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REVIEW ARTICLE

Vacuolar calcium channels

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Abstract

The central vacuole is the largest Ca^{2+} store in a mature plant cell. Ca^{2+} release from this store contributes to Ca^{2+}-mediated intracellular signalling in a variety of physiological responses. However, the routes for vacuolar Ca^{2+} release are not well characterized. To date, at least two voltage-dependent and two ligand-gated Ca^{2+}-permeable channels have been reported in plant vacuoles. However, the so-called VVCa (vacuolar voltage-gated Ca^{2+}) channel most probably is not a separate channel but is identical to another voltage-dependent channel—the so-called SV (slow vacuolar) channel. Studies in the last few years have added a new dimension to our knowledge of SV channel-mediated ion transport and the mechanisms of its regulation by multiple natural factors. Recently, the SV channel was identified as the product of the *TPC1* gene in *Arabidopsis*. In contrast, the TPC1 channel from other species was thought to be localized in the plasma membrane. A re-evaluation of this work under the assumption that the TPC1 channel is generally a vacuolar channel provides interesting insights into the physiological function of the TPC1/SV channel. Considerably less is known about vacuolar Ca^{2+} channels that are supposed to be activated by inositol 1,4,5-trisphosphate or cADP ribose. The major problems are controversial reports about functional characteristics, and a remarkable lack of homologues of animal ligand-gated Ca^{2+} channels in higher plants. To help understand Ca^{2+}-mediated intracellular signalling in plant cells, a critical update of existing experimental evidence for vacuolar Ca^{2+} channels is presented.

Key words: Calcium channel, calcium release, signal transduction, SV channel, tonoplast, vacuole.

Introduction

While calcium can make up to 5% of the dry weight of a plant (Broadley *et al*., 2003), its cytosolic free concentration is extremely low, <1 μM. A large portion of the total Ca^{2+} is bound to cell walls and anionic macromolecules inside the cell. The water-soluble Ca^{2+} in plant cells is compartmentalized into organelles functioning as Ca^{2+} stores, with the central vacuole containing most of the water-soluble Ca^{2+}. The huge Ca^{2+} concentration differences between Ca^{2+} stores and surrounding cytosol are the basis for the function of Ca^{2+} as second messenger in intracellular signal transduction. Since the vacuole is the largest Ca^{2+} pool in a typical plant cell, vacuolar Ca^{2+} channels play a critical role in Ca^{2+}-mediated signal transduction as well as in Ca^{2+} homeostasis (Bush, 1995; Hetherington and Brownlee, 2004). In this article, the evidence for different voltage-gated and ligand-gated vacuolar Ca^{2+} channels is reviewed, and—where information is available—their regulation, structure, and possible physiological functions are discussed.

Ca^{2+} transport across the vacuolar membrane

To understand the function of vacuolar Ca^{2+} channels, it is instructive to have a look at the driving forces for vacuolar Ca^{2+} transport. The free Ca^{2+} concentration inside vacuoles is typically ~1000-fold higher than in the surrounding cytosol (Evans *et al*., 1991; Bush, 1993). The electrical potential difference across the vacuolar membrane ranges from 0 mV to −30 mV (Bethmann *et al*., 1995; Walker *et al*., 1996). Both the Ca^{2+} concentration gradient and the membrane potential therefore drive Ca^{2+} efflux from the vacuole—via Ca^{2+} channels—while Ca^{2+} uptake into the vacuole requires energy.

Cytosolic free Ca^{2+} concentrations, as measured with ion-selective microelectrodes and fluorescent dyes, range from 100 nM to 350 nM at rest (Felle, 1989; Bethmann

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et al., 1995; Felle and Hepler, 1997; Plieth, 2001). During Ca^{2+}-mediated signal transduction processes, cytosolic free Ca^{2+} concentrations may transiently reach 1 μM and more, but they always remain low compared with those in the vacuole. In higher plants, vacuolar free Ca^{2+} concentrations of 1.5–2.3 mM have been measured with ion-selective microelectrodes (Felle, 1988). As in the cytosol, there can be a large difference between the total and the free vacuolar Ca^{2+} concentration, due to Ca^{2+} binding by proteins and organic acids.

Ca^{2+} uptake into vacuoles, i.e. active transport against the electrochemical potential gradient, is mediated by P-type Ca^{2+} pumps (Geisler *et al*., 2000; Sze *et al*., 2000) and H^+/Ca^{2+} antiporters (Shigaki and Hirschi *et al*., 2006). Primary Ca^{2+}-pumps (*Arabidopsis ACA*-gene family) mediate high-affinity (K_m=0.2–1.0 μM) low-turnover Ca^{2+} uptake, whereas H^+/Ca^{2+} antiporters (*Arabidopsis CAX*-gene family) mediate low-affinity (K_m ~10 μM) high-capacity Ca^{2+} uptake. It was therefore speculated that the two vacuolar Ca^{2+} uptake systems may be suited for operation at different levels of cytosolic Ca^{2+} (Maeshima, 2001).

Because of the huge cytosol-directed electrochemical gradient for Ca^{2+}, the opening of any Ca^{2+}-permeable channel will result in Ca^{2+} release from the vacuole that has to be very tightly regulated. At least four different vacuolar Ca^{2+} channels have been described, two voltage-dependent Ca^{2+} channels (VVCa and SV) and two ligand-gated channels (White, 2000; Sanders *et al*., 2002).

