A Perspective on the Slow Vacuolar Channel in Vacuoles from Higher Plant Cells

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Received May 27, 2005

INTRODUCTION

Historically, the vacuole has been an important system for the application of the patch-clamp technique to plant cells, and not only for reasons of simple preparation and the purity of its membrane. The vacuole is an intracellular organelle that can occupy up to 90% of the cellular volume of mature plant cells. For the plant cell, the tonoplast represents an essential crossing point between the cytosol and the vacuolar lumen containing a variety of transport systems.[1] Not only do these allow the storage of ions, metabolites, and xenobiotics, they also contribute to the homeostasis of cytosolic parameters, since the homeostasis of a given ion can be maintained by its release or uptake across the vacuolar membrane. In addition, the vacuole is likely to be involved in cellular signal transduction and can be an excellent model system for the study of calcium release from internal stores, leading to increases in cytosolic calcium. It is clear that the regulation of these processes is based on the permeability properties of the vacuolar membrane.

Since its initial characterization,[2] the slowly activating vacuolar (SV) channel has been found in all tissue and plant species investigated so far. The SV channel represents the dominating conductance of the tonoplast at elevated cytosolic calcium concentrations. Interestingly, hypotheses about its physiological role have taken into consideration all the vacuolar functions mentioned above. Recent experimental evidence[3] from *Arabidopsis thaliana* strongly indicates that the structure of the SV channel is determined by the TPC1 gene (two-pore channel 1). This work will briefly summarize the principal characteristics of this channel, concentrating on recent findings and otherwise by referring to previous reviews.[4−7]

DISTRIBUTION

The SV channel was initially described in barley leaf vacuoles[8] but received its name in a subsequent study on sugar beet tap root vacuoles.[2] It was then identified in many cell types, like cells of the root[9] and leaf mesophyll,[10] guard cells,[11] aleurone cells,[12] and cultured cells,[13,14] as well as in many plant species: in land plants such as *Plantago* ssp.,[15] carrot,[16] tomato,[17] and *Arabidopsis thaliana*;[18] in the sweet-water pond plant *Eichhornia crassipes*,[19] and in the marine plant *Posidonia oceonica*.[20] In summary, it is generally accepted that the SV is a ubiquitous channel in higher plants.

Figure 1A shows typical macroscopic SV-type currents elicited by a series of positive voltage steps (cytosol with respect to the vacuolar lumen, according to the convention of Bertl et al.[21]), recorded in isolated vacuoles derived from leaves of the tree *Citrus resini*. The *I* versus *V* plot shown in Figure 1B reveals the characteristics of a strongly outward-rectifying channel.

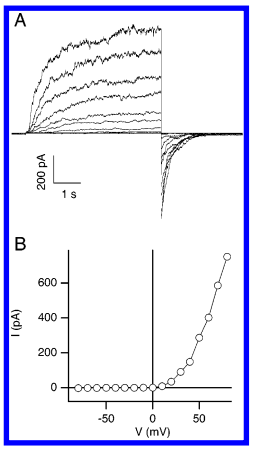

Figure 1. Macroscopic SV currents recorded in vacuoles from the leaf of *Citrus resini*. (A) SV currents elicited by a series of voltage steps ranging from −80 to +80 mV in 10 mV steps. Holding and tail potentials were at −70 mV. (B) SV current−voltage characteristic, obtained by plotting the mean value of the steady-state current versus the applied membrane potential. Symmetric ionic solutions were (in mM) KCl, 150; HEPES/KOH, 10; and pH 7.0. In these experiments, the calcium concentration was not buffered. It was estimated to be in the micromolar range by atomic absorption spectroscopy.

CALCIUM DEPENDENCE

The SV channel is activated by an increase in cytosolic calcium; in the sugar beet, channel activation can already be observed at concentrations above 150 nM.[2] As a matter of fact, there are contrasting reports on calcium concentrations activating the channel: in the sugar beet, the calcium dependence was described by a Michaelis−Menten function with a K_m value of ∼1.5 μM,[2] while in radish vacuoles, experimental data were fitted with a Hill function yielding a K_h value of ∼0.9 mM; in this system, an interaction of at least three calcium ions with the channel protein was hypothesized.[22] However, these differences are probably ascribable to different working conditions or to the specific properties of the two plant species. Interestingly, the calcium sensitivity of the SV channel depends on the cytosolic magnesium concentration.[23,24]

GATING AND VOLTAGE DEPENDENCE

The SV channel shows a complex gating behavior: it needs to step through at least two closed states before reaching the open state.[9,25−27] Cyclic multistate kinetic models

