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Regulation of the Slow Vacuolar Channel by Luminal Potassium: Role of Surface Charge

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Abstract. Voltage-dependent activation of slow vacuolar (SV) channels has been studied on isolated patches from red beet (*Beta vulgaris* L.) vacuoles. Isoosmotic variation of vacuolar K^+ from 10 to 400 mM in Ca^{2+}-free solutions at the vacuolar side shifted the SV channel activation threshold to more positive voltages. The effect of K^+ could be mimicked by additions of choline or *N*-methyl D-glucamine and could be explained by unspecific screening of the negative surface charge. Fitting the dependence of voltage shift on K^+ concentration to the Gouy-Chapman model yields a surface charge density of 0.36 ± 0.05 e^-/nm^2. Negative surface potential also tended to increase the local concentration of permeable ions (K^+), resulting in anomalously high single-channel conductance, ~200 pS in 10 mM KCl. An increase of ionic strength due to addition of impermeable cations greatly reduced the unitary conductance. Large positive shift of the SV channel voltage dependence, caused by physiological (0.5 mM) free vacuolar Ca^{2+}, was partly ameliorated by increasing luminal K^+. We interpreted these results as follows: K^+ induced a reduction of surface potential, hence i) causing a positive shift of the voltage dependence and ii) a dilution of Ca^{2+} in the membrane vicinity, thus reducing the inhibitory effect of vacuolar Ca^{2+} and causing a negative shift of the SV channel voltage dependence, with a sum of the two shifts being negative.

Key words: Slow vacuolar channel — Voltage gating — Calcium — Potassium — Patch clamp — Vacuole

Introduction

In non-halophyte plants, K^+ is the most abundant cation species and the principal component of cellular osmoticum. In contrast to the cytosolic K^+ level, vacuolar K^+ concentration displays much larger variability. Studies with ion-selective microelectrodes on barley plants revealed vacuolar potassium activity (a_K) of ~120 mM in roots (epidermis or cortex) and ~230 mM in leaves (epidermis or mesophyll), whereas the cytosolic a_K value was ~75 mM for all these tissues. Moreover, K^+ deprivation resulted in a drastic decrease (down to 10 mM) of vacuolar a_K in root cortical cells, whereas cytosolic a_K decreased by only 15% (Walker, Leigh & Miller, 1996). Similarly, in mesophyll cells of salinized plants, cytosolic a_K decreased by ~15%, whereas vacuolar a_K decreased from 235 to 150 mM, due to K^+ replacement by Na^+ (Cuin et al., 2003). Thus, maintenance of a relatively constant K^+ activity in the cytosol of metabolically active cells is incurred at the expense of the vacuolar K^+ pool. Depending on the nutrient availability and metabolic status of the plant, vacuolar K^+ may be substituted by other cations or by non-electrolytes (Leigh, 1997). For instance, at midday, K^+ concentration in the open stomata transiently drops by about 3–fold, and K^+ is replaced by sucrose to balance the decrease in turgor (Talbott & Zeiger, 1996). During closure of stomata in *Commelina communis*, K^+ concentration in guard cells decreased from 448 to 95 mM, whereas a parallel measurement in epidermal cells reports an increase of K^+ from 73 to 448 mM (Penny & Bowling, 1974).

Potassium is accumulated in the vacuole due to K^+/H^+ antiport (via Na^+/H^+ antiporter capable of transporting K^+ ions; Apse, Sottosanto & Blumwald, 2003). Under K^+ starvation conditions, K^+ is transported actively from vacuole to cytosol, possibly via a yet elusive K^+/H^+ symport mechanism (Walker et al., 1996). There are three cation-selective channels in the vacuolar membrane, slow vacuolar (SV), fast vacuolar (FV) and vacuolar potassium (VK), mediating passive K^+ exchange across the tonoplast (*see* Allen & Sanders, 1997, for a

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review). Potassium distribution between the vacuole and the cytosol, therefore, depends on a coordinated function of these channels and transporters. But do vacuolar channels not only mediate but also sense changes in K^+ redistribution across the tonoplast? In a previous study, we have reported substantial effects of K^+ on the voltage-dependent open probability of FV channels (Pottosin & Martinez-Estevez, 2003). In this work we have investigated the effects of luminal K^+ on a dominant tonoplast cation channel of the SV type. The SV channel is ubiquitous in terrestrial plants (Hedrich et al., 1988). Besides monovalent cations (K^+, Na^+), it also conducts divalent ones (Ca^{2+}, Mg^{2+}) with a little preference (Pottosin, Dobrovinskaya & Muñiz, 2001). Due to a huge cytosol-directed Ca^{2+} gradient across the tonoplast, the activity of this channel has to be reduced to a low level, so that Ca^{2+} accumulation in the vacuole, via H^+/Ca^{2+} antiport and Ca^{2+}-pumping (Pittman & Hirschi, 2003) may balance Ca^{2+} release by SV channels. The key factor down-regulating the SV channel activity, is the vacuolar Ca^{2+} itself (Pottosin et al., 2004 and references therein). Therefore, the effects of luminal K^+ have been studied also in combination with those of vacuolar Ca^{2+}.

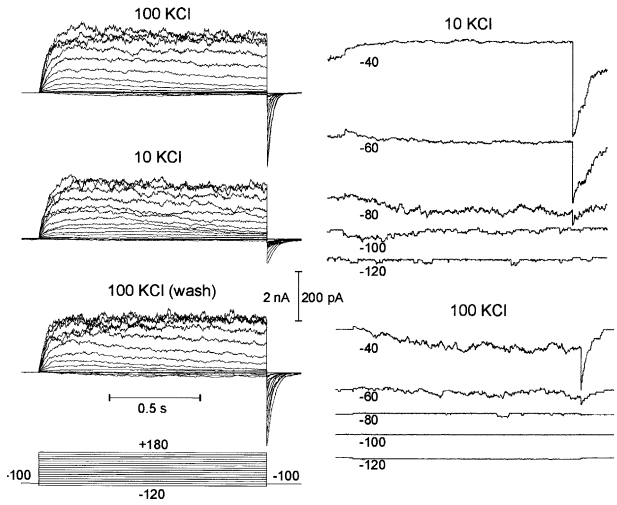

Fig. 1. Effect of luminal K^+ on the voltage-dependent activation of the SV channels in red beet vacuoles at zero vacuolar Ca^{2+}. Left panel presents SV currents in response to a series of voltage steps from −120 to +180 mV, in 20 mV increments, registered on a large inside-out (vacuolar side-out) patch at two different levels of vacuolar K^+, 100 and 10 mM (vacuolar Ca^{2+}~12 nM). Pipette (cytosolic) solution contained 100 mM KCl, 0.5 mM free Ca^{2+}. Records were filtered and sampled at 1 kHz. Voltage difference is defined as cytosol minus vacuole. To demonstrate the effect of K^+ on the activation threshold, current traces for the voltage steps between −120 and −40 mV are amplified 10-fold and display in the right panel.