The VVCa channel

In contrast to the SV channel which is activated at positive tonoplast potentials, the VVCa (vacuolar voltage-gated Ca^{2+}) channel is gated open at negative tonoplast potentials. One might argue that the opening of a vacuolar Ca^{2+}-permeable channel at physiological conditions (negative membrane potentials, millimolar luminal Ca^{2+}) is hard to reconcile with the cytosolic Ca^{2+} homeostasis.The VVCa Ca^{2+} channel was characterized by single-channel recordings on isolated patches from vacuoles of *Beta vulgaris* tap roots (Johannes *et al*., 1992; Johannes and Sanders, 1995) and of *Vicia faba* guard cells (Allen and Sanders, 1994*b*). Amazingly, when comparing the properties of the SV channel (see below) and the VVCa channel, these show striking similarities, especially when data from the same species are taken. Table 1 and Fig. 1 summarize published data obtained with vacuoles of *B. vulgaris* tap roots. Obviously, the single-channel conductance for 50 mM K^+ or for 10 mM Ca^{2+}, or Mg^{2+}, or Ba^{2+} is identical for the SV channel and the VVCa channel within error limits, and both channels show a high affinity (submillimolar K_m value) for Ca^{2+} and a low affinity for K^+ (Table 1). The SV channel is activated by Ca^{2+} (Hedrich and Neher, 1987) and inhibited by H^+ (pK ~6.8) from the cytosolic side (Schulz-Lessdorf and Hedrich, 1995), while the so-called VVCa channel is activated by Ca^{2+} (Johannes *et al*., 1992) and inhibited by H^+ (pK ~6.5) from the vacuolar side (Allen and Sanders, 1994*b*). In other words, the VVCa channel has the properties of an SV channel that is inserted in the vacuolar membrane the 'other way round'—or was measured in an isolated membrane patch that was oriented the 'other way round'. The so-called VVCa channel has a high density—like the SV channel—and isolated patches always contained multiple channels, but whole-vacuole recordings from the VVCa channel do not seem to exist. To test further the possibility that the VVCa channel might be identical to the SV channel, channel activation by Ca^{2+} was compared. As shown in Fig. 1, increasing Ca^{2+} concentrations had the same effect on the voltage-dependent open probability of the SV channel and the VVCa channel. It seems unlikely that there exist two Ca^{2+} channels with identical functional properties but opposite orientation in the vacuolar membrane, while there are no recordings documenting both channels at the same time—even though both have a rather high density. It is therefore postulated that the so-called VVCa channel is not a separate Ca^{2+} channel of the vacuolar membrane but is identical to the SV channel—recorded the 'other way round'. In the discussion of the SV channel following below, data from the so-called VVCa channel are included.

The SV channel

The SV (slow-activating vacuolar) channel is by far the best described vacuolar ion channel. Earlier reports of

Table 1. *Comparison of single-channel properties of the SV channel and the so-called VVCa channel from* Beta vulgaris *taproots*

	Unitary conductance (pS)				K_m values (mM)	
	50 mM K^+	10 mM Ca^{2+}	10 mM Mg^{2+}	10 mM Ba^{2+}	K^+	Ca^{2+}
SV	167[a]	12.3[a]	18.4[a]	16.7[b]	103±14[a]	0.165±0.032[a]
VVCa	188[c]	11.7±1.2[c]	17.4±1.2[c]	17.0±1.5[c]	143 (11.8)[d]	0.244 (0.044)[d]

[a] Data from Pottosin *et al*. (2001)
[b] Data from Pantoja *et al*. (1992)
[c] Data from Johannes and Sanders (1995)
[d] Data from Gradmann *et al*. (1997) were calculated assuming either a rigid pore model or a flexible pore model (data in parentheses).

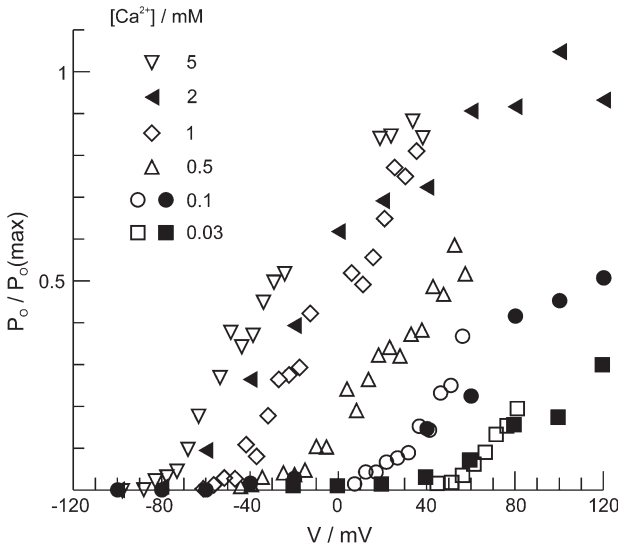

Fig. 1. Effect of Ca^{2+} on open probability. The voltage dependence of the SV channel at 0.03 mM (filled squares), 0.1 mM (filled circles), and 2 mM (filled triangles) cytosolic Ca^{2+} is compared with the voltage dependence of the VVCa channel at 0.03 mM (open squares), 0.1 mM (open circles), 0.5 mM (open triangles), 1 mM (open diamonds), and 5 mM (open inverted triangles) Ca^{2+}. For the SV channel (filled symbols), whole vacuole currents were recorded in symmetrical 100 mM KCl, pH 7.5, with nominal 0 Ca^{2+} inside the vacuole as described in Pottosin *et al.* (2004). Whole vacuole currents were divided by corresponding single-channel currents and normalized to maximum activity to calculate $P_O/P_O(max)$. Data points for the VVCa channel were taken from Figure 6A of Johannes and Sanders (1995) and are based on amplitude histograms of single-channel recordings. After inverting the membrane voltage, VVCa channel data points (open symbols) were superimposed to SV channel data. It is obvious that the voltage dependence at 0.03 mM and 0.1 mM Ca^{2+} shows no significant difference between the SV channel and the VVCa channel, and the voltage dependence of the SV channel at 2 mM Ca^{2+} is just in between the voltage dependence at 1 mM and 5 mM of the VVCa channel.