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10.1021/ci050218a CCC: $30.25
Published on Web 11/08/2005

have been proposed to explain the mechanisms of activation and deactivation that have been revealed experimentally.[9,25,26]

Several lines of evidence prove that the SV channel has an intrinsic voltage dependence. Experiments performed using a rapid perfusion system indicate that the movement of an intrinsic voltage sensor is the rate-limiting step controlling channel gating.[24] Furthermore, outward rectification is not determined by a voltage-dependent block caused by vacuolar di- or polyvalent cations driven into the pore by negative potentials, as is the case in animal potassium channels.[28] Indeed, the SV channel exhibits its usual voltage dependence in the absence of divalent ions on the vacuolar side[29] as well as in the absence of divalent ions on both sides of the membrane.[24] It should be noted that, in conditions of reduced vacuolar calcium or of asymmetrical potassium, the SV channel is able to conduct inward currents.[8,10,23,29]

THE PERMEATION PORE

The SV channel is permeable both to monovalent and to divalent cations.[30] For a discussion on the possible permeation of anions through the SV channel, refer to Schulz-Lessdorf and Hedrich[31] and Miedema and Pantoja.[32]

As in the majority of ion-selective channels,[28,33] potassium permeates through the SV pore by a single-file multi-ion mechanism. Several types of measurements on the SV channel support this property: (i) conductance measurements varying cytosolic potassium in sugar beet vacuoles[34] reached a peak at potassium concentrations between 200 and 300 mM; (ii) an anomalous mole fraction effect, typical of a single-file multi-ion pore, was recorded in measurements of the reversal potential[35] and the conductance[29] of the SV channel when varying the calcium to a potassium mole fraction.

One approach to the estimation of the pore size is the substitution of cytosolic potassium with equimolar concentrations of other monovalent cations, as reported in Figure 2, which indicates a pore size between 3.4 and 5.6 Å. On the basis of the permeation of TMA^+ and large organic cations at elevated positive potentials, Dobrovinskaya et al.[36] suggested a larger SV channel pore size of about 7 Å. This estimation is in agreement with the permeation of magnesium,[10,30,36] which is characterized by a strong hydration shell and a slow dehydration rate.[28] It is interesting to note that the channel's permeability for NH_4^+ is definitely lower than that for K^+ (Figure 2B), while its selectivity for both ions, derived from measurements of reversal potentials,[16,19] is comparable. Even though models for calcium permeation through the SV channel have been proposed (e.g., ref 35), a direct measurement of the fraction of total current which is due to calcium permeation is missing.

SINGLE-CHANNEL CONDUCTANCE

The SV channel displays a main conductance which depends on potassium concentration. In sugar beet vacuoles, values of 40−300 pS at K^+ concentrations ranging from 50 to 600 mM were determined.[34] Figure 3A shows an example of single-channel openings with a conductance of around 95 pS at 150 mM K^+. As suggested by the size of the permeation pore, the conductance of the cation-selective SV channel is large in relation to the values reported for highly selective channels.[28]

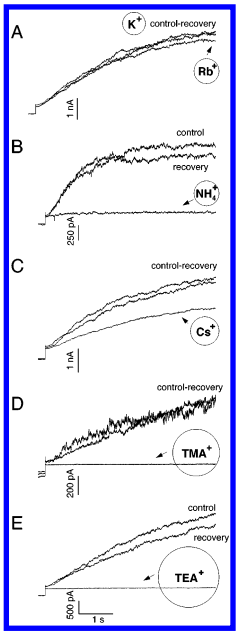

Figure 2. Macroscopic SV currents mediated by different monovalent cations. Currents, elicited by voltage steps to +80 mV from a holding potential of 0 mV, were recorded in the whole-vacuole configuration using vacuoles isolated from carrot (*Daucus carota*) cultured cells. The bath solution containing 100 mM K^+ (control, recovery) was exchanged with solutions containing equimolar concentrations of Rb^+ (A), NH_4^+ (B), Cs^+ (C), TMA^+ (D), and TEA^+ (E). Circles indicate the relative dimension of the respective cations. Absolute values of the respective ionic diameter radius (in Å) are K^+, 1.33; Rb^+, 1.48; NH_4^+, 1.5; Cs^+, 1.67; TMA^+, 2.8; and TEA^+, 3.7. The ionic solution in the pipette was (in mM) KCl, 200; $MgCl_2$, 2; $CaCl_2$, 0.1; MES/Tris, 10; and pH 6.0. In the bath: KCl, 100; $MgCl_2$, 2; $CaCl_2$, 1; DTT, 1; HEPES/Tris, 10; pH 7.0. The osmolarity of the solutions was adjusted to 670 mOsm by the addition of D-sorbitol.