Materials and Methods

Preparation and Media

Vacuoles were prepared from *Beta vulgaris* taproot slices as described previously (Pottosin et al., 2001). The osmolality of solutions was adjusted by sorbitol to isotonic or slightly hypertonic with respect to the vacuolar sap (range 570–730 mOs) as verified by a cryoscopic osmometer (OSMOMAT 030, Gonotec, Germany). A few released vacuoles were collected by a micropipette (5–10 μl volume) and transferred to the experimental chamber (300 μl volume). Experiments were carried out at room temperature (23–25°C). Solutions contained 0.5 mM free Ca^{2+} at the cytosolic side to activate SV channels (and inhibit the FV ones) and either zero (+2 mM EGTA, free Ca^{2+} ~12 nM) or 0.5 mM free Ca^{2+} at the vacuolar side. Cytosolic and vacuolar pH was adjusted to 7.5 and 6.5 with HEPES and MES (5–15 mM), respectively. The solution at the cytosolic side (normally, pipette solution) contained 100 mM K^+. Vacuolar solutions with 10, 30, 100, and 400 mM K^+ were prepared by addition of the corresponding amount of KCl, taking into account the K^+ introduced by KOH titration of EGTA and HEPES/MES. In some experiments, K^+ at the vacuolar side was partly substituted by choline ([2−hydroxyethyl] trimethyl-ammonium) or NMDG (*N*-methyl-D-glucamine); choline was added as choline chloride, NMDG was titrated by HCl to neutral pH before use. All chemicals were analytical grade (Sigma Chemical Co, St Louis, MO).

Patch-clamp Protocols and Analyses

Patch pipettes were fabricated as described previously (Pottosin et al., 2001). Current measurements were performed using an Axopatch 200A Integrating Patch-Clamp amplifier (Axon Instruments, Foster City, CA). The reference AgCl electrode was connected to the bath via a 3% agar bridge filled with 100 mM KCl. The sign of voltage referred to the cytosolic side, and positive (outward) currents represented an efflux of cations from the cytosol into the vacuole. Liquid junction potentials between reference electrode and solutions containing 10 mM K^+ plus 90 mM choline or $NMDG^+$, measured as described by Ward and Schroeder (1994), were +4.8 ± 0.3 mV or +7.3 ± 0.1 mV (bath positive). Large (C = 0.5–1 pF) inside-out (Hamill et al., 1981) tonoplast patches were examined to test the vacuolar K^+ on the SV current; K^+ concentration was changed by bath perfusion. Due to technical problems in obtaining inside (vacuolar side)-out patches from red beet vacuoles, some outside-out patches were included in the concentration dependence (*see* Fig. 4).

Two basic voltage protocols were applied. To study the voltage-dependent activation of SV channels, voltage was stepped for 1.25 or 3.0 s from an appropriate holding potential to potentials in the range −100 to +200 mV in 20 mV increments. Unitary current-voltage (I/V) relationships at different ionic conditions were obtained by application of 15 ms-long voltage ramps between +200 and −200 mV. Records were filtered at 5 kHz (voltage ramps) or 1 kHz (voltage steps) by 4−pole low-pass Bessel filter (80 dB per decade), digitized using a DigiData 1200 Interface (Axon Instruments), and recorded directly on a hard disk of an IBM-compatible PC at 20 kHz (ramps) or 1 kHz (steps) sampling rate. The command voltage protocols were applied and the analyses carried out using the pClamp 6.0 software package (Axon Instruments). Unitary I/V relations were obtained by subtracting successful ramp records (SV channel is open during the ramp) by leak currents (no channels open during the voltage ramp), *see* Pottosin et al. (2001) for details. Macroscopic time- and voltage-dependent SV currents were measured as the difference between current value at moment t = 0 after application of a voltage step and steady-state current (or peak current, in case of the presence of inactivation, e.g., traces in Fig. 1). To obtain the voltage dependence of SV

channels, first the macroscopic current, $I(V)$, was divided by the single-channel current, $i(V)$, yielding the mean number of open channels at a given potential, NP_o, where N is the number of channels, present in the patch, P_o is voltage-dependent open probability. Due to uncertain values of N, the NP_o values were normalized to maximum value, NP_o (max) for each macropatch, yielding P/P_{max} as a function of membrane voltage. For each ionic condition, P/P_{max} values as a function of voltage were averaged for a number of individual patches and final results were presented as mean ± SD. It has been shown (Pottosin et al., 2004), that the minimal gating scheme for the SV channels is C2-C1-O, where C2 and C1 are two separate closed states, and O is the open state. The voltage-dependent transitions between C2 and C1, and C1 and O are characterized by Boltzmann functions, with different midpoint potential values, V_2 and V_1, respectively, and different gating charge, $z_2 = 2.9$ and $z_1 = 0.7$ elementary charges, so that the voltage-dependent open probability can be expressed as:

$$P_o = \frac{1}{1 + \exp\left(-z_1(V - V_1)\frac{F}{RT}\right) \cdot \left(1 + \exp\left(-z_2(V - V_2)\frac{F}{RT}\right)\right)} \quad (1)$$