vacuolar Ca^{2+} channels mediating Ca^{2+} uptake into the vacuole (Pantoja *et al*., 1992; Ping *et al*., 1992*a*, *b*) can probably be explained as recordings from SV channels at a time when the Ca^{2+} permeability of the SV channel was not yet understood (Ward and Schroeder, 1994). The SV channel is the most abundant tonoplast channel. Based on patch-clamp recordings, channel densities of ~1 SV channel per μm^2 and higher have been calculated (Schulz-Lessdorf and Hedrich, 1995; Pottosin *et al*., 1997). Proteomic characterization of the vacuolar membrane proteins of *Arabidopsis* (Carter *et al*., 2004; Szponarski *et al*., 2005) revealed the SV channel (TPC1), but no other vacuolar ion channels. The SV channel seems to be ubiquitous among terrestrial plants (*Embryophytes*) including ferns and liverworts (Hedrich *et al*., 1988).

Molecular identity

Patch-clamp recordings on isolated vacuoles of *Arabidopsis* knock-out mutants lacking KCO1 (*kco1*) showed decreased slow-activating currents. This was interpreted as an involvement of KCO1 in the formation of SV channels (Schönknecht *et al*., 2001), while it might have been a pleiotropic effect. When KCO1 was expressed in yeast, it formed a voltage-independent, Ca^{2+}-activated, K^+-selective ion channel (Bihler *et al*., 2005). An *Arabidopsis* knock-out mutant lacking TPC1 (*tpc1-2*) does not show any SV channel activity, and *TPC1*-overexpressing lines have increased SV channel activity, demonstrating that the *TPC1* gene of *Arabidopsis* encodes the SV channel (Peiter *et al*., 2005). TPC stands for two-pore channel, a family of voltage-gated cation channels consisting of two homologous domains with six transmembrane helices and one pore domain each (Fig. 2). Originally discovered in rat kidney, TPC channels can be understood as an evolutionary intermediate between single-domain, Shaker-type K^+ channels and the family of voltage-dependent Ca^{2+} and/or Na^+ channels from animals consisting of four homologous domains (Ishibashi *et al*., 2000). In higher plants, the TPC channel is highly conserved; especially the pore loops (White *et al*., 2002), and the membrane-spanning parts largely consist of identical or conserved amino acids (Fig. 2). In *Arabidopsis*, AtTPC1 (At4g03560) is the only member of the TPC family (Furuichi *et al*., 2001), indicating that the SV channel might be formed by a TPC1 homodimer. Only a single gene or mRNA homologous to AtTPC1 has been detected in rice (Kurusu *et al*., 2004), while in tobacco (*Nicotiana tabacum*) BY-2 cells two highly homologous (97.1% amino acid identity) NtTPCs were identified (Kadota *et al*., 2004). In this context, it is interesting that it had been observed that the single-channel conductance of SV channels in guard cells exceeds the single-channel conductance in other cell types (Schulz-Lessdorf *et al*., 1995). It now should be possible to determine whether the different unit conductance goes back to different gene products or is caused by post-translational or post-transcriptional modifications.

Intracellular localization

While SV channel activity in patch-clamp recordings only has been registered from vacuolar membranes, most of the published work about TPC1 in plants has been interpreted assuming that TPC1 is a plasma membrane channel. For AtTPC1, localization in the vacuolar membrane has been demonstrated by green fluorescent protein (GFP) constructs, antibody binding, a correlation between *TPC1* expression level and SV channel activity (Peiter *et al*., 2005), and by proteomic analysis of vacuolar membranes (Carter *et al*., 2004; Szponarski *et al*., 2005). Even though, when first described, AtTPC1 was suggested to be a plasma membrane channel (Furuichi *et al*., 2001), its tonoplast localization now seems to be established (Peiter *et al*., 2005). The reported targeting of AtTPC1–GFP fusion proteins to the plasma membrane of BY-2 cells (Kawano *et al*., 2004) might be indicative of mistargeting, as has been observed

