Redox-dependent modulation of the carrot SV channel by cytosolic pH

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Abstract Currents mediated by a slow vacuolar (SV) channel were recorded and characterized in vacuoles from cultured carrot cells. The carrot channel shows the typical functional characteristics reported for channels of the SV category previously identified in other plants, i.e., slow voltage-dependent activation kinetics, current activation favoured by cytosolic calcium and permeability to different monovalent cations. The carrot channel is strongly activated by cytosolic reducing agents (such as dithiothreitol, DTT, and glutathione, GSH) and has a peculiar dependence on cytosolic pH, which, in turn, is affected by the concentration of cytosolic reducing agents. Specifically, in 1 mM DTT or GSH the channel displayed a maximum conductance at neutral pH. The normalized conductance did not depend significantly on DTT concentration at acidic pH, while at alkaline pH the attenuation of the normalized conductance declines with increasing DTT concentration. Our results suggest two pH-titratable groups within the carrot SV channel, one of these depending on cysteine residues exposed to the cytosolic side of the vacuole.

Keywords: Plant vacuole; SV channel; Cytosolic pH; Redox

1. Introduction

The slow vacuolar (SV) channel has been identified in the tonoplast of all plant species and tissues investigated so far, thereby displaying well-conserved properties. It has been suggested that the SV channel is a calcium-induced calcium-release channel [1], activated by cytosolic calcium and positive *trans*-membrane potentials [2–6], and modulated by a variety of physiological parameters such as cytoplasmic and luminal pHs [7], vacuolar calcium concentration [8,9], as well as reducing/oxidizing agents [10–12]. Actually, the well-conserved basic properties displayed by SV channels from various sources do not help to hypothesize differential roles for this channel in the different conditions experienced in a large variety of tissues and plant species.

The redox potential of the cytosol modulates many cellular functions in plants [13], affecting also cytoplasmic pH [14]; recently, it has become evident that reducing and oxidizing agents are potent modulators of plant ion channels (for review see [15]). In this study, we investigate the effects of pH_{cyt} and reducing agents focusing on their possible interaction in carrot SV channel modulation. We also take advantage of the possibility to patch vacuoles from both cultured carrot cells and carrot roots.

2. Materials and methods

2.1. Vacuole isolation

Vacuoles were obtained from carrot cell cultures in a two-step procedure: first, suspension cells were subjected to an enzymatic treatment using 0.8% cellulase (Onozuka) and 0.08% pectolyase (Sigma) dissolved in 1 mM $CaCl_2$, 500 mM D-sorbitol, 0.5 % (w/v) polyvinylpyrrolidone (PVP-10), 0.5% (w/v) BSA and 5 mM MES, pH 5.5. After incubation for 45 min at 30 °C, resulting protoplasts were washed two times and resuspended in standard bath solution. Vacuoles were released into the recording chamber by hyposmotic shock treatment of protoplasts in 100 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT and 5 mM Tris–MES, pH 7.5, adjusted to $\Pi = 300$ mOsm with D-sorbitol. After settling of the vacuoles, hypotonic solution was carefully replaced by standard bath solution. Instead, vacuoles from carrot roots were readily extruded into the recording chamber by gently slicing the root cortex tissue into the standard bath solution.

2.2. Patch-clamp recordings

Patch-clamp experiments were performed on isolated vacuoles using the whole-vacuole and excised cytosolic side-out patch configurations. The latter configuration was frequently used in experiments requiring a fast perfusion of the vacuoles with different bath solutions. Unless otherwise indicated, experiments were performed on cultured carrot cells. The diameter of patched vacuoles typically ranged from 10 to 15 μm. Standard ionic solutions were in the pipette: 200 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 MES–Tris, pH 6.0; in the bath: 100 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM DTT and 10 HEPES–Tris, pH 7.0. The osmolarity of the bath and pipette solution was adjusted to 720 and 670 mOsm, respectively, by the addition of D-sorbitol. According to the redox milieu investigated, vacuoles were kept in the same conditions, from the release in the hypotonic solution, then in the bath, and also during perfusion. The bathing medium surrounding the tonoplast was exchanged using a "fast perfusion system" based on five perfusion pipettes (with a diameter in the order of 30 μm), filled with the standard bath solution (control) and the solutions to be investigated [11]. One of the pipettes was manually positioned by means of a hydraulic manipulator in front of the vacuole within a fews [12]. A peristaltic pump, together with a gravity driven "slow perfusion system", allowed smooth and slower (~1 min) exchange of the whole bath solution. Ionic currents were recorded with a List EPC7 current–voltage amplifier. Data were digitized using a 16 bit Instrutech A/D/A board (Instrutech, Elmont, NY, USA), interfaced to a MacIntosh PC, which generated the voltage stimulation protocol and archived the current response. Patch pipettes were pulled from thin-walled borosilicate glass (Clark Electrochemical