At low open probabilities the SV channel mainly resides in C2 and activation is dominated by strongly voltage-dependent processes. The weakly voltage-dependent transition between C1 and O was interfered with on some occasions by inactivation processes, causing large errors in the definition of V_1, and, consequently, in V_2 values. Therefore, to describe the shift of voltage dependence caused by variation of ionic strength, instead of two parameters (V_1, and V_2) we have evaluated a single one ($V_{1\%}$), i.e., the voltage at which 1% of maximal SV channel activity was reached ($P/P_{max} = 0.01$). Dependence of $V_{1\%}$ on ionic strength (vacuolar $[K^+]$) was described by Gouy-Chapman formalism (Latorre, Labarco & Naranjo, 1992):

$$V_{1\%}(C) = V_{1\%(C=\infty)} + \phi_o(C) = V_{1\%(C=\infty)} + (2RT/F) * \ln(X + (X^2+1)^{1/2}) \quad (2)$$

where $V_{1\%\ (C=\infty)}$ is the $V_{1\%}$-potential value at infinite cation (K^+) concentration; ϕ_o (C) (in mV) is the potential at zero distance from the luminal membrane surface as a function of K^+ concentration; $X = 1.36 \cdot \sigma \cdot C^{-1/2}$, σ is surface charge (in e^-/nm^2), and C is concentration (moles/liter); F, R and T have their usual meanings.

Results

Effects of Monovalent Cations at Zero Vacuolar Calcium

Effects of vacuolar K^+ variation on the SV channels were studied first in the virtual absence of Ca^{2+} at the vacuolar side (free Ca^{2+} ~ 12 nM). A typical experiment is presented in Fig. 1. A large inside-(vacuolar side)-out tonoplast patch was initially bathed in symmetric 100 mM KCl, supplemented by 0.5 mM Ca^{2+} at the cytosolic side to activate SV channels (and inhibit FV currents). First openings of SV channels could be detected at potentials around −80 mV at these ionic conditions (expanded current traces, right panel). Substitution of the vacuolar solution to 10 mM KCl resulted in a shift of activation threshold to potentials as low as −120 mV. The reversal potential of time-dependent current shifted to ~ −50 mV (note the change of polarity of currents activated at −60 and −40 mV). The maximal activity, observed at high positive potentials, was not affected, as evidenced by the unchanged amplitude of outward currents. The effect of K^+ decrease on the SV channel voltage dependence was completely reversible; the return of 100 mM KCl solution in the bath restored the initial threshold potential level.

The effects of K^+ variation on single-channel currents and on the open probability have to be separated. Therefore, we applied fast (15 ms) voltage ramps between −200 and +200 mV to obtain current-voltage (I/V) relationships of an open SV channel at different ionic conditions. Due to the presence of 0.5 mM Ca^{2+} at the cytosolic side, the outward (vacuole-directed) current, carried mainly by K^+, was partly suppressed. Reducing cytosolic Ca^{2+} to subnanomolar levels practically abolished the single-channel current rectification (Fig. 2*A*), but the activation threshold at these conditions shifted to very high positive potentials (> +120 mV, *not shown*), thus, high (0.5 mM) cytosolic Ca^{2+} was kept throughout the study. Asymmetric decrease of $[K^+]$ from 100 to 10 mM at the vacuolar side shifted the reversal potential to −50 mV (cytosol more negative), due to cationic selectivity of the SV channel. At voltages above +80 mV, outward currents through the SV channel for 100/100 mM KCl and 100/10 mM KCl cytosol/vacuole gradients were practically indistinguishable, because in both cases, the current was dominated by a flow of K^+ from the cytosolic side (100 mM KCl pipette solution). Curiously, a ten-fold decrease of K^+ at the vacuolar side caused only ~10% decrease of the single-channel conductance, from 222 ± 7 pS to 197 ± 8 pS (evaluated in the voltage range from −75 to −200 mV). The unitary conductance of ~200 pS in low saline (10 mM KCl) is far too high to account for any meaningful pore diameter, as will be shown in the Discussion. This contradiction can be explained, assuming that, due to a fixed negative charge in the pore vicinity, the local K^+ concentration is much higher than in the bulk solution.

If the above proposal is correct, screening of the surface charge by large impermeable cations would reduce the unitary conductance. Results, presented in Fig. 2*B*, corroborate this prediction. When bath (vacuolar) solution (10 mM KCl) was replaced by 10 mM KCl supplemented by either 90 mM choline ($Chol^+$) or *N*-methyl D-glucamine (NMDG) chlorides, the single-channel conductance decreased by about 10-fold. Neither $NMDG^+$ nor $Chol^+$ displayed a measurable permeability in SV channels, as no significant shift of reversal potential was observed.

To quantify the effect of K^+ and organic cations ($NMDG^+$, $Chol^+$) on the SV channel voltage-dependent open probability, we have divided mac-