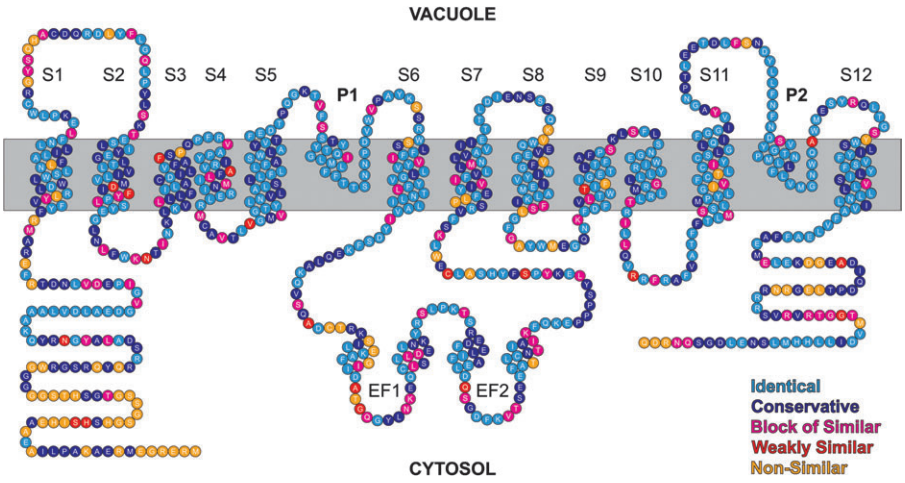

Fig. 2. Model of OsTPC1. The NCBI Conserved Domain Search (Marchler-Bauer *et al*., 2005) was used to identify functional domains of plant TPC channels. S1–S12 indicate the 12 putative transmembrane α-helices, P1 and P2 indicate the two pore loops each consisting of the pore helix and the selectivity filter, EF1 and EF2 indicate the Ca^{2+}-binding EF-hand motifs, and the grey band indicates the lipid bilayer of the vacuolar membrane. The amino acid sequence of OsTPC1 (Hashimoto *et al*., 2004; Kurusu *et al*., 2004) was aligned with the amino sequences of HvTPC (GI:39545849; *Hordeum vulgare*), TaTPC1 (Wang *et al*., 2005), AtTPC1 (Furuichi *et al*., 2001), and NtTPC1A and NtTPC1B (Kadota *et al*., 2004) in AlignX (Invitrogen Corp.). Identical amino acids are indicated in cyan, conserved substitutions in blue, blocks of similar amino acids in magenta, weakly similar amino acids in red, and non-similar amino acids in orange.

with C-terminal GFP fusions of other integral membrane proteins (Tian *et al*., 2004). GFP fusion proteins of OsTPC1 from rice (*Oryza sativa*, GFP–OsTPC1) and TaTPC1 from wheat (*Triticum aestivum*, TaTPC1–GFP) were reported to localize in the plasma membrane of onion epidermal cells (Kurusu *et al*., 2005; Wang *et al*., 2005). Keeping in mind that it might be hard to distinguish between plasma membrane localization and vacuolar membrane localization of GFP in intact onion epidermal cells, and including the possibility of mistargeting of GFP fusion proteins (Tian *et al*., 2004), it is believed it might be worth reconsidering some of the experimental results obtained with NtTPC1 (Kadota *et al*., 2004), OsTPC1 (Hashimoto *et al*., 2004; Kurusu *et al*., 2004, 2005), and TaTPC1 (Wang *et al*., 2005) under the assumption that these might be SV channels of the vacuolar membrane (see below).

Not knowing the tertiary structure of the SV channel, useful information about its pore dimensions can be obtained by applying blocking cations of different size and length (Dobrovinskaya *et al*., 1999*a*). The outcome of such an approach is summarized in Fig. 3. It appears that substances with a diameter of ⩽7 Å (the size of a fully hydrated Mg^{2+} ion) can permeate the pore. In line with such a pore diameter, the SV channel has a high permeability for alkali cations (Amodeo *et al*., 1994; Paganetto *et al*., 2001) as well as alkali earth cations (Pantoja *et al*., 1992; Ward and Schroeder, 1994; Pottosin *et al*., 2001). Considering only physiologically abundant cations, the SV channel can mediate passive exchange of K^+, Na^+, NH_4^+, Ca^{2+}, and Mg^{2+} between the vacuole and cytosol. Early studies suggested a significant anion permeability of the SV channel (Hedrich *et al*., 1986; Hedrich and Kurkdjian, 1988; Schulz-Lessdorf *et al*., 1995). More recent analyses have shown, however, that anion (Cl^-) permeability of the SV channel is immeasurably low (Ward *et al*., 1994; Pottosin *et al*., 2001). Negative surface charges at the SV channel pore entrances probably contribute to the charge-selecting mechanism, attracting cations and rejecting anions (Pottosin *et al*., 1999, 2001, 2005).

Theoretical calculations based on physiologically relevant electrochemical ionic gradients across the tonoplast show that SV channel-mediated currents are dominated by K^+, while Ca^{2+} currents are rather small. At zero voltage, 1 mM luminal and 1 μM cytosolic Ca^{2+}, the single-channel Ca^{2+} current is ~100 fA and 400 fA for SV channels from *Beta* taproots and *Vicia* guard cells, respectively (Gradmann *et al*., 1997; Allen *et al*., 1998). Nevertheless, with only a few open SV channels per vacuole, Ca^{2+} release approaches the pA range, which is comparable with estimated maximum rates of Ca^{2+} uptake into the vacuole. The main route of vacuolar Ca^{2+} uptake is via proton motive force-driven Ca^{2+}/H^+ exchange. The proton motive force is built up by tonoplast H^+ pumps that generate whole-vacuole currents of 10–20 pA (~30 μA cm^{-2}) (Hedrich and Kurkdjian, 1988; Hedrich *et al*., 1989). Obviously Ca^{2+} uptake into the vacuole cannot exceed H^+ pump currents over an extended time period. This implies that active Ca^{2+} uptake into the vacuole can only compensate for passive Ca^{2+} release by a very small fraction of the thousands of SV channels per vacuole. Assuming just a 1 pA net Ca^{2+} release into a typical cytoplasmic volume of 1 pl, cytosolic free Ca^{2+} would reach 1 μM in ~1 min, even with only 1 out of 10 000 cytoplasmic Ca^{2+} ions being in a free form. Most vacuoles examined contain several thousand SV channel copies