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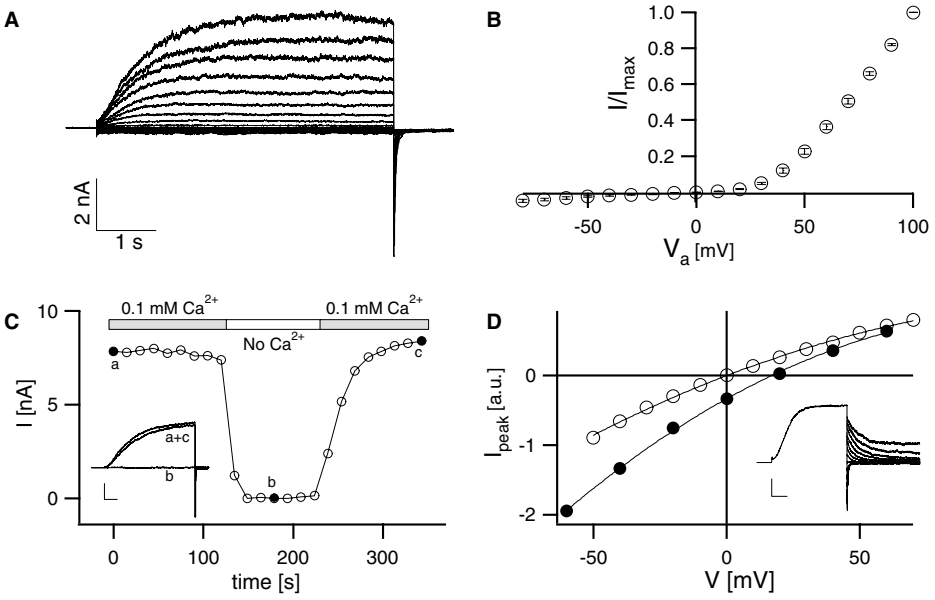

Fig. 1. Macroscopic Slow Vacuolar currents in the tonoplast of cultured carrot cells. (A) SV currents elicited by a series of voltage steps ranging from +100 to −80 mV in 10 mV steps. Holding and tail potentials were at 0 and −50 mV, respectively. (B) SV current–voltage characteristic, obtained by plotting the mean value of the steady state current versus the applied membrane potential. (C) Macroscopic steady-state currents measured in the presence of 0.1 mM $CaCl_2$ (control/recovery) and in the absence of calcium (No Ca^{2+}, corresponding to a free calcium concentration of about 20 nM) are plotted as a function of time. Applied potential: +80 mV. The three filled circles correspond to the three curves displayed in the inset (scale bars 1 s/3 nA). (D) Instantaneous tail currents plotted as a function of the tail potential. Empty circles refer to tail currents recorded in symmetric ionic conditions (see inset), the filled circles represent the instantaneous tail currents obtained in the same vacuole in asymmetric conditions. The interpolation of the instantaneous tail current gave a reversal potential $V_{Rev} \approx 0$ mV and +17.8 mV in symmetric and asymmetric conditions, respectively. Inset: SV currents were activated by pulses to +100 mV, followed by tail potentials ranging from −50 to +70 mV in 10 mV increments; symmetric ionic solutions (i.e., standard ionic solutions with the exception of symmetric 100 mM KCl); scale bars 1 s/0.5 nA.

Instruments, Pangbourne, Reading, UK). The voltage convention is that proposed by Bertl et al. [16].

3. Results

Fig. 1A shows typical outward currents mediated by a time- and voltage-dependent slowly activating channel in the tonoplast of cultured carrot cells. The current–voltage characteristics of the channel displaying steep voltage-dependent activation summarized in Fig. 1B prompted us to investigate the behaviour of the channel as a function of cytosolic calcium concentrations, in order to verify whether these currents are possibly mediated by a SV type channel [2]. Indeed, the carrot currents were efficiently modulated by cytosolic calcium. Fig. 1C illustrates the time course of the current steady state at low and large Ca^{2+} concentrations; an example of the reversible decrease of the slow vacuolar current when Ca^{2+}_{cyt} was reduced from 0.1 mM (control/recovery) to about 20 nM (No Ca^{2+}) is given in the inset of Fig. 1C.