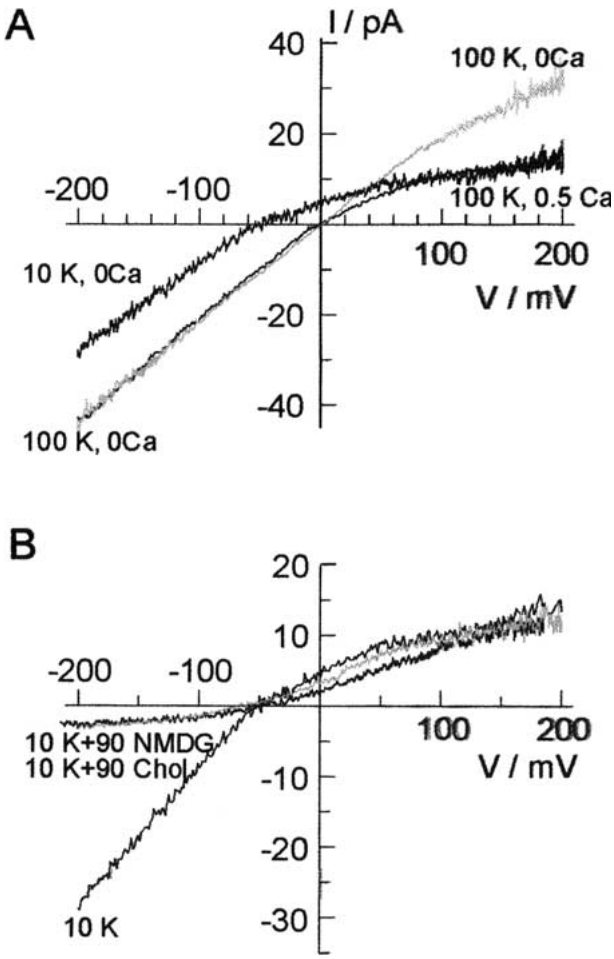

Fig. 2. Rectifying current-voltage relations of the SV single channels at different ionic conditions. (*A*) Continuous current-voltage (*I/V*) relations were obtained by application of ramp-wave (±200 mV, 15 ms) voltage protocols. An almost linear *I/V* relation was obtained in symmetric 100 mM KCl, 0 Ca (+2 mM EGTA, free Ca^{2+} ≤ 12 nM), *grey curve*. Addition of 0.5 mM free Ca^{2+} at the cytosolic side decreased the outward (vacuole-directed) current without any effect on the inward current (*black curve*). Decrease of vacuolar K^+ from 100 to 10 mM shifts the reversal of single-channel current to ~−50 mV, with only 10% decrease of conductance at negative potentials. Data are presented as mean ± SD, with a number of individual *I/V* curves averaged, $n = 15$ (symmetric 100 mM KCl, 0 Ca), $n = 76$ (100 mM KCl +0.5 mM Ca/100 mM KCl, 0 Ca cytosolic/vacuolar sides), $n = 28$ (100 mM KCl +0.5 mM Ca/100 mM KCl + 0 Ca, cytosol/vacuole). (*B*)The curve for 100 mM KCl +0.5 mM Ca^{2+}/10 mM KCl + 0 Ca (cytosol/vacuole) was replotted from *A* (10 K, no error bars) and compared with *I/V* relations, obtained after addition of either 90 mM NMDG-Cl ($n = 18$) or 90 mM Chol-Cl ($n = 9$) at the vacuolar side. The reversal of single-channel current did not change. The unitary conductance of the inward current (voltage range from −75 to −200 mV) decreased to 18 ± 4 pS ($NMDG^+$) or to 22 ± 3 pS ($Chol^+$).

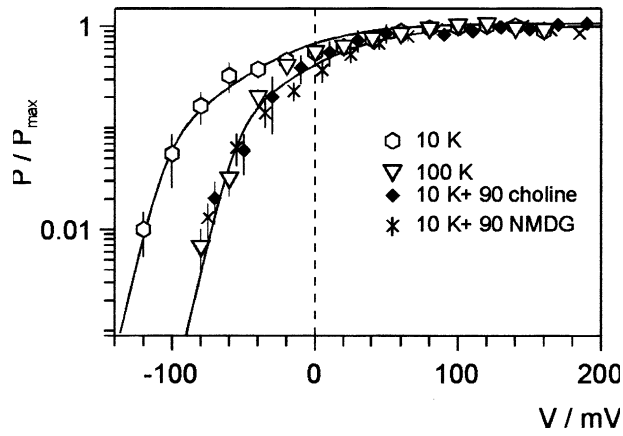

Fig. 3. K^+-induced shift of the SV channel voltage dependence is due to the change of ionic strength. Voltage dependence of SV currents in inside (vacuolar side)-out patches, exposed to either 10 mM KCl ($n = 4$ patches) solution, 100 mM KCl + 90 mM NMDG-Cl ($n = 6$), 10 mM KCl + 90 mM NMDG-Cl ($n = 6$), or 10 mM KCl + 90 mM Chol-Cl ($n = 5$), free vacuolar Ca^{2+} level was ~12 nM. Pipette solution contained 100 mM KC1 and 0.5 mM Ca^{2+}. Macroscopic SV currents in response to a series of voltage steps (as in Fig. 1) were divided by respective single-channel currents at correspondent potentials to obtain the mean number of open SV channels (NP_0, where N is number of channels in the patch, P_0 - open probability) as a function of membrane voltages. NP_0 values were normalized to a maximal value obtained for each patch, and the normalized data (P/P_{max}) for different patches were averaged and presented as mean ± SD. Solid lines are drawn in accordance with Equation (1), with $z_1 = 0.75$ and $z_2 = 2.90$ (fixed), and V_1, V_2 of −21 ± 4, −105 ± 2 mV (10 K) or +11 ± 7, −55 ± 3 mV (other).

roscopic SV currents by respective single-channel currents (*see* Materials and Methods for details). Figure 3 shows the results for four different vacuolar solutions. Increase of vacuolar K^+ from 10 to 100 mM shifted the voltage dependence of the SV channel open probability by ~50 mV to more positive potentials. It could be seen from this plot, that the effect of vacuolar K^+ on the voltage dependence was unspecific and could be mimicked by impermeable cations, i.e., a single curve described the data for 100 mM KCl and 10 mM KCl plus 90 mM $NMDG^+$ or $Chol^+$. Therefore, it is likely that screening of negative surface charge by either permeable (K^+) or impermeable ($NMDG^+$, $Chol^+$) cations was the cause of the voltage-dependence shift. We have analyzed the concentration dependence of the K^+ effect on the SV channel voltage dependence in more detail, testing more patches and two additional vacuolar K^+ concentrations, 30 and 400 mM. The summary of these results is presented in Fig. 4*A*. Increase of vacuolar K^+ caused a parallel shift of the voltage dependence in the region of low voltage probabilities. Therefore, to describe the effect by a single parameter, we have chosen the potential at which 1% of maximal SV channel activity was registered ($V_{1\%}$ Fig. 4*A*, dashed line). The mean values of $V_{1\%}$ have been plotted against vacuolar [K^+] (Fig. 4*B*). The datapoints could be described by a simple Gouy-Chapman formalism (Eq. 2, Materials and Methods), yielding a surface-charge density of 0.36 ± 0.05 e^-/nm^2 and $V_{1\%\ (C=\infty)} = -8 \pm 6$ mV as the upper