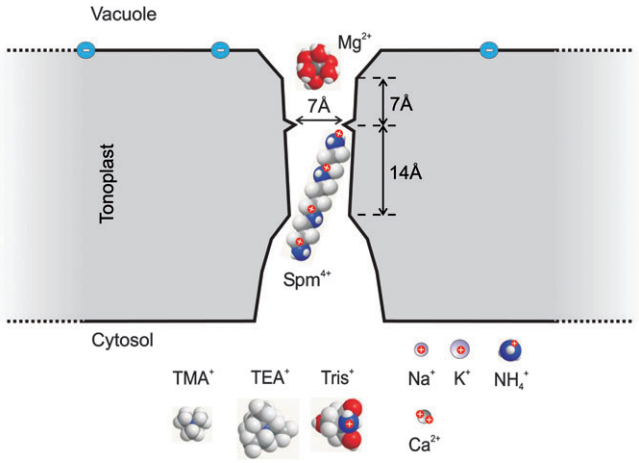

Fig. 3. SV channel pore architecture. Silhouettes of physiologically important permeable cations are presented at the right hand side of the pore. A significant SV channel conductance for Mg^{2+} can only be explained if this strongly hydrated cation (positioned inside the pore) preserves the first hydration shell while crossing the pore. Organic blockers ($Tris^+$, TMA^+, TEA^+, and polyamines) meet a major energy barrier (depicted as a pore constriction) on their way through the pore. Data on the voltage-dependent blockage imply that this barrier is located close to the extracytosolic (vacuolar) side. Applying a large positive voltage from the side of cation application pushed all blockers except TEA^+ to pass to the opposite membrane side. Based on this 'cut-off' limit, the pore diameter at its narrowest place is ~7 Å, just sufficient to allow the passage of hydrated Mg^{2+} or $Tris^+$ cations. Using blocking cations of extended length and equally spaced positive charges (shown here is $Spermine^{4+}$) allowed the definition of the physical length of the cytosolic and vacuolar pore tunnels. It appears that the cytosolic region of the pore can accommodate one spermine or two putrescine molecules (~14 Å), whereas the vacuolar region distance is ⩽7 Å, which is the length of a putrescine molecule (Dobrovinskaya *et al.*, 1999*b*). At the vacuolar membrane surface in the vicinity of the SV channel protein, a significant negative surface charge was detected, with an average distance between elementary charges of ~17 Å; this charge tends to concentrate permeable and blocking cations in the neighbourhood of the pore entrance (Pottosin *et al.*, 2005).

(Schulz-Lessdorf and Hedrich, 1995; Pottosin *et al.*, 1997; Dobrovinskaya *et al.*, 1999*b*). Therefore, <0.1% of all SV channels can be open at rest, implying a very strict control of SV channel gating.

Voltage dependence and regulation

Since its discovery, the SV channel has been known to be activated at positive tonoplast potentials and by elevated cytosolic Ca^{2+} (Hedrich and Neher, 1987). The strict voltage dependence, also preserved in the virtual absence of Ca^{2+} (Cerana *et al.*, 1999; Carpaneto *et al.*, 2001), implies an intrinsic voltage sensor. Increase of cytosolic Ca^{2+} levels has a dual effect on SV channel activity, an increase in the maximal number of open SV channels at high positive potentials and a shift of the voltage dependence to less positive potentials (Hedrich and Neher, 1987; Schulz-Lessdorf and Hedrich, 1995; Pottosin *et al.*, 1997). The sensitivity of the SV channel to cytosolic Ca^{2+} is enhanced several fold by calmodulin (Bethke and Jones, 1994). A negative shift of the voltage dependence is also observed at increasing cytosolic Mg^{2+} levels (Pei *et al.*, 1999; Carpaneto *et al.*, 2001). To explain the overlapping effects of Ca^{2+} and Mg^{2+}, Pei and co-workers (1999) proposed two cytosolic binding sites, a Ca^{2+}-selective one, binding Ca^{2+} with high affinity, and another site binding Ca^{2+} and Mg^{2+} with comparable affinity in the submillimolar to millimolar range. Binding of divalent cations to the latter site stabilizes the SV channel in its open state, shifting the activation threshold to less positive potentials. At physiological conditions (low cytosolic Ca^{2+}, submillimolar cytosolic Mg^{2+}), this site is preferentially occupied by Mg^{2+}. In an attempt to evaluate the impact of cytosolic Ca^{2+} and Mg^{2+} on vacuolar Ca^{2+} release, the non-invasive MIFE technique was applied to isolated vacuoles (Wherrett, 2006). In the absence of divalent cations on the cytosolic side, vacuolar Ca^{2+} release was <1 pA per vacuole (average diameter 40 μm), which is close to the detection limit. An increase of cytosolic Ca^{2+} to 20–50 μM increased Ca^{2+} release to a few pA per vacuole; Ca^{2+} release was doubled upon addition of 1 mM Mg^{2+}. These Ca^{2+} fluxes were inhibited by ~80% after addition of 0.1 mM Zn^{2+}, a known SV channel blocker (Hedrich and Kurkdjian, 1988), indicating that the measured Ca^{2+} fluxes were largely mediated by the SV channel.

Reducing agents such as dithiothreitol (DTT) or glutathione increase the open probability of the SV channel (Carpaneto *et al.*, 1999; Scholz-Starke *et al.*, 2004). Further cytosolic factors affecting SV channel activity are reversible protein phosphorylation exerting either positive or negative control, depending on the phosphorylation site (Allen and Sanders, 1995; Bethke and Jones, 1997), and 14-3-3 proteins that reduce SV currents without affecting their voltage dependence (van den Wijngaard *et al.*, 2001).

Several physiologically relevant cations, such as heavy metal ions (Zn^{2+} and Ni^{2+}) and polyamines, inhibit the SV channel at micromolar concentrations (Hedrich and Kurkdjian, 1988; Dobrovinskaya *et al.*, 1999*a*, *b*; Paganetto *et al.*, 2001; Carpaneto, 2003). Some of them, such as polyamines, act solely via binding within the channel pore, blocking the flow of permeable cations (Dobrovinskaya *et al.*, 1999*b*), whereas others, such as Ni^{2+}, also modify channel gating (Carpaneto, 2003). An interesting example of such a dual action on the SV channel was recently demonstrated for the aminoglycoside antibiotic neomycin. Neomycin applied from the cytosolic side was shown to block the current through an open SV channel but at the same time activated SV channels by shifting their voltage dependence towards negative potentials (Scholz-Starke *et al.*, 2006). This was interpreted as an indication that the SV channel can be activated at physiologically relevant (i.e. negative) tonoplast potentials by special regulatory molecules (Scholz-Starke *et al.*, 2006). The stimulatory effect of neomycin on the SV channel may explain the observation that in the presence

of neomycin, a voltage-evoked Ca^{2+} increase is followed by intracellular Ca^{2+} release in guard cells (Grabov and Blatt, 1999).

