably cause irreversible alterations in the cytosolic Ca2+.

Therefore, although the activation of SV channels by cytosolic Ca2+ provides a positive feedback system, i.e. the more Ca2+ released via the SV channel, the higher the activity of this and neighbouring SV channels, a strict negative SV channel control by physiological factors (see Pottosin and Schönknecht, 2007, for more details) effectively prevents this chain reaction at the whole cell level.

As Ranf et al. (2008) used a cytosolic aequorin reporter, their data set aside the potential role of the SV channel in local Ca2+ signalling, because this could not be detected by their method. Such local Ca2+ signals, the so-called Ca2+ sparks and puffs, have been well studied in animal cells. For instance, in tight junctions of striated muscle, a cross-talk between sarcoplasmic ryanodine receptor channels (RyRs) and voltage-dependent Ca2+ channels of t tubules is initiated. This occurs due to a clustering of RyRs and their positive regulation by cytosolic Ca2+, thereby generating a synchronous response of several RyRs: a Ca2+ spark (Franzini-Armstrong and Protasi, 1997). It should be noted that, with respect to high Ca2+ microdomains, a free Ca2+ concentration at a distance of ≈0.1 μm from the pore entrance of a Ca2+ conducting channel may reach the hundred micromole range (Demuro and Parker, 2006; Rizutto and Pozzan, 2006). Due to its vast area, the tonoplast is often in contact with several intracellular stores, as well as the plasma membrane. Furthermore, tonoplast SV channels are very abundant. Based on the surface density (Fig. 5C), the average distance between neighbouring channels is less than 1 μm. Thus, there should almost certainly be several SV channel copies in the contact zones of the tonoplast with other membranes. Provided the SV channels are clustered so as to increase the cross-activation by Ca2+ ions released from the vacuole into the cytosol, and that at least one channel of each cluster is at a short (≈100 nm) distance from a Ca2+-permeable channel of another membrane (e.g. plasma- or ER membrane), the conditions for Ca2+ spark formation will be met. Therefore, a functionally large vacuole would be split in multiple small circuits, and the vacuolar Ca2+ release would be asynchronous and restricted to local contact zones with other organelles and the plasma membrane. This hypothesis should be addressed in further studies employing such techniques as high-resolution confocal or TIRF microscopy on intact cells (Demuro and Parker, 2006).

In conclusion, a combination of MIFE Ca2+ flux measurements and patch-clamp evaluations of the SV channel activity in isolated vacuoles suggests that only a minor contribution to global Ca2+ signalling is made by SV channel-mediated vacuolar Ca2+ release. Whether the vacuolar SV channels are capable of modulating local Ca2+ responses remains to be elucidated.

**Acknowledgements**

This study was supported by CONACyT project 38181N to IP, VP received the fellowship from the same project. ARC funding to SS is gratefully acknowledged. The authors are indebted to Dr TA Cuin for a critical review of the paper.
References

Allen GJ, Sanders D, Gradmann D. 1998. Calcium–potassium selectivity: kinetic analysis of current–voltage relationships of the open, slowly activating channel in the vacuolar membrane of *Vicia faba* guard-cells. *Planta* 204, 528–541.

Carpaneto A, Cantu AM, Gambale F. 2001. Effects of cytoplasmic Mg$^{2+}$ on slowly activating channels in isolated vacuoles of *Beta vulgaris*. *Planta* 213, 457–468.

Carter C, Pan S, Zouhar J, Avila EL, Girke T, Raikhel NV. 2004. The vegetative vacuole proteome of *Arabidopsis thaliana* reveals predicted and unexpected proteins. *The Plant Cell* 16, 3285–3303.

Chen Z, Newman I, Zhou M, Mendham N, Zhang G, Shabala S. 2005. Screening plants for salt tolerance by measuring K$^+$ flux: a case study for barley. *Plant, Cell and Environment* 28, 1230–1246.

Cuin TA, Miller AJ, Laurie SA, Leigh RA. 2003. Potassium activities in cell compartments of salt-grown barley leaves. *Journal of Experimental Botany* 54, 657–661.

Demuro A, Parker I. 2006. Imaging single-channel calcium microdomains. *Cell Calcium* 40, 413–422.

Felle II. 1988. Cytoplasmic free calcium in *Riccia fluitans* L. and *Zea mays* L.: interaction of Ca$^{2+}$ and pH? *Planta* 176, 248–255.

Franzini-Armstrong C, Protasi F. 1997. Ryanodine receptors of striated muscles: a complex channel capable of multiple interactions. *Physiological Reviews* 77, 699–729.

Gambale F, Cantu AM, Carpaneto A, Keller BU. 1993. Fast and slow activation of voltage-dependent ion channels in radish vacuoles. *Biophysical Journal* 65, 1837–1843.

Gradmann D, Johannes E, Hansen U-P. 1997. Kinetic analysis of Ca$^{2+}$/K$^+$ selectivity of an ion channel by single-binding-site models. *Journal of Membrane Biology* 159, 169–178.

Hedrich R, Barbier-Brygoo H, Felle II, et al. 1988. General mechanisms for solute transport across the tonoplast of plant vacuoles: a patch-clamp survey of ion channels and proton pumps. *Botanica Acta* 101, 7–13.

Hedrich R, Kurkdjian A. 1988. Characterization of an anion-permeable channel from sugar beet vacuoles: effects of inhibitors. *The EMBO Journal* 7, 3661–3666.

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

Iwashikina N, Hedrich R. 2005. K$^+$ currents through SV-type vacuolar channels are sensitive to elevated luminal sodium levels. *The Plant Journal* 41, 606–614.

Kuratomi S, Matsuoka S, Sarai N, Powell T, Noma A. 2003. Involvement of Ca$^{2+}$ buffering and Na$^+$/Ca$^{2+}$ exchange in the positive staircase of contraction in guinea-pig ventricular myocytes. *Pflügers Archiv—European Journal of Physiology* 446, 347–355.

Martinez-Serrano A, Blanco P, Satrustegui J. 1992. Calcium binding to cytosol and calcium extrusion mechanisms in intact synaptosomes and their alterations with aging. *Journal of Biological Chemistry* 267, 4672–4679.

Miller AJ, Cookson SJ, Smith SJ, Wells DM. 2001. The use of microelectrodes to investigate compartmentation and the transport of metabolized inorganic ions in plants. *Journal of Experimental Botany* 52, 541–549.

Newman IA. 2001. Ion transport in roots: measurement of fluxes using ion-selective microelectrodes to characterize transporter function. *Plant, Cell and Environment* 24, 1–14.

Pottosin II, Dobrovinskaya OR, Muñiz J. 2001. Conduction of monovalent and divalent cations in the slow vacuolar channel. *Journal of Membrane Biology* 181, 55–65.

Shabala S. 2004. The vegetative vacuole proteome of *Beta vulgaris* L.: interaction of Ca$^{2+}$ and pH? *Planta* 218, 528–541.

Walker DJ, Leigh RA, Miller AJ. 1996. Potassium homeostasis in vacuolate plant cells. *Proceedings of the National Academy of Sciences, USA* 93, 10510–10514.

Ward JM, Schroeder JL. 1994. Calcium-activated K$^+$ channels and calcium-induced Ca$^{2+}$ release by slow vacuolar ion channels in guard cell vacuoles implicated in the control of stomatal closure. *The Plant Cell* 6, 669–683.

Wherrett T, Ryan PR, Delhaize E, Shabala S. 2005. Effect of aluminium on membrane potential and ion fluxes at the apices of wheat roots. *Functional Plant Biology* 32, 199–208.