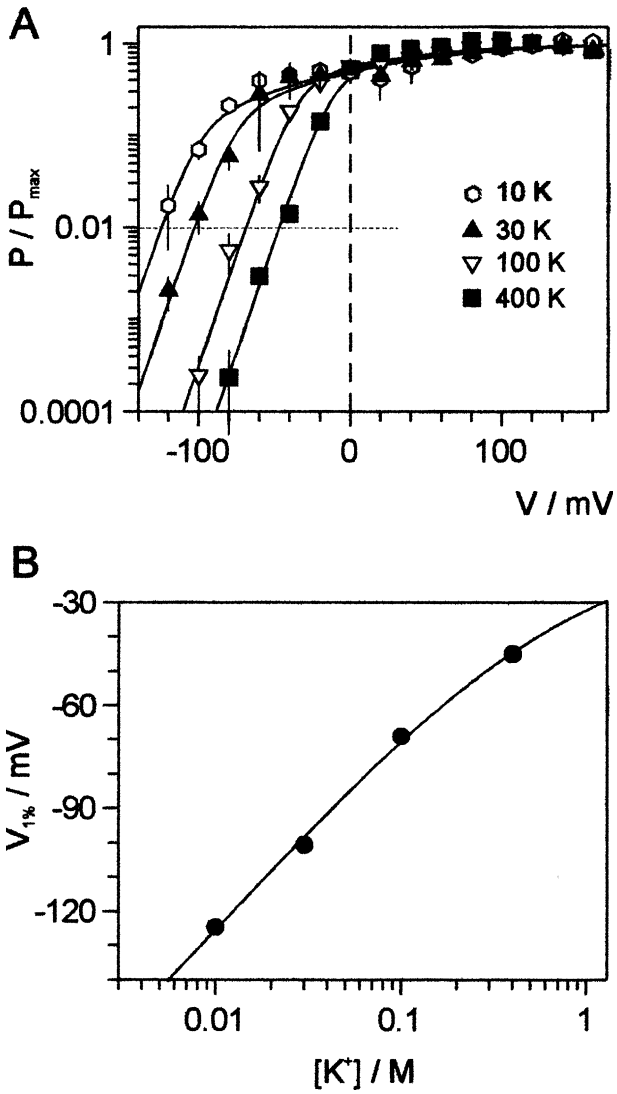

Fig. 4. K^+-induced shift of the SV channel voltage dependence and evaluation of the surface charge. (*A*) Voltage dependence of SV currents in excised patches at different vacuolar K^+ concentrations. Data are presented as mean ± SD with the number of explored patches in parentheses: 10 mM K^+ (n = 7), 30 mM K^+ (n = 4), 100 mM K^+ (n = 14) and 400 mM K^+ (n = 7). Data are adjusted to Eq. 1 (*solid lines*). Dashed line represents a 1% threshold level (intercepts are mean potentials, at which 1% of maximal SV channel activity was observed). (*B*) Voltage, at which 1% of SV channels are active (intercepts from *A*) as a function of vacuolar K^+ concentration. Solid line is a fit to the Gouy-Chapman model (Eq. 2) with a mean surface charge density, $\sigma = 0.36 \pm 0.05$ e^-/nm^2, and threshold voltage at infinite $[K^+]$, $V_1\%_{(c=\infty)} = -8 \pm 6$ mV.

limit. According to this fit, a significant (about −60 mV) surface potential exists at a typical vacuolar K^+ concentration of about 100 mM. This implies an up to 10-fold and 100-fold increase of the local concentration of monovalent and divalent cations, respectively, at the luminal surface of tonoplast. Among divalent cations, vacuolar Ca^{2+} was identified as a principal negative regulator of the SV channel (Pottosin et al., 2004). Therefore, it was important to test vacuolar K^+ effects on the background of physiological vacuolar Ca^{2+} concentration.

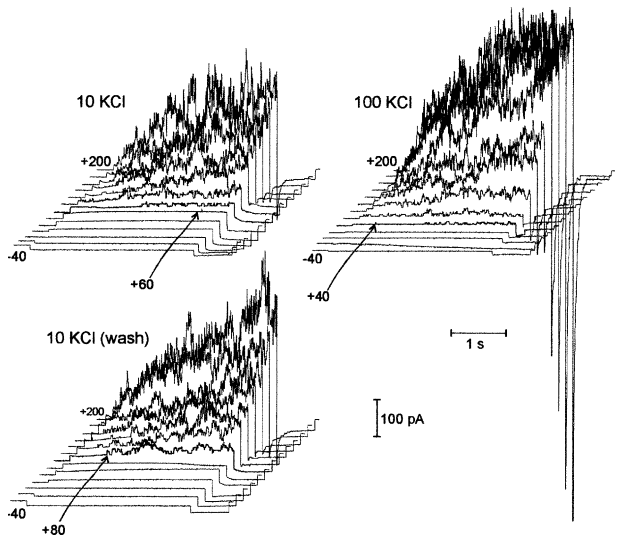

Fig. 5. Vacuolar K^+ effect on the SV-channel voltage-dependent activation is inverted in the presence of Ca^{2+} in the vacuole. Example of original records (sampled at 0.2 kHz) from an inside (vacuolar side)-out patch, subjected to a series of pulses from a holding potential of 0 mV to voltages between −40 and +200 mV in 20 mV increments; after each test pulse the potential was transiently switched to −100 mV, evoking channel deactivation (tail currents). The patch was initially bathed in symmetric 0.5 mM free Ca^{2+}, 10 and 100 mM K^+ at vacuolar and cytosolic sides, respectively. Then, K^+ in the bath (vacuolar side) was changed to 100 mM (100 KCl), and returned to 10 mM (10 KCl, washout). Potentials, at which first SV channel openings were detected, are indicated by arrows.