The composition of the vacuolar compartment is much more variable than the cytosolic composition (Leigh, 1997). Several vacuolar factors have been shown to control SV channel function. Lowering the vacuolar pH decreases SV channel activity (Schulz-Lessdorf and Hedrich, 1995; Pottosin *et al*., 1997). Even more efficient is the variation of vacuolar Ca^{2+} levels. Vacuolar Ca^{2+} competes with H^+ and Mg^{2+} for the same binding sites. Removal of vacuolar Ca^{2+} at neutral pH results in a dramatic negative shift of the SV channel voltage dependence, and a threshold for activation as low as −100 mV is observed. Vacuolar Mg^{2+} is much less efficient compared with Ca^{2+}, in terms of both binding affinity (millimoles versus micromoles for Ca^{2+}) and voltage shift (Pottosin *et al*., 1997, 2004). Binding of vacuolar Ca^{2+} and Mg^{2+} causes stabilization of the channel's closed states and shift of the activation threshold to unphysiological, positive potentials—the opposite effects compared with the action of these ions at the cytosolic side. An increase of vacuolar Ca^{2+}, therefore, albeit increasing the driving force for Ca^{2+} release, closes the Ca^{2+}-permeable SV channel. Extrapolation to physiologically relevant electrochemical gradients for Ca^{2+} across the tonoplast yielded an SV channel open probability of <0.03% (Pottosin *et al*., 1997). Other vacuolar cations potently shifting the SV channel voltage dependence to more positive potentials are Na^+ (Ivashikina and Hedrich, 2005) and Al^{3+} (Wherrett *et al*., 2005). The inhibitory effects of multivalent cations (e.g. Ca^{2+}) on the SV channel at the vacuolar side are decreased at increasing ionic strength (Pottosin *et al*., 2005).

The SV channel is unquestionably the best characterized vacuolar ion channel, yet its physiological role is still unclear. Based on its voltage dependence, the SV channel could mediate uptake of cations into the vacuole. However, for most physiologically important cations, the direction of the electrochemical potential gradient only allows passive release from the vacuole. The SV channel, once gated open, will mediate the efflux of vacuolar Na^+ (especially under salt stress), Mg^{2+}, and Ca^{2+}.

Initially the SV channel was postulated to allow the equilibration of K^+ across the vacuolar membrane (Colombo *et al*., 1988; Amodeo *et al*., 1994; Paganetto *et al*., 2001) and to be involved in turgor regulation (Hedrich and Schroeder, 1989). Under adequate K^+ nutrition, cytosolic and vacuolar K^+ concentrations are comparable (Bethmann *et al*., 1995; Walker *et al*., 1996), and a high K^+ permeability of the tonoplast probably keeps the electrical potential low. The SV channel might contribute to this K^+ permeability. Under K^+-replete and K^+-deficient conditions, in contrast, significant K^+ gradients are established across the tonoplast to maintain a stable cytosolic K^+ concentration while the vacuolar K^+ concentration changes according to external availability (Walker *et al*., 1996). To establish significant trans-tonoplast K^+ gradients, SV channel activity has to be regulated down to allow effective K^+ compartmentalization. It is known that K^+ starvation causes an increase in cellular putrescine content (Richards and Coleman, 1952; Smith, 1985), to levels which block the SV channel (Dobrovinskaya *et al*., 1999*b*). Moreover, low vacuolar K^+ concentrations down-regulate SV channel activity (Pottosin *et al*., 2005). In non-halophytic plant cells, K^+ is a major cellular osmoticum. Hence, control of SV channel activity by vacuolar K^+ is likely to contribute to turgor regulation. It is known that $^{86}Rb^+$ (which is chemically similar to K^+) release during stomatal closure is controlled in a feedback manner by the remaining vacuolar cation content; this $^{86}Rb^+$ release depends, at least in part, on the elevation of cytosolic Ca^{2+} with a high threshold, suggesting an involvement of the SV channel (MacRobbie, 1995, 1998).

An important strategy of plants to adapt to salt stress is the effective compartmentalization of cytotoxic Na^+ into the vacuole. Under these conditions, all routes allowing passive Na^+ release from the vacuole—such as an open SV channel—have to be closed. Growth under salt stress resulted in reduced SV channel activity in two *Plantago* species, and only for the salt-tolerant species (*P. maritima*) was a complete suppression of SV channel activity observed (Maathuis and Prins, 1990). Moreover, the SV channel is down-regulated by vacuolar Na^+ (Ivashikina and Hedrich, 2005) and blocked by increasing levels of the polyamines spermidine and spermine, which are induced by salt stress (Smith, 1985; Erdei *et al*., 1990; Dobrovinskaya *et al*., 1999*b*).

Early on, the SV channel was postulated to be involved in Ca^{2+} uptake into the vacuole (Pantoja *et al*., 1992) or Ca^{2+}-mediated Ca^{2+} release from the vacuole (Ward and Schroeder, 1994). Meanwhile it seems to be clear that the huge trans-tonoplast Ca^{2+} gradient only allows channel-mediated Ca^{2+} release, and not uptake, at physiologically attainable tonoplast potentials (see above). Hence, the SV channel operates as a vacuolar Ca^{2+}-release channel. Under some circumstances, this Ca^{2+} release might be autoinducible because the SV channel is Ca^{2+} activated. As discussed above, even the opening of just a tiny fraction of the thousands of SV channels per vacuole inevitably results in a considerable increase of the cytosolic free Ca^{2+} concentration, pointing to an involvement in Ca^{2+}-mediated intracellular signal transduction. There are some indications from recent publications as to in which intracellular Ca^{2+} signalling pathways the SV channel might or might not be involved.

