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Calcium channels in the vacuolar membrane of plants: multiple pathways for intracellular calcium mobilization

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SUMMARY

An increasing number of studies imply that Ca^{2+} mobilization from intracellular stores plays an important role in stimulus evoked elevation of cytosolic free calcium during signal transduction in plants. It is believed that Ca^{2+} is released mainly from the vacuole, which contains a high Ca^{2+} concentration in a large volume, and can be regarded as the principal Ca^{2+} pool in mature higher plant cells. The large size of the organelle confers unique experimental advantages to the study of endomembrane ion channels. The patch-clamp technique can be directly applied to isolated vacuoles to characterize Ca^{2+} release pathways at the single channel level and confirm their membrane location. Using radiometric, ligand-binding and electrophysiological techniques we characterized two different pathways by which Ca^{2+} can be mobilized from the vacuole of *Beta vulgaris* tap roots.

Inositol 1,4,5 trisphosphate ($InsP_3$)-elicited Ca^{2+} release from tonoplast enriched vesicles is dose-dependent, highly specific for $InsP_3$, and is competitively inhibited by low M_r heparin ($K_i = 34$ nM). This striking resemblance to the animal counterpart which is probably located in the ER is further reflected by the binding properties of the solubilized $InsP_3$ receptor from beet, which bears similarities to the $InsP_3$ receptor of cerebellum. Thus, $InsP_3$ and heparin bind to a single site with sub-micromolar K_ds, whereas other inositol phosphates have affinities in the supra-micromolar range.

The second Ca^{2+} channel in the beet tonoplast is voltage-sensitive and channel openings are largely promoted by positive shifts in the vacuolar membrane potential over the physiological range. Channel activity is neither affected by $InsP_3$ addition nor by alteration of cytosolic free calcium, and from a large range of Ca^{2+} antagonists tested, only Zn^{2+} and the lanthanide Gd^{3+} proved to be effective inhibitors. With Ca^{2+} as a charge carrier the maximum unitary slope conductance is about 12 pS and saturation occurs at $\leqslant 5$ mM vacuolar Ca^{2+}. The channel has an approximately 20-fold higher selectivity for Ca^{2+} over K^+ which is achieved by a Ca^{2+} binding site in the channel pore. The unique properties of this novel Ca^{2+} release pathway suggests that it is specific for plants. The presence of both $InsP_3$-gated and voltage-gated Ca^{2+} channels at the vacuolar membrane implies flexibility in the mechanism of intracellular Ca^{2+} mobilization in plant cells.

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1. INTRODUCTION

Cytosolic free calcium ($[Ca^{2+}]_c$) is becoming increasingly recognized as a key element in stimulus-response coupling in plant cells. Low levels of $[Ca^{2+}]_c$ are maintained in the resting state by Ca^{2+}-ATPases at the plasma membrane and endoplasmic reticulum (Evans *et al.* 1991) and by H^+ gradient-driven Ca^{2+} uptake into the large central vacuole (Blackford *et al.* 1990). Investigations with various species indicate that a variety of environmental stimuli elicit rapid elevation of $[Ca^{2+}]_c$, including dark-induced inhibition of photosynthetic activity (Miller & Sanders 1987), growth regulators (Felle 1988*a*), hypotonicity of the external medium (Okazaki & Tazawa 1990), touch and low temperature (Knight *et al.* 1991). Independent studies have indicated a number of potential regulatory sites at which these elevated levels of $[Ca^{2+}]_c$ might act, including the enzymes of intermediary metabolism (Brauer *et al.* 1990), Ca^{2+}-dependent protein kinases (Harper *et al.* 1991), ion channels (Hedrich & Neher 1987; Schroeder & Hagiwara 1989; Tester & MacRobbie 1990) and gene expression (Braam and Davis 1990).

It is widely assumed that elevation of $[Ca^{2+}]_c$ is the result of activation of Ca^{2+} channels. Originally, such arguments were based primarily on the finding that $[Ca^{2+}]_c$-mediated signal transduction in animal cells involves entry of Ca^{2+} to the cytosol from the extracellular medium or from intracellular stores, rather than mere inhibition of energy-coupled efflux (Williamson & Monck 1989; Berridge & Irvine 1989;

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Tsien & Tsien 1990). Against this background, a growing number of reports have yielded pharmacological (Graziana *et al.* 1988; MacRobbie & Banfield 1988; Brosnan & Sanders 1990; Tester & MacRobbie 1990) and electrophysiological (Beilby 1984; Shiina & Tazawa 1987; Tester & Harvey 1989; Alexandre *et al.* 1990; Schroeder & Hagiwara 1990; Cosgrove & Hedrich 1991) evidence for the presence of Ca^{2+} channels in plant cells (for reviews, see Johannes *et al.* 1991; Schroeder & Thuleau 1991).