Combined Effect of Vacuolar K^+ and Ca^{2+} on the SV channel

At 0.5 mM free Ca^{2+} on the vacuolar side, the SV channels were silent at (cytosol) negative voltages and started to activate only at positive ones. As can be seen from the records, presented in Fig. 5, the activation threshold was further modified by the vacuolar K^+ level: an increase of vacuolar K^+ from 10 to 100 mM resulted in the activation of SV channels at lower potentials. The effect was reversed by returning to low-K^+ solution in the bath. Using the same approach as above, we first obtained unitary I/V relations at 10 and 100 mM vacuolar K^+ (Fig. 6*A*). Vacuolar Ca^{2+} reduced the single-channel conductance at negative potentials; the effect was more pronounced at low (10 mM) K^+, *cf.* with Fig. 2*A*. Due to Ca^{2+} permeability of the SV channel, the reversal potential of the single-channel current (∼ −25 mV) deviated significantly from the K^+ equilibrium potential (−55 mV for 100/10 mM K^+ gradient). The macroscopic SV current was divided by single-channel currents to obtain a mean number of open SV channels (NP_o) as a function of voltage.

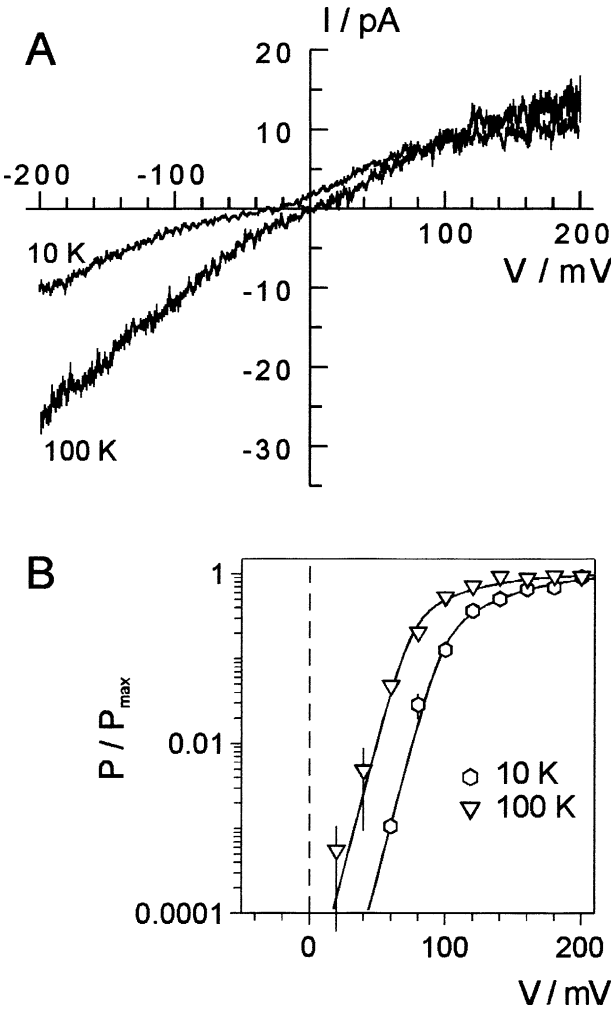

Fig. 6. Vacuolar Ca^{2+} modifies single-channel current-voltage relationships and the effect of vacuolar K^+ on the SV channel voltage dependence. (*A*) Single SV channel current-voltage (*I/V*) relationships in symmetric 100 mM KCl, 0.5 mM Ca^{2+} (*100 K*) and with 10 mM KCI (*10K*) at the vacuolar side. Data are mean ± SD, obtained after averaging of *n* individual *I/V* relationships; $n = 7$ (100/100 mM K^+) and $n = 10$ (100/10 mM K^+ cytosol/ vacuole). (*B*) Voltage dependence at 0.5 mM free vacuolar Ca^{2+} and two concentrations of vacuolar K^+, 100 mM (*100 K*, $n = 4$ patches) and 10 mM (*10 K*, $n = 6$ patches). Solid lines are drawn in accordance with Equation (1), with $z_1 = 0.75$ and $z_2 = 2.90$ (fixed), and V_1, V_2 of +139 ± 6, +99 ± 2 mV (*10 K*) or +109 +6, ±69 ± 3m*V* (*100K*).

Normalized plots of voltage dependence of the open probability at two vacuolar K^+ concentrations are presented in Fig. 6*B*. Increasing vacuolar K^+ from 10 to 100 mM shifted the voltage threshold for activation (at the level of 1% of maximal open probability, $V_{1\%}$) by 25 mV, from +74 to +49 mV. This shift was smaller and in the opposite direction compared to that produced by an equivalent K^+ change in the virtual absence of vacuolar Ca^{2+} (from −125 mV to −69 mV, Fig. 4*B*). Therefore, at physiological vacuolar Ca^{2+}, the increase of luminal K^+ promoted the SV channel opening, rather than impeding it.

Discussion

Surface Charge, Ca^{2+} and Voltage-dependent Activity of SV Channels

This study summarizes the evidence for the presence of a significant negative surface charge at the luminal interface of the tonoplast, in the proximity of SV channels. Screening of surface charge by increasing the ionic strength in the vacuolar compartment caused a positive shift of the SV channel voltage dependence (Fig. 3 and Fig. 4*A*) and decreased the single-channel conductance (Fig. 2*B*). Indeed, unitary conductance of 200 pS for the 10 mM KCl solution (Fig. 2, this study) is anomalously high. A simple calculation, assuming an unrestricted diffusion into the channel pore, cylindrical pore shape and resistivity of 1 kΩ·cm (Hille, 2001), for the SV channel with a pore length of about 2 nm (Dobrovinskaya, Muñiz & Pottosin, 1999) and unitary conductance of 200 pS, yields an average pore diameter of 3.2 nm, above any meaningful limit for a selective channel. However, our estimate of the surface potential (∼ −120 mV at 10 mM KCl, Fig. 4*B*) implies up to a 100-fold increase in local concentration of K^+ in the vicinity of the membrane as compared to the bulk solution, i.e., much lower resistivity and a higher diffusion rate. Shift of the voltage dependence by impermeable cations, $NMDG^+$ or $Chol^+$ (Fig. 3) was accompanied by an approximately 10-fold decrease of single-channel conductance (Fig. 2*B*), and both effects were consistent with a reduction of surface potential by ∼50 mV. Previously, we have shown that negative voltage charge at the cytosolic end of FV channels in the same membrane greatly affected their voltage dependence (Pottosin & Martínez-Estévez, 2003). The contribution of the surface potential from the vacuolar side was less obvious in the case of the FV channels, or at least its possible effect was less pronounced than that on the SV channels. This comparison could support the notion that the large portion of surface charge is retained in the exposed groups of the channel protein rather than entirely associated with negatively charged heads of phospholipids (Hille, 2001). On the other hand, a specific phospholipid microenvironment and/or the relative position of the channels with respect to the membrane surface may underlie the differences in surface potentials sensed by functional groups of different channel proteins.