Al^{3+} stress causes a sustained elevation of cytosolic Ca^{2+} possibly via reactive oxygen species-dependent activation of the SV channel (Kawano *et al*., 2004). The Al^{3+}-induced Ca^{2+} increase is higher in Al^{3+}-sensitive

wheat plants compared with Al^{3+}-resistant genotypes (Zhang and Rengel, 1999). At the same time, Al^{3+}-resistant wheat plants, which show a smaller cytosolic Ca^{2+} increase in response to Al^{3+}, show a higher degree of SV channel inhibition by Al^{3+} compared with an Al^{3+}-sensitive wheat genotype (Wherrett *et al*., 2005). Disturbance of cytosolic Ca^{2+} homeostasis (Rengel, 1992) due to sustained SV channel activation may be an important part of Al^{3+} toxicity, and a more effective blockage of the SV channel by vacuolar Al^{3+} may contribute to increased Al^{3+} tolerance (Wherrett *et al*., 2005).

An H_2O_2-induced increase in cytosolic Ca^{2+} in tobacco BY2 cells was inhibited by co-suppression of NtTPC1A/B (NtTPC1A/B encode two highly homologous SV channels in tobacco), and enhanced by overexpression of AtTPC1 (Kawano *et al*., 2004; Kadota *et al*., 2005), suggesting that vacuolar Ca^{2+} release by the SV channel is an important part of oxidative stress-induced signal transduction. (As discussed above, in contrast to the original publications, a vacuolar localization of all *TCP1* gene products is assumed here.) A sucrose-induced cytosolic Ca^{2+} increase was slightly enhanced by overexpression of AtTPC1 in *Arabidopsis* leaves, while suppression of TPC1 expression resulted in inhibition of the cytosolic Ca^{2+} increase in response to sucrose (Furuichi *et al*., 2001; Kadota *et al*., 2004). In contrast, the cytosolic Ca^{2+} increase in tobacco BY2 cells caused by a hypo-osmotic shock was not affected by co-suppression of NtTPC1A/B, whereas overexpression of AtTPC1 in the same cells resulted in an enhanced Ca^{2+} increase (Kadota *et al*., 2004; Kawano *et al*., 2004). While the SV channel seems to play a critical role in sucrose-induced Ca^{2+} increase, its contribution to the hypo-osmotic shock-induced Ca^{2+} increase is less clear.

There is evidence that the SV channel is an essential component of elicitor-induced signal transduction and programmed cell death in both monocotyledonous and dicotyledonous plants. In tobacco BY2 cells, co-suppression of NtTPC1A/B caused a reduced response to the fungal elicitor cryptogein, consisting of a smaller cytosolic Ca^{2+} increase and less defence-related gene expression and programmed cell death (Kadota *et al*., 2004). In suspension-cultured rice cells, an insertional knock-out mutant of OsTPC1 severely suppressed elicitor (*Trichoderma viride xylanas*)-induced activation of a mitogen-activated protein kinase (MAPK) and programmed cell death, while OsTPC1 overexpression caused enhanced elicitor sensitivity with an elevated oxidative burst, and increased activation of MAPK and programmed cell death (Kurusu *et al*., 2004).

Whereas *Arabidopsis* plants lacking or overexpressing AtTPC1 do not seem to display an obvious phenotype (Peiter *et al*., 2005), rice plants lacking OsTPC1 grew to slightly smaller size, and OsTPC-overexpressing plants showed reduced growth and greening of roots (Kurusu *et al*., 2004). In *Arabidopsis* knock-out lines lacking AtTPC1 (*Attpc1*), the plant hormone abscisic acid (ABA) is less effective at inhibiting germination, while ABA sensitivity of germination is increased in AtTPC1 overexpression lines (Peiter *et al*., 2005). In contrast to this, ABA-induced stomatal closure was affected neither by AtTPC1 overexpression nor by knock-out (Peiter *et al*., 2005). However, stomatal closure induced by high external Ca^{2+} (10 mM) could not be observed in *Attpc1* knock-out mutants, while AtTPC1 overexpression lines showed Ca^{2+}-induced stomatal closure comparable with that of the wild type (Peiter *et al*., 2005). *Arabidopsis* lines overexpressing TaTPC1 (*T. aestivum*) exhibited reduced stomatal apertures compared with wild-type plants at high external Ca^{2+} (Wang *et al*., 2005). Whereas the SV channel seems to be an essential component of ABA-induced inhibition of seed germination and stomatal closure under high external Ca^{2+}, ABA-induced stomatal closure does not seem necessarily to require a functional SV channel. To explain the latter, it has been postulated that stomatal ABA signalling is robust, meaning that the loss of AtTPC1 is compensated by recruiting alternative cation release pathways (Peiter *et al*., 2005).