INTERACTING FACTORS

The SV channel is modulated by a variety of factors, such as calmodulin,[12,31,37] phosphatases,[37] 14-3-3 proteins,[38] and polyamines,[39] and presents a complex pharmacology including inhibitors of diverse channel classes (see refs 4−6 and references therein).

Recently, the effects of cytosolic pH on the SV channel have been studied in detail.[16] The authors demonstrated that the carrot SV channel presents maximum conductance at neutral pH, suggesting the presence of two pH-titratable groups within the channel protein. Interestingly, this maximum is affected by the redox state of the channel, as it shifts to more basic pH values at elevated (up to 10 mM) concentrations of the reducing agent dithiothreitol (DTT). In addition, reducing and oxidizing agents[40] directly affect the activity of the SV channel.[41] Single-channel experiments on sugar beet vacuoles revealed that DTT increases the total open probability (NP_o) rather than the conductance of the channel (Figure 3A). Figure 3B shows complete and irreversible inhibition by the oxidizing agent chloramine-T, applied from the cytosolic side. It has been suggested that cysteine residues may be the molecular determinant of this sensitivity; indeed, the SH-group modifying agent phenylarsine oxide reversibly reduces SV currents.[16]

Interestingly, SV channels are also modulated by cytosolic heavy metal ions.[9,31,42] In radish vacuoles, Carpaneto[22] showed that nickel (Ni^{2+}) reversibly inhibits the channel by binding to a site different from the calcium binding site. Furthermore, SV channels from different species, examined

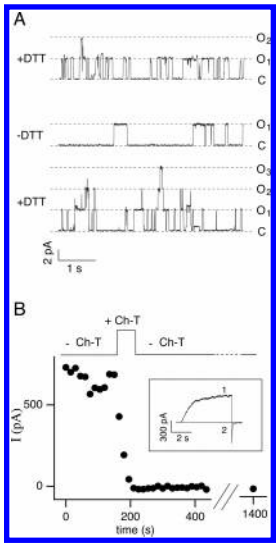

Figure 3. (A) DTT increases the open probability of SV channels in sugar beet vacuoles. Single-channel openings observed in the presence of 1 mM DTT (upper trace) and after the removal of DTT (middle trace). Lower trace: recovery. The closed state (C) and the different opening levels (O1, O2, and O3, indicating the opening of 1, 2, or 3 channels, respectively) are indicated by dotted lines. Applied potential, +40 mV; sampling rate, 4 ms; filter, 100 Hz. (B) Chloramine-T irreversibly inhibits SV currents in *Posidonia oceanica* vacuoles. Substitution of DTT by 1 mM chloramine-T (Ch-T) determines an irreversible decrease of SV currents. The inset shows the currents elicited by voltage steps to +60 mV from a holding potential of 0 mV in the presence of DTT, before (1) and after (2) the addition of Ch-T. The two curves in the inset were obtained by averaging 11 traces. The ionic luminal and cytosolic solutions adopted for sugar beet experiments were (in mM) KCl, 150; $MgCl_2$, 2; HEPES/KOH, 15; and pH 7.2 with $CaCl_2$ equal to 0.1 in the cytosol and equal to 1 in the vacuole. The solutions to patch the *P. oceanica* vacuoles were symmetrical, i.e., KCl, 400; $MgCl_2$, 5; $CaCl_2$, 0.1; HEPES/KOH, 25; and pH 7.2. The solutions were adjusted (by the addition of D-sorbitol) to values slightly larger than the osmotic pressure of the sugar beet taproot and to 900 mOsm for *P. oceanica* vacuoles.

in identical working conditions, exhibit different affinities for Ni^{2+}: the channel from *E. crassipes* was significantly less-sensitive to the action of nickel ($K_m = 99 \pm 20$ μM) than the *Beta vulgaris* channel ($K_m = 25 \pm 2$ μM).[19] Also, SV channels from *E. crassipes* remained functional in the absence of reducing agents,[19] thus, appearing to be more resistant to cytosolic oxidative conditions than others, for instance, *P. oceanica* channels.[41] These findings may indicate sequence modifications in the channel proteins of different species, presumably as a result of an adaptation of the plant to specific environmental conditions.

In the context of a possible role of the SV channel in the calcium-induced calcium-release mechanism, it should be noted that the cationic dye ruthenium red was shown to block the SV channel from sugar beets at micromolar concentrations.[43] In the animal field, this compound is known as an inhibitor of this mechanism and is used to reveal calcium mobilization from intracellular stores (ref 43 and references therein).