Strongly voltage-dependent SV channels are situated in the tonoplast, which does not experience large changes of potential difference. However, the voltage dependence of the SV channel could be specifically modulated by several factors. Thus, within a narrow physiologically attainable range of membrane voltages, the activity of SV channels could

be changed greatly. Increase of cytosolic Ca^{2+} and/or Mg^{2+} shifts the SV activation to more negative voltages (Schulz-Lessdorf & Hedrich, 1995; Pottosin et al., 1997; Pei, Ward & Schroeder, 1999; Carpaneto, Cantu & Gambale, 2001). In contrast, vacuolar Ca^{2+} and/or Mg^{2+} shift the voltage dependence to more positive potentials (Pottosin et al., 1997, 2004). Both vacuolar and cytosolic binding sites for divalent cations display a preference for Ca^{2+} over Mg^{2+}. In contrast to Ca^{2+}, there is no such large gradient for Mg^{2+} across the tonoplast (Shaul, 2002). Thus, in the vacuole, the free Ca^{2+} to Mg^{2+} ratio is higher than in the cytosol, and Ca^{2+} becomes a crucial regulator of the SV channel activity.

Therefore we have re-evaluated the effect of ionic strength in the presence of physiological concentrations of Ca^{2+} at the luminal side. There is a single study on higher plant cells (Felle, 1988), defining free vacuolar Ca^{2+} levels, 2.3 mM in *Riccia* rhizoids and 1.5 mM in maize roots (*Zea mays*). Our own study on red beet vacuoles revealed a lower level of free luminal Ca^{2+} (~0.25 mM, unpublished). For the present work we have chosen an intermediate value (0.5 mM). In the presence of 0.5 mM Ca^{2+} at the vacuolar side, the shift of voltage dependence, induced by increasing vacuolar K^+ from 10 to 100 mM, was smaller in magnitude and opposite to that produced by the same K^+ change in the absence of Ca^{2+} (Fig. 6B). Our interpretation of this result is depicted in Fig. 7. Assuming that in the presence of Ca^{2+}, K^+-induced reduction of the surface potential at the vacuolar side is approximately the same as at zero Ca^{2+}, i.e., ~56 mV (Fig. 4*A*)[1].the voltage dependence of the SV channel would be shifted to more *positive* (cytosol minus vacuole) potentials by this extent. However, negative surface potential tends to concentrate Ca^{2+} ions in the proximity of the membrane. Reduction (by absolute value) of surface potential by 56 mV will diminish the local concentration of Ca^{2+} approximately 100-fold. From our previous study (Fig. 6 in Pottosin et al, 2004) it follows that the activation threshold for the SV channel is shifted by

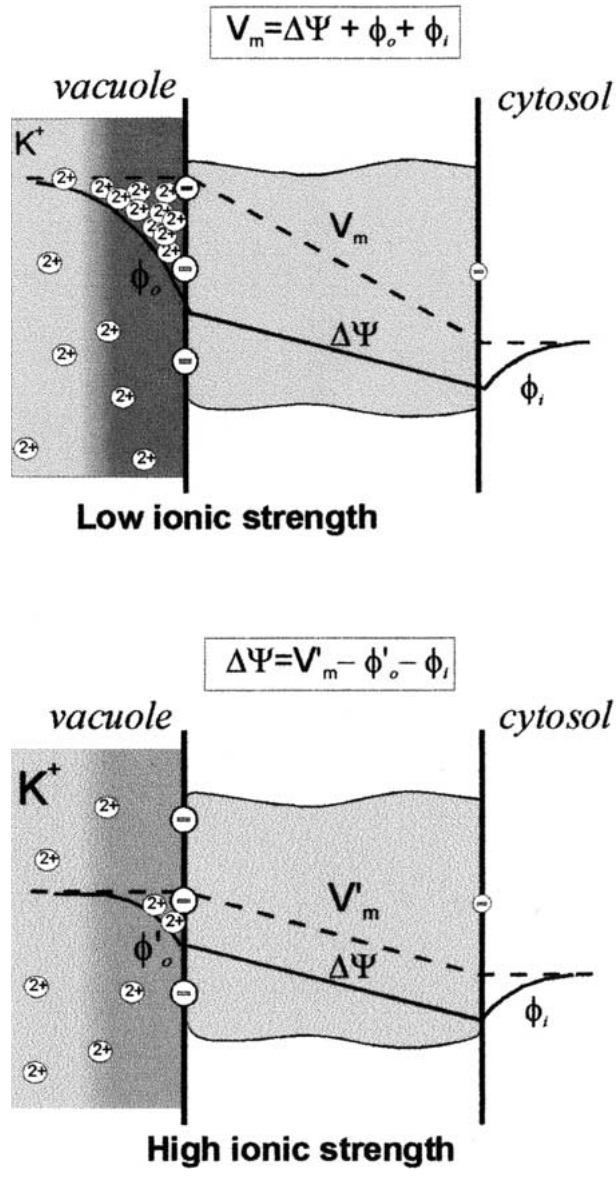

Fig. 7. Reduction of surface potential at the vacuolar side affects the transmembrane voltage difference ($\Delta\psi$) and local Ca^{2+} concentration at the membrane interface. The potential fixed between bulk phases (V_m) is composed of transmembrane potential difference ($\Delta\psi$) and a pair of surface potentials (ϕ_0 and ϕ_i for vacuolar and cytosolic interfaces, respectively). Negative surface potential ϕ_0 tends to concentrate divalent cations (Ca^{2+}) in the vicinity of the membrane. It is supposed that a membrane-embedded ionic channel could sense only the transmembrane voltage difference ($\Delta\psi$) fraction of clamped voltage (V_m). Screening of the surface charge at increased ionic strength (K^+) reduces ϕ_0. To keep the $\Delta\psi$ constant, one requires an equivalent change of clamped potential (V_m); in this particular case, a less vacuolar side-positive (more positive cytosol minus vacuole) potential must be applied to compensate a reduction of negative vacuolar surface potential. Thus, an apparent *positive* shift of the channel's voltage-dependence (open probability vs V_m) results. However, as the SV channel senses vacuolar Ca^{2+}, which shifts the voltage dependence more positive, a dilution of local Ca^{2+}, caused by reduction of negative surface potential, will cause an opposite, *negative* shift of the voltage dependence. The direction net effect on the channel's voltage dependence will depend on the relative strength of partial effects (*see* text for the discussion).