Moreover, using a tail current protocol we verified that the slowly activating currents are mediated by a cation selective channel. The peak of the tail currents, after a pre-pulse to +100 mV, is plotted (Fig. 1D) against the tail voltage in symmetrical (empty circles) and asymmetrical (filled circles) potassium solutions (K^+_{cyt}/K^+_{vac} 100/100 and 100/200, respectively). The fact that the reversal voltage obtained in asymmetric conditions, $V_{Rev} = 17.8 \pm 0.7$ mV, is closer to the Nernst potential for potassium ($V_{Nernst} = +15.6$ mV) than to the one for chloride ($V_{Nernst} = -14.9$ mV) indicates that the channel is highly cation selective.

In order to extend further the characterization of the channel, we investigated its permeability to different monovalent cations (summarized in Table 1). Substituting the external potassium with an equimolar solution of a different monovalent cation, we obtained the following permeation sequence: $K^+ > Rb^+ > Cs^+ > NH_4^+ \gg TMA^+ \approx TEA^+$. Interestingly, reversal voltages derived from tail protocols like those shown in Fig. 1D, gave the following selectivity sequence: $K^+ \approx NH_4^+ > Rb^+ > Cs^+ \gg TMA^+ \approx TEA^+$ (see Table 1). From a comparison of the permeation and selectivity sequences, it is interesting to note that the channel displays a high selectivity (almost comparable to that of potassium) for ammonium, which nevertheless displays a much lower permeation rate through the pore. This is possibly due to a high affinity of this ion for some binding sites in the permeation pathway. These measurements suggest that the slowly acti-

Table 1
Channel selectivity to different monovalent cations derived from reversal potentials

Ion	Ionic radius (Å)	V_{Rev} (mV)	P_{ion}/P_{K+}	N
K^+	1.33	$+17.8 \pm 0.7$	1	7
Rb^+	1.48	$+24.6 \pm 0.4$	0.75	5
Li^+	0.68	$+26.4 \pm 0.8$	0.70	4
Cs^+	1.67	$+35.1 \pm 0.5$	0.49	4
NH_4^+	1.5	$+17.2 \pm 0.6$	~1	4
TMA^+	2.8	≫17.8	0	3
TEA^+	3.7	≫17.8	0	3

Channel selectivity (P_{ion}/P_{K+}) derived, according to the Goldmann–Hodgkin–Katz equation [28], on the basis of the corresponding reversal potential V_{Rev}, for the various monovalent cations investigated in this paper. N is the number of experiments performed.

vating current in carrot vacuoles is mediated by a SV type channel with selectivity properties similar to those reported for other SV channels [2,3,5,17,18] and with a large pore size (i.e., $\geqslant 4$ Å), comparable to that already proposed for SV channels in other plants [18,19].

Studying the effect of cytosolic pH, we verified that in accordance with other studies [7] the current decreases with cytosolic acidification from pH 7 to pH 6 (Fig. 2A, top). Interestingly, we found that also alkalinization of the cytosol from pH 7 to pH 8 decreased channel activity (Fig. 2A, bottom). Replacing DTT in the standard bath solution with glutathione (GSH = 1 mM; Fig. 2B), one of the most important reducing agents in plant cells, this current reduction at alkaline pH was even more pronounced. In Fig. 2C, the mean value of the stationary current level (normalized with respect to the current at pH 7) is plotted against cytosolic pH. Data follow a bell-shaped function with a maximum activity at around pH 7.0. In the voltage range investigated, between 40 and 100 mV, this effect does not depend on the membrane potential (data not shown). For comparison between cultured cells and carrot plant tissues, measurements were also performed on vacuoles from carrot root cortex cells in the presence of 1 mM DTT leading to comparable results (diamonds in Fig. 2C). As it has been demonstrated that Tris inhibits the SV channel from red beet vacuoles [19], we verified in control experiments with KOH-adjusted solutions that the carrot current decrease at alkaline pH is not due to the higher Tris concentration in the bath solution (data not shown). Based on the results shown above, suggesting a differential influence of reducing agents DTT and GSH on pH modulation of the current, experiments were also done in the presence of higher (10 mM) and lower (0.1 mM) DTT concentrations. While variation of the DTT concentration yielded comparable normalized currents in the acidic pH range, it significantly affected the sensitivity of the SV channel towards alkaline pH. In 0.1 mM DTT, the normalized current decrease at alkaline pH was higher compared to 1 mM DTT (Fig. 2C). Instead, at a high DTT concentration (10 mM) SV current decreased to a lower extent at pH 8.0 and even increased slightly at pH 7.5 (Fig. 2C), shifting the current maximum to more alkaline pH values.