In addition to these modulations from the cytosolic side, the SV channel also presents interactions with vacuolar factors; in a detailed study, Pottosin et al.[27] demonstrated that the channel has two different binding sites for vacuolar divalent cations and suggested that vacuolar Ca^{2+} may be a dominant factor in controlling the channel's voltage-dependent open probability.

MOLECULAR IDENTITY

The first hypothesis regarding the molecular identity of the SV channel came from an analysis of *Arabidopsis* plants lacking the expression of the putative K^+ channel gene KCO1.[44] Patch-clamp studies on vacuoles isolated from these plants revealed a reduction in SV current density, suggesting a regulatory role rather than an essential, structural one. Recently, strong evidence has indicated that the SV channel protein in *A. thaliana* is encoded by the TPC1 (two-pore channel 1) gene.[3] Vacuoles of a tpc1 knockout mutant without detectable levels of TPC1 protein did not show any SV-type currents. The TPC1 gene, the only representative of the TPC gene family in *Arabidopsis*,[45] encodes a protein of 733 amino acids (Figure 4A), which is localized in the vacuolar membrane[3] and is predicted to form a calcium-permeable channel. As shown in Figure 4B, the protein is made up of two linked Shaker-like units, each composed of six transmembrane regions. In accordance with the functional data, the respective S4 segments contain a series of basic residues probably representing the channel's voltage sensor. The protein loops predicted to face the cytosolic side present two EF hand regions, four cysteine residues, and two histidine residues presumably conferring sensitivity toward cytosolic calcium, redox agents, and pH, respectively. Mutagenesis experiments will help to associate functional aspects of the SV currents to the structure of the TPC1 protein.

In view of the differences in single-channel conductance values reported for different SV sources,[46] it has been suggested that the channel may comprise a multigene family.[29] Moreover, a high variability in its susceptibility toward redox agents has been found in vacuoles from carrot cultured cells.[16] As only one or two TPC1-like sequences per genome have been found in the plant species examined so far,[3] the observed variabilities may depend on the presence of additional regulatory proteins.

PUTATIVE PHYSIOLOGICAL FUNCTIONS AND CONCLUSIONS

At present, the precise physiological role of SV channels has yet to be identified. A first indication of possible functions has been provided by an analysis of tpc1 knockout plants revealing differences in the abscissic acid (ABA) dependent repression of seed germination and the stomatal response to extracellular calcium.[3] Interestingly, mutant plants are viable and able to complete their life cycle also in the absence of the only TPC1 gene copy, indicating that the function of this gene can be compensated by other genes.

The functional properties of the channel have suggested an implication in a calcium-induced calcium-release mechanism (CICR) based on the channel's calcium-dependent activation and calcium permeability.[11] Ward and Schroeder proposed CICR in the context of ABA control of stomatal closure in guard cells. An analysis of the tpc1 mutant, however, showed no pronounced phenotype in stomatal movements induced by ABA, thus, raising doubts on the involvement of the SV channel. Other hypotheses that have been made concern (i) the channel's involvement in the homeostasis of cytosolic potassium on the basis of its gating properties[29] and (ii) its involvement in Na^+ storage inside the vacuole on the basis of the finding that the channel allows the passage of Na^+ into the vacuole but not in the opposite direction.[29]