[1] Ca^{2+} affects/reduces the surface potential in two ways: i) screening by bulk Ca^{2+} ions and multivalent cations is far more potent as compared to that of monovalent ones (K^+); ii) partial neutralization of the surface charge due to a specific Ca^{2+} binding to ionizable sites within the so-called Stern layer, with the affinity for Ca^{2+} being 10^1 to 10^2 stronger as compared to alkali metal cations (Latorre et al., 1992; Hille, 2001). However, at a Ca^{2+} concentration of 0.5 mM, its effects on the surface potential are relatively small. As an example, in frog node of Ranvier, Ca^{2+} as a sole cation present, at 0.5 mM concentration reduced the surface potential by only 5 mV (Fig. 20.11 in Hille, 2001). This could be considered an upper limit, because i) the surface charge density in our case is 3 times lower and ii) the effects of Ca^{2+} on the surface charge/potential will be less pronounced on the background of K^+. Therefore, the reduction of the tonoplast surface potential by luminal K^+ increase will be almost unchanged in the presence of 0.5 mM Ca^{2+}

~40 mV per 10-fold increase of free vacuolar Ca^{2+} concentration (in the range 10 μM to 10 mM Ca^{2+}). Thus, a 100-fold decrease of local Ca^{2+} will cause ~80 mV shift of the SV voltage dependence to more *negative* potentials. The algebraic sum of these two shifts is 56–80 = −24 mV, exactly as it was observed in the experiment (Fig. 6*B*).

Physiological Implications of Channels' Modulation by Luminal Cations

Maintenance of ionic homeostasis in plant cells is critical for metabolically active compartments. Under K^+ deficiency, an almost constant cytosolic K^+ level in metabolically active cells is maintained at the expense of vacuolar K^+. When vacuolar K^+ drops to a very low level, thermodynamically its transport from vacuole to cytosol has to be active, probably via K^+/H^+ symport mechanism (Walker et al, 1996). The latter caused the acidification of cytosol at severe K^+ starving. The situation may be even worse (more acidification and futile ATP consumption by the H^+-pump powering coupled transports), if K^+ were to re-enter the vacuole passively through cation channels of the tonoplast. Negative control by low luminal K^+ of the major vacuolar cation channels of SV and FV types (Pottosin & Martínez-Estévez, 2003; this paper) could be a part of the protective mechanism.

Salt stress provides another situation, where cellular K^+ decreases and is replaced by Na^+. However, accumulation of Na^+ in the vacuole, especially in salt-tolerant species like red beet, would be larger in magnitude than the loss of K^+, thus, the total ionic strength in the lumen has to increase. Therefore, one might expect an increase of the SV channel activity under salt stress. This obviously comes in conflict with the observed accumulation of Na^+ into the vacuole against the electrochemical gradient for this ion and maintenance of high vacuole: cytosol Na^+ ratio. Previously, we have proposed that an increase of polyamine content in Na^+-stressed cells could reduce the leak of cations through non-selective tonoplast channels, FV and SV (Pottosin et al., 2003). Up-regulation of Ca^{2+}/H^+ antiport by CAX1 (Cheng et al., 2004) might be another plausible possibility, as accumulation of Ca^{2+} in the vacuole strongly inhibits both FV and SV channels (Tikhonova et al., 1997; Pottosin et al., 1997, 2004). Finally, a recent study on the vacuoles from a salt-sensitive species (*Arabidopsis thaliana*) has shown that K^+ and Na^+ are not equivalent in their action on the SV channels, and that a replacement of luminal K^+ by Na^+ caused an ~40 mV shift (at zero vacuolar Ca^{2+}) of the SV activation threshold to non-physiological positive potentials, thus preventing Na^+ leak from vacuole via SV channels (Ivashikina & Hedrich, 2005). It would be interesting, therefore, to test whether luminal Na^+ exerts a similar specific effect on SV channels of salt-tolerant species (e.g. *Beta vulgaris*) at physiological Ca^{2+} range.

Sensing of vacuolar ionic (K^+) content by tonoplast channels might be important for stomata function. Flux measurements in response to the best studied "closing" signal ABA revealed that the release of solute from vacuole is always terminated after a loss of a constant quantity of tracer; variation of ABA concentration could only modify the time course of this transient (MacRobbie, 1995). This was interpreted as evidence for participation of some stretch-activated channels in the vacuolar solute release (MacRobbie, 1998). However, cessation of the solute release might be simply due to a decreased activity of tonoplast cation (FV and SV) channels at the lower limits of vacuolar K^+ (Pottosin & Martínez-Estévez, 2003; this study). During stomata closure, ABA activates multiple Ca^{2+} fluxes, triggering tracer release. One of the components of the release, with a high cytosolic Ca^{2+} threshold, was tentatively attributed to SV channels (MacRobbie, 2000). The SV channels are Ca^{2+}-permeable and Ca^{2+}-activated channels, hence, could be regulated by Ca^{2+} in a feedforward manner: the more Ca^{2+} released in cytosol, the higher the SV channel activity. Reducing the SV channel open probability by low vacuolar K^+ might interfere with this auto-activating mechanism, i.e., does not allow cytosolic Ca^{2+} elevation beyond the threshold for the SV channels activation. Finally, concentration-dependent regulation of major tonoplast cation channels by monovalent cations (primarily by K^+) present in the vacuolar lumen may have a more universal role. This mechanism could operate similarly to a safety valve, preventing over-accumulation of K^+, hence, controlling volume and turgor pressure in a variety of plant cells.

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