However, the identity of channels responsible for mediating Ca^{+} influx to the cytosol during specific types of signal transduction in plant cells is far from clear. This uncertainty is best exemplified in the most thoroughly studied system for which Ca^{2+}-mediated stimulus–response coupling has been described for plants – that of stomatal guard cell closure. Thus, while electrophysiological analysis has suggested enhancement of Ca^{2+} influx across the plasma membrane during closure via opening of relatively non-specific cation channels (Schroeder & Hagiwara 1990), digital imaging of the changes in $[Ca^{2+}]_c$ which ensue closing stimuli demonstrates that intracellular stores of Ca^{2+} dominate in their contribution to the overall elevation of $[Ca^{2+}]_c$ to supra-micromolar levels (Gilroy *et al.* 1991).

It might well emerge that the relative contributions of plasma membrane and endomembrane channels to $[Ca^{2+}]_c$ signalling is a function of environmental conditions, and in particular of the extent to which a significant extracellular pool of Ca^{2+} is available for mobilization. In terrestrial higher plants, by contrast with animal cells and aquatic plant cells which are normally bathed in media containing millimolar levels of free Ca^{2+}, this extracellular pool is likely to be both limited and variable: although Ca^{2+} is normally present in significant quantities in the cell wall, its activity is lowered substantially as a result of chelation by the carboxyl groups of wall polymers. Variation of apoplastic pH will affect the extent of this chelation (Dainty & Richter 1989). Extracellular Ca^{2+} therefore appears *a priori* unlikely to constitute a principal source of Ca^{2+} for intracellular signalling in terrestrial higher plants.

Intracellular pools of Ca^{2+} in terrestrial plants are, by contrast, significant and stable. In particular, the contents of the large central vacuole, which in mature cells occupy between 70% and 90% of intracellular volume, comprise a simple salt solution with free Ca^{2+} maintained in the millimolar range (Felle 1988*b*). This intracellular Ca^{2+} pool represents a potential major source of mobilizable Ca^{2+}. These *a priori* considerations have been given added relevance by the finding that one ligand responsible for intracellular Ca^{2+} mobilization in animal cells – inositol 1,4,5-trisphosphate (InsP_3) – releases Ca^{2+} from vacuolar membrane vesicles and intact vacuoles from higher plants (Schumaker & Sze 1987; Ranjeva *et al.* 1988).

The vacuoles of higher plants offer unique advantages as an experimental system for investigation of intracellular Ca^{2+} mobilization. Unlike all intracellular compartments in animal cells, intact vacuoles are sufficiently large to permit the ready application of the patch-clamp technique. This enables confirmation of the location of ion channels without the uncertainties relating to contamination which accompany membrane fractionation techniques. Furthermore, the properties of ion channels can be studied in the native membrane under conditions which allow full experimental control over ionic composition on each side of the membrane, thereby permitting detailed analysis of properties such as selectivity, permeation, gating and pharmacology.

Cells in the storage root of beet (*Beta vulgaris* L.) are highly vacuolated, and comprise an excellent starting material both for the preparation of isolated intact vacuoles and of vesicles formed from the vacuolar membrane (tonoplast). We have exploited these attributes in order to enable the joint application of biochemical and electrophysiological approaches to the study of Ca^{2+} channel properties in a single biological system.

2. THE VACUOLAR InsP_3-GATED CHANNEL

(*a*) *Properties of InsP$_3$-elicited Ca^{2+} release*

Membrane vesicles from beet can be loaded with Ca^{2+} in the presence of ATP. Calcium uptake can be measured radiometrically using vesicle filtration, and is defined as the Ca^{2+} ionophore-sensitive Ca^{2+} associated with the vesicles. (Ionophore-insensitive Ca^{2+} retention on the filter typically accounts for around 25% of the total steady state retention.) Ca^{2+} uptake displays the established characteristics of transport across the tonoplast: it is insensitive to the plasma membrane and ER Ca^{2+}-ATPase inhibitor vanadate, as well as to the mitochondrial H^{+}-ATPase inhibitor azide, but is abolished by NO_3^-, which inhibits the vacuolar H^{+}-ATPase, and by the protonophore FCCP. These findings are therefore all consistent with H^{+}-coupled Ca^{2+} uptake driven by the vacuolar H^{+}-ATPase.