Ligand-gated Ca^{2+} channels

A variety of physiological responses in plants is mediated by intracellular ligands such as IP_3 (inositol 1,4,5-trisphosphate, a product of phosphoinositol hydrolysis by phospholipase C) or cADPR (cADP ribose, an NAD metabolite), both known to activate distinct Ca^{2+} release channels in animal cells (Ehrlich *et al*., 1994; Guse *et al*., 1999). Photolysis of caged IP_3 or microinjection of cADPR into guard cells produces stomatal closure (Blatt *et al*., 1990; Leckie *et al*., 1998). Internal levels of IP_3 and cADPR in guard cells are increased upon ABA treatment (Lee *et al*., 1996; Wu *et al*., 1997). Voltage-evoked transient increases in cytosolic Ca^{2+} in guard cells are inhibited by high concentrations of ryanodine, implying an important contribution of cADPR-mediated signalling (Grabov and Blatt, 1999). At the same time, blocking of phospholipase C activity abolished ABA-induced cytosolic Ca^{2+} oscillations and stomatal closure (Staxen *et al*., 1999). Inhibitor analysis of vacuolar solute loss during ABA-induced stomatal closure revealed that cADPR- and IP_3-linked pathways together make a significant or even dominant (at low ABA doses) contribution (MacRobbie, 2000). In *Arabidopsis* roots, hyperosmotic or NaCl treatment induced IP_3 production and, simultaneously, an intracellular Ca^{2+} mobilization; both processes were blocked by the phospholipase C inhibitor U-73122 (DeWald *et al*., 2001). It appears that chilling also provokes a Ca^{2+} response including an IP_3-mediated component (Knight *et al*., 1996). Therefore, both cADPR and IP_3 are involved in intracellular Ca^{2+} release in plants.

The nature of plant intracellular ligand-gated Ca^{2+} release channels and their organelle location is less clear. Early studies on microsomes from *Chenopodium album* (Lommel and Felle, 1997) and *B. vulgaris* (Allen *et al*., 1995) indicated that cADPR- and IP_3-induced Ca^{2+} release has a vacuolar origin. However, a more detailed study made on separated membrane fractions from cauliflower florets revealed that the contribution of IP_3-induced Ca^{2+} release from vacuoles is minor compared with release from non-vacuolar stores (Muir and Sanders, 1997) and that cADPR-mobilized Ca^{2+} release originates mainly from rough endoplasmic reticulum vesicles (Navazio *et al*., 2001). In *C. rubrum*, high affinity IP_3-binding sites were located exclusively in the endoplasmic reticulum fraction (Martinec *et al*., 2000).

At first glance, patch-clamp measurements on isolated vacuoles should give a definitive answer as to whether IP_3- or cADPR-sensitive Ca^{2+}-release channels are located in the tonoplast or not. Yet, the results of such studies are not consistent. In contrast to the promising work by Alexandre and co-workers (Alexandre *et al*., 1990; Alexandre and Lassalles, 1992), who reported IP_3-dependent single-channel activity in red beet vacuolar membranes, later studies from different laboratories (Chasan and Schroeder, 1992; Ping *et al*., 1992*a*; Gelli and Blumwald, 1993) could not reproduce these results. Recordings on isolated tonoplast patches of *B. vulgaris* showed current fluctuations at negative potentials in the presence of 1 μM IP_3 (Allen and Sanders, 1994*a*), 100 nM cADPR (Allen *et al*., 1995), or high (10 μM) concentrations of ryanodine (Muir *et al*., 1997). These current fluctuations, however, did not show obvious conductance levels, and the IP_3-dependent current fluctuations (Allen and Sanders, 1994*a*) greatly differed from the single-channel recordings presented by Alexandre and co-workers (Alexandre *et al*., 1990; Alexandre and Lassalles, 1992). Both IP_3 and cADPR have been reported to enhance instantaneous currents at the whole vacuole level (Allen and Sanders, 1994*a*; Allen *et al*., 1995; Leckie *et al*., 1998). However, little attempt has been made to separate these instantaneous currents from an unspecific leak or the instantaneous FV current. In particular, the whole vacuolar currents activated by cADPR in *V. faba* guard cells display the same voltage dependence as the intrinsic FV current, and were suppressed by submicromolar cytosolic Ca^{2+} (Leckie *et al*., 1998) as the FV current is (Allen and Sanders, 1996). The non-invasive MIFE technique is a very sensitive method to measure Ca^{2+} fluxes from individual plant cells (Shabala *et al*., 2006). This technique was applied to the large central vacuoles from red beet taproots. An average Ca^{2+} current of ~1 pA per vacuole was measured at resting conditions, and no significant increase was observed upon application of 2 μM IP_3 (Wherrett, 2006).

Keeping in mind the strong evidence that IP_3 and ryanodine/cADPR-type receptor Ca^{2+} channels play an important role in Ca^{2+}-mediated signal transduction in plant cells, it is surprising that no corresponding genes have yet been identified in plants (Nagata *et al*., 2004). This may be explained by very weak homology between plant and animal Ca^{2+} release channels due to an early evolutionary divergence (Maathuis, 2004). Alternatively, homoplasy may be an explanation, since functionally similar Ca^{2+} signal transduction pathways may well have evolved from different molecular building blocks (Bothwell and Ng, 2005). In light of the importance of intracellular Ca^{2+} signalling, the identification of genes encoding the ion channels responsible for cADPR- or IP_3-induced Ca^{2+} release in plants, along with the biochemical and physiological characterization of their products, is a priority task. In parallel, the search for corresponding single channels in different intracellular membranes (large central vacuole, small vacuoles, and different endoplasmic reticulum fractions) for a biophysical and pharmacological characterization should be reinforced.

Note added in proof

In a recent review article entitled 'Inositol trisphosphate receptor in higher plants: is it real?' Krinke *et al*. (*Journal of Experimental Botany* **58,** 361–376, 2007) summarize the current knowledge about IP_3 receptor Ca^{2+} channels in plants. A recent publication by Bonaventura *et al*. (*The Plant Journal* **49,** 889–898, 2007) indicates that a gain-of-function allele of TPC1 activates oxylipin biogenesis after leaf wounding in *Arabidopsis*.

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