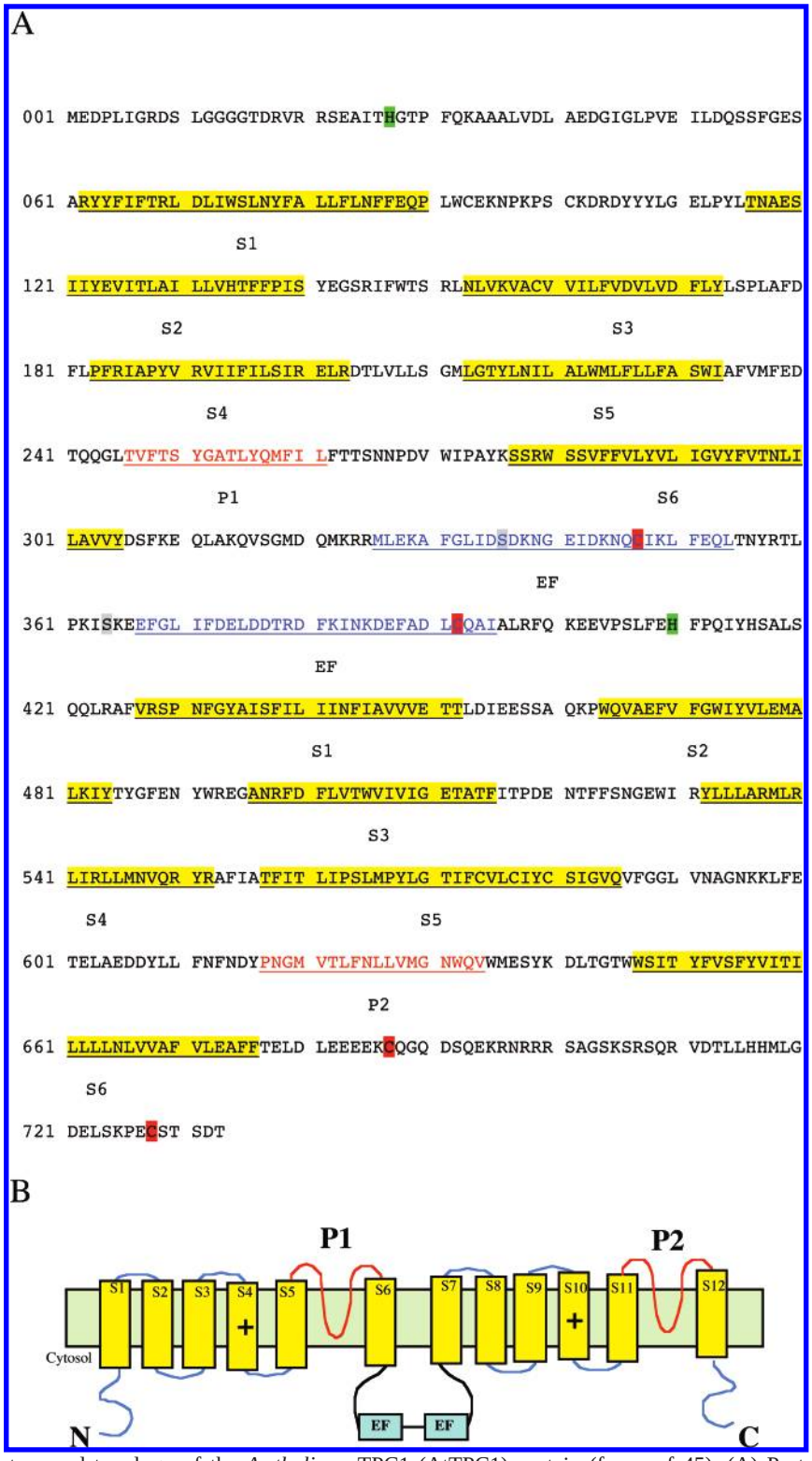

Figure 4. Primary structure and topology of the *A. thaliana* TPC1 (AtTPC1) protein (from ref 45). (A) Protein sequence of AtTPC1 presenting putative transmembrane segments S1−S12 (highlighted yellow), putative pore domains P1 and P2 (red), EF hands (blue, underlined), cysteine (C, highlighted red), and histidine (H, highlighted green) residues putatively exposed to the cytosol and putative protein kinase C-specific phosphorylation sites (S, highlighted gray). (B) Putative topology of AtTPC1 showing putative pore domains P1 and P2 (red), basic residues in S4 and S10 (+), and two EF hands (blue).

Nevertheless, above all, the fact remains that the channel is prevalently in the closed state at physiological tonoplast potentials, even at elevated Ca_{cyt}^{2+} concentrations (1 μM). Recent reports have proposed that (i) vacuolar calcium could play an essential role in shifting the open probability of the channel[27] or (ii) the channel could perform its physiological function during hyperpolarization-evoked deactivation.[26] This mechanism, however, still requires prior SV-channel activation by depolarization of the tonoplast, which has not been demonstrated so far. A simple hypothesis may be a hitherto unknown "helper factor",[10] whether in the form of an organic molecule or a regulatory protein, capable of shifting the open probability of the channel toward physiological potentials. In the absence of this factor, as a result of cellular down-

regulation or removal during vacuole isolation for patch-clamp studies, it is reasonable that the channel be safely closed in the range of physiological potentials.

ACKNOWLEDGMENT

The authors thank Dr. Franco Gambale for helpful discussions and suggestions, Dr. Monica Bregante for critical comments on the manuscript, Stella de Robertis for English language assistance, and Dr. Francois Luro for providing *Citrus resini* plants. This work was supported by the EU-RTN project "Novel ion channels in plants" (HPRN-CT-2002-00245) and by Mis 3.1-PIC Interreg III-Italia-Francia "Isole 2000/2006".

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CI050218A