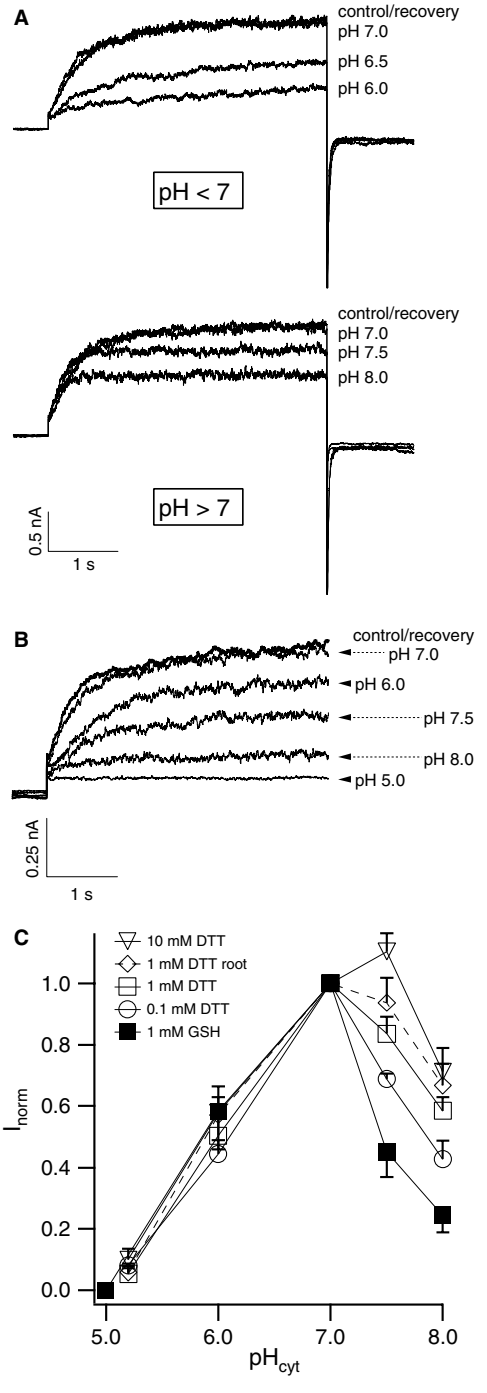

Fig. 2. Redox-dependent modulation of SV currents by cytosolic pH. (A) Representative recordings of macroscopic currents from a large cytoplasmic side-out excised patch. Currents were elicited by a voltage step to +80 mV; holding and tail potentials were at 0 and −80 mV, respectively. Starting from pH 7.0 (control), the cytosolic face of the patch was consecutively perfused with solutions of more acidic (top) or alkaline (bottom) pH values, then returning to pH 7.0 (recovery). Every trace represents the mean of at least 3 traces. All solutions contained 1 mM DTT. (B) Representative recordings of macroscopic excised patch currents at different cytosolic pH values in the presence of 1 mM glutathione. Currents were elicited by a voltage step to +80 mV, holding potential was at 0 mV. Every trace represents the mean of at least 3 traces. (C) Steady state currents recorded (upon a voltage step to +80 mV) at different pH_{cyt} values in the presence of 0.1, 1 and 10 mM DTT (open symbols) or 1 mM glutathione (GSH; closed squares) were normalized to the current at pH 7.0 ($I_{norm} = I/I_{pH7.0}$) and plotted versus cytosolic pH. Currents derived from carrot root cortex vacuoles in the presence of 1 mM DTT are indicated by open diamonds and dashed line. Data points represent means of 3–7 independent experiments, error bars represent SEM.