Vesicles which have accumulated Ca^{2+} to a steady level release $21 \pm 1\%$ of intravesicular Ca^{2+} on addition of 10 μM InsP_3. This release is transient unless conducted in the presence of a protonophore which eliminates re-uptake (Brosnan & Sanders 1990). Further doses of InsP_3 fail to elicit additional Ca^{2+} release, and since InsP_3-gated Ca^{2+} channels do not inactivate spontaneously (Alexandre *et al.* 1990), the simplest explanation for this observation is that the InsP_3- sensitive Ca^{2+} pool has been depleted. The existence of separate InsP_3-sensitive and -insensitive pools in this preparation need not imply heterogeneous origin of the vesicles, however. Quantitative considerations based on limited channel density in the native membrane and the high surface:volume ratio of vesicles are consistent with the notion that only a minority of tonoplast vesicles possess an InsP_3-gated Ca^{2+} channel (Brosnan 1990).

The InsP_3-elicited release of Ca^{2+} is also rapid, being completed within the time (10–15 s) at which the first sample can be collected after InsP_3 addition. It is not therefore possible to measure the rate of Ca^{2+} loss without stopped-flow techniques. Nevertheless, the

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total extent of Ca^{2+} loss exhibits a Michaelian dependence on InsP_3 concentration ($K_{1/2} = 0.54 \pm 0.11$ μM: Brosnan & Sanders 1990). This value is in accord with those determined from other plant systems (0.6 μM in oat root tonoplast vesicles: Schumaker & Sze 1987; 0.2 μM in *Acer*: Ranjeva *et al.* 1988). Similar values have also been reported from animal cells (Berridge & Irvine 1984).

Why should the total Ca^{2+} loss be dose-dependent? It might, after all, be anticipated that sub-maximal doses of InsP_3 would merely induce Ca^{2+} loss at a lower rate, but to the same end-point. This dose-related (or 'quantal') Ca^{2+} release has been repeatedly observed in animal systems and might arise as a result of interaction between the InsP_3 binding site and regulatory Ca^{2+} binding sites in the vesicle lumen (see Irvine (1990*a*) and references therein). This implies that InsP_3-elicited Ca^{2+} loss is controlled by the intra-luminal Ca^{2+} concentration. However, a definitive account of the underlying mechanism of this response is still lacking.

Other inositol phosphates (inositol 1,4-bisphosphate, inositol 1,3,4,5-tetrakisphosphate) and GTP are ineffective in releasing Ca^{2+} from beet membrane vesicles and have no significant effect on the magnitude of InsP_3-elicited Ca^{2+} release. These results suggest that the InsP_3-gated Ca^{2+} channel from plants is highly specific for InsP_3, again in accord with findings for the InsP_3 receptor from animal cells. While whole cell studies have revealed that InsP_4 and GTP can have a role in refilling InsP_3 labile Ca^{2+} stores in animals (Tsien & Tsien 1990), it seems clear that this response is unrelated to the properties of the InsP_3-gated Ca^{2+} channel per se.

The pharmacological properties of the InsP_3 channel also bear a strong resemblance to those of the animal counterpart. Thus, potential antagonists which have proved ineffective against InsP_3-elicited Ca^{2+} release from beet vesicles include nifedipine (an inhibitor of L-type Ca^{2+} channels in mammalian plasma membranes), ruthenium red (which blocks Ca^{2+} uptake into mitochondria) and ryanodine (an antagonist of the sacroplasmic reticulum Ca^{2+} channel). By contrast low molecular mass (4–6 kDa) heparin, which is a potent inhibitor of InsP_3-elicited Ca^{2+} release in animal cells, eliminates 96% of the comparable response of beet vesicles at a concentration of 1 μM (Brosnan & Sanders 1990). The effect is competitive with InsP_3 and the derived K_i for heparin is 34 nM (figure 1). The potency of heparin is reduced dramatically as the degree of polymerization is increased. Thus, the $K_{1/2}$ for inhibition by high molecular mass heparin (6–20 kDa) in the presence of 10 μM InsP_3 is 1400 nM, which contrasts with a value of 86 nM for the lower molecular mass form in identical conditions. Very similar effects have been reported for animal tissues (Chopra *et al.* 1989).