The unitary conductance (γ) of the SV channel was determined in membrane patches obtained from carrot root vacuoles (Fig. 3A and B). After a prepulse to −50 mV followed by the ramp protocol shown in Fig. 3A, it is possible to record traces mediated by a few single channels (Fig. 3A). In control conditions (pH 7.0), close to the SV reversal potential (i.e., in the voltage range between +10 and +30 mV), we estimated $\gamma = 95 \pm 4$ pS (mean slope ± standard deviation of the slope) in standard bath solution (Fig. 3B). Channel conductance at pH 8.0 perfectly matched this value at small voltages ($\gamma = 94 \pm 5$ pS), displaying only a small deviation at more positive membrane potentials (i.e., >+60 mV, see legend and Fig. 3B). However, this difference cannot account for the macroscopic current decrease at pH 8.0 illustrated in the inset of Fig. 3B as well as in Fig. 2C.

As one important difference between our experiments and those by other authors [7] may possibly be the presence of

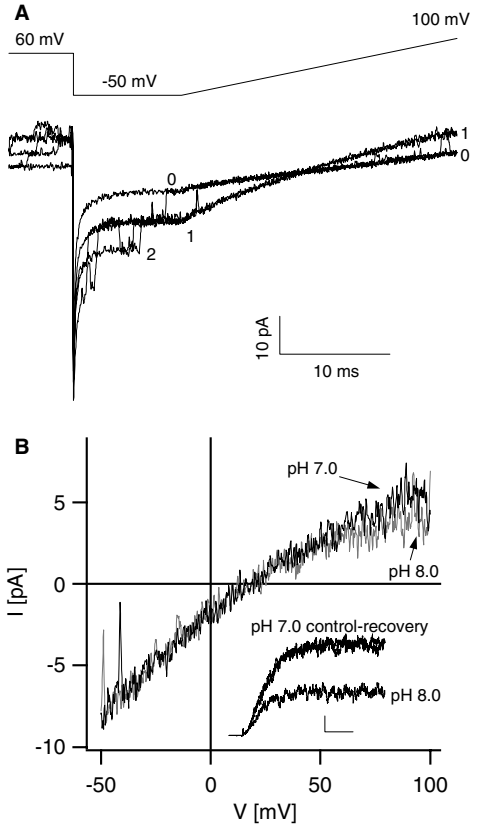

Fig. 3. SV single channel recordings in vacuoles from the carrot root cortex. (A) Single channels were recorded from a cytoplasmic side-out excised patch by applying the voltage protocol shown at the top. Note the pre-ramp potential (at −50 mV), applied in order to isolate single channel transitions. Numbers 0, 1, 2 refer to the number of open channels recorded. Standard bath solution, pH 7.0. (B) An example of two single channel traces recorded during the application of the voltage ramp at pH 7.0 (black) and pH 8.0 (grey), after leakage subtraction. Both traces intersect the voltage axis at $+18 \pm 2$ mV. The slope conductance around this reversal voltage (i.e., from 10 to 30 mV range) was 95 ± 4 and 94 ± 5 pS at pH 7.0 and pH 8.0, respectively. The chord conductance at +80 mV (evaluated fitting the experimental data between +60 and +100 mV with the equation $\gamma = I/(V - V_{Rev})$, with $V_{Rev} = +18$ mV) was 74 ± 1 pS and 56 ± 1 pS at pH 7.0 and pH 8.0, respectively. These values correspond to a decrease of the single channel conductance of 24% at pH 8.0. Inset: macroscopic currents from the same cytoplasmic side-out excised patch showing a reversible reduction of 52% at pH 8.0. Currents were elicited by a voltage step to +80 mV; holding potential was at 0 mV. Scale bars 0.2 s/50 pA.

DTT in the bath solution, we performed measurements in the absence of this reducing agent. Unfortunately, it was not possible in our experimental conditions to remove DTT during the preparation of the vacuoles because the decrease of current amplitude and the lower stability of patches did not allow to perform reliable measurements. Therefore, we decided to work with DTT in the bath and to perfuse the vacuole (excised cytoplasmic side-out patch) with solutions of different pH omitting DTT either for short or longer periods of time. Upon fast removal of DTT, a decrease of the SV current amplitude was apparent (Fig. 4A); this decrease presented high variability of both speed and extent, i.e. within the first minute ($n = 25$ different vacuoles tested) 32% of excised patches were not sensitive (decrease < 5%), 40% were sensitive (5% < decrease < 50%), 28% were very sensitive (decrease > 50%), without altering the pH dependence. Prolonged absence of DTT from the bath solution resulted in a current reduction to a very low level (in Fig. 4A: tenfold reduction compared to DTT conditions). Comparing the effects of alkaline pH, the SV channel apparently loses some of its sensitivity after long-term absence of DTT (Fig. 4A), which might simply be due to a lower signal-to-noise ratio and current stability at these small amplitudes.

The susceptibility of SV currents and their pH modulation towards reducing agents strongly suggested cysteine residues within the channel protein to be exposed to the cytosolic side of the tonoplast and possibly involved in the modulation by cytosolic pH. To support this hypothesis, we tested the effect of the SH-group modifying agent phenylarsine oxide (PAO) on SV currents. PAO is an agent known to react specifically with closely spaced sulfhydryl groups in proteins forming cyclic adducts [20]. As shown in Fig. 4B, inhibition of SV currents upon addition of PAO (100 μM) in the bath solution is rapid and complete. Note that recovery of currents is accomplished only in the presence of DTT, which is in line with previous findings that ring formation by PAO can be reversed by thiols like DTT [21,22]. In experiments applying 1 or 10 μM PAO, current reduction was smaller, recovered partly already in control solution in the absence of DTT and almost completely in control solution containing 1 mM DTT (data not shown).

4. Discussion

We have identified a SV type slow-activating, calcium- and voltage-dependent channel, highly selective for cations in the tonoplast of cultured cells and root cortex cells of *Daucus carota*.

We demonstrate that this channel presents a maximum of current conductance as a function of cytosolic pH. This maximum is typically observed at neutral pH and is affected by the redox state of the channel being shifted towards more basic pH values at elevated concentrations of the reducing agent DTT. Interestingly, only the alkaline branch of the current-pH curve responds to variations in DTT concentration. In the presence of physiological reducing compounds, like GSH, the pH dependence displays the same peculiar characteristics observed in DTT. These findings suggest the existence of at least two pH titrable groups within the channel protein, one of these being controlled by cysteine residues sensitive to the action of reducing agents. Since at pH 8.0, the conductance of SV single channels is not sufficiently altered and preliminary results derived from macroscopic currents suggest that also the open probability of the SV channel does not change appreciably (data not shown), we propose the mechanism of SV modulation by alkaline pH to be based mainly on a reduced recruitment of active channels.

Regarding the behaviour of the carrot SV channel at alkaline pH, our results diverge from those obtained on the sugar beet channel showing increasing activity in this pH range [7]. This may be due to differences in plant species or working conditions (primarily, absence of reducing agents in the bath solution). As we confirmed our results also in the absence of reducing agents, the redox-dependent pH behaviour described in this work might indeed be species-specific for carrot.

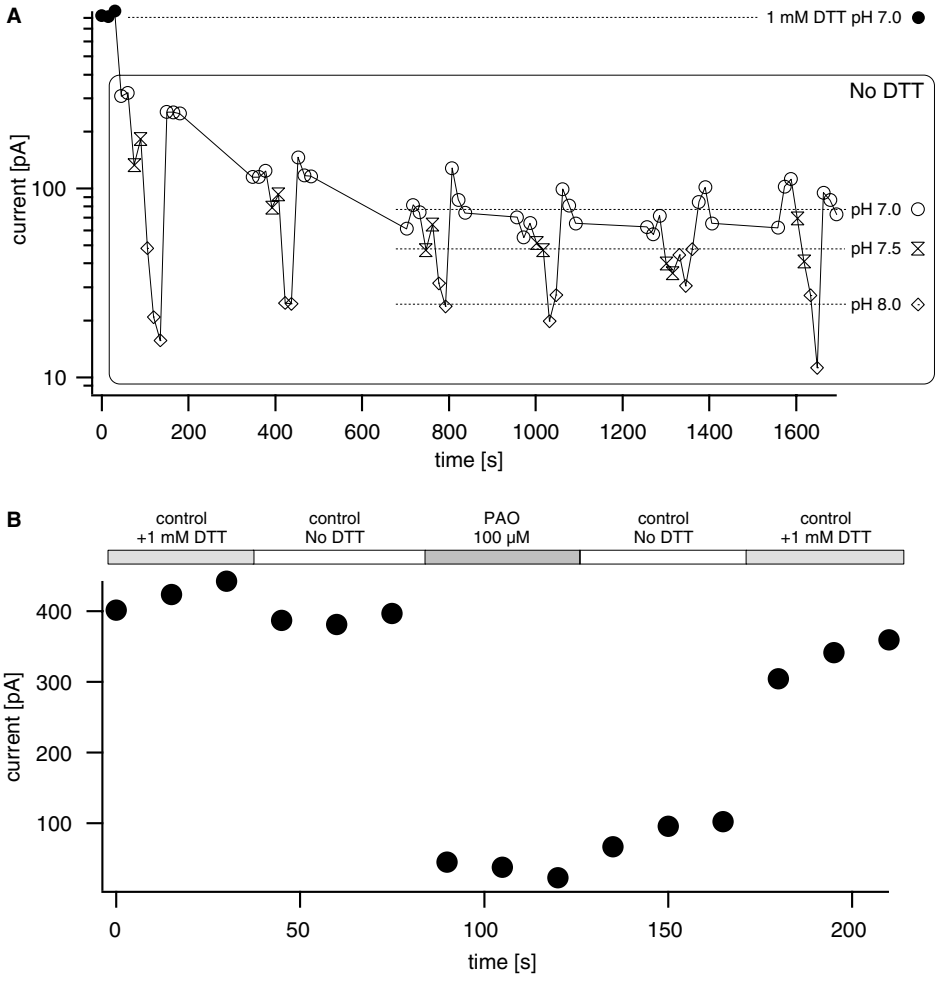

Fig. 4. The carrot SV channel exposes cysteine residues to the cytoplasmic side of the tonoplast. (A) Semilogarithmic plot of the time course of SV currents (from carrot root vacuoles) during prolonged absence of DTT in the bath solution. Starting from control conditions (+1 mM DTT; filled circles), the excised patch was continuously perfused with control solution not containing DTT (open circles). At intervals of about 5 min, currents (elicited by a voltage step to +80 mV) were recorded at neutral and alkaline pH values in the absence of DTT. (B) Macroscopic steady state currents (from carrot root vacuoles) measured in the presence of DTT (control +1 mM DTT), in the absence of DTT (control No DTT) and in the presence of phenylarsine oxide (PAO 100 μM) are plotted as a function of time. Applied potential: +80 mV. Equivalent results were also obtained in vacuoles from cultured carrot cells.

Furthermore, reducing agents are crucial to preserve functionality of the carrot SV channel, as the current amplitude decreases dramatically in their absence, confirming previous findings in sugar beet and *Posidonia oceanica* [11]. Application of the oxidant chloramine T, on the other hand, inactivated the channel irreversibly (data not shown; [11]). Again, cysteine residues appear to be the molecular determinants, being the target of reducing agents and the SH-group modifying agent phenylarsine oxide, which was shown to reduce SV currents in a rapid and reversible manner. Considering the great variability of current decrease upon removal of DTT on the one side and on the other side the high reproducibility of current reduction at alkaline pH (even in the absence of reducing agents), we suggest that these two phenomena might not be directly correlated, but based on two different redox-sensitive groups exposed to the cytosolic side of the tonoplast.

In the light of our results, the activity of the carrot SV channel is predicted to be sensitive to the redox environment of the cytoplasm. In highly reducing conditions, it shows maximal activity at physiological pH (≈7.5). Lowering the cellular antioxidant status, i.e., in conditions of oxidative stress, leads to a decrease of SV activity at alkaline pH concomitant with an optimum shift to neutral pH. Indeed, acidification of the cytosol (to pH 7.0) upon pathogen attack [14] could stimulate SV channel mediated calcium influx from the vacuole, providing a possible mechanism for the reported rise in cytosolic calcium upon acidification [23]. In plant cells, a wide range of biotic and abiotic stress factors leads to an increased formation of reactive oxygen species (ROS). Interestingly, in *Plantago* species grown in salt stress conditions, SV channel activity was reduced due to a decrease of open probability [24]. Thus, SV downregulation in response to salinity could be mediated by ROS that are produced under such conditions [25], either by a direct oxidative effect on the channel protein or indirectly by a decrease of the cellular antioxidant pool. Finally, the phytohormone abscisic acid (ABA) is known to cause increased ROS production [26] and cytosolic alkalinization [27] in guard cells. Since both parameters decrease SV channel activity, it is probably not involved in ABA-induced calcium increases in carrot.

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