In all of the properties of InsP_3-elicited Ca^{2+} release described so far for beet vesicles – including InsP_3 specificity, quantal release, and pharmacology – the characteristics of the plant system appear to mirror faithfully those described for animal cells. The significance of these findings is twofold. First, the InsP_3-gated Ca^{2+} channel in animals is clearly located on a different membrane: either a sub-population of ER (Ross *et al.* 1989) or specialized organelles termed 'calciosomes' (Krause 1991). Our results therefore indicate that despite the disparate membrane location and the taxonomic divide between plants and animals, each cell type possesses a common Ca^{2+} channel which presumably evolved before the two kingdoms split. Second, with compelling evidence for a physiological role of InsP_3 in plant cell signal transduction still lacking (Irvine 1990*b*; Brosnan & Sanders 1992), the capacity for high-affinity InsP_3-elicited Ca^{2+} release in plants suggests that the ligand has a bona fide function in vacuolar Ca^{2+} mobilization.

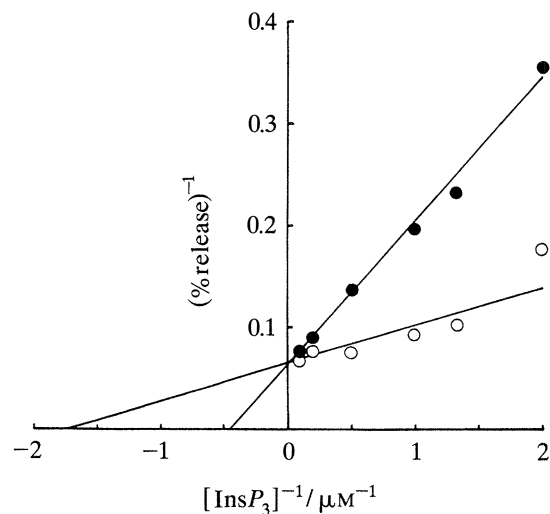

Figure 1. Lineweaver–Burke plot showing competitive inhibition of InsP_3-induced Ca^{2+} release by low molecular mass heparin. Ca^{2+} release from tonoplast enriched microsomes elicited by a range of InsP_3 concentrations (0.5–10 μM) was measured in the absence (open circles) or presence (filled circles) of 100 μM heparin. The derived K_i for heparin is 34 nM. 100% Ca^{2+} release corresponds to 1.2 nmol mg^{-1} membrane protein.

(*b*) *Ligand binding to the InsP_3 receptor*

The studies summarized above imply the existence of an InsP_3 receptor on the vacuolar membrane of plants. More detailed characterization of this receptor must lie in a description of its ligand binding properties.

Attempts to characterize InsP_3-specific binding to beet membranes were unsuccessful. Formate-anion exchange chromatography (Rincón *et al.* 1989) revealed that, in the conditions of the Ca^{2+} release assays, a substantial fraction of added InsP_3 is degraded by phosphatases to InsP_3 or possibly inositol monophosphates. In the light of these findings, we developed a method described by Maeda *et al.* (1990) for assaying the binding properties of the solubilized InsP_3 receptor with minimal interference from membrane-associated phosphatases. After solubilization of membranes in 1% Triton X-100, followed by centrifugation at 20000 *g* for 20 min, equilibrium binding of [^{3}H]InsP_3 in the supernatant was determined at 4°C.

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The reaction was stopped by addition of polyethylene glycol (PEG), and radioactivity was assayed in the PEG precipitate following centrifugation at 10 000 *g* for 5 min. Specific InsP_3 binding was defined as that displaced by addition of 10 μM unlabelled InsP_3.

When assayed in material derived from microsomes, InsP_3-specific binding accounts for $43 \pm 6\%$ of total binding. Specific binding sites are of low density (typically 8 to 10 fmol mg^{-1}) but they do appear to be localized primarily on the tonoplast. Thus, the specific activity of binding is three- to fourfold higher when assayed on material derived from density gradient-enriched tonoplast.

In common with studies from animal cells (Worley *et al.* 1987; Guillemette & Segui 1988; Rossier *et al.* 1989; Challis *et al.* 1991), InsP_3-specific binding to beet microsomal material is sensitive to neutral or acid pH, with an optimum at pH 8.0. Non-specific binding is not pH-sensitive in animals (Challis *et al.* 1991) or in beet.

The $[^3H]$InsP_3 displacement assay described above has been used to measure the affinity of the receptor for InsP_3 as well as its specificity for other ligands. InsP_3 binds to a single site with a $K_d = 56 \pm 6$ nM, which compares well with reported values of 20 to 100 nM for the InsP_3 receptor from rodent cerebellum (Supattapone *et al.* 1988; Maeda *et al.* 1990; Challis *et al.* 1991). Significantly, the K_d for InsP_3 binding is an order of magnitude lower than the $K_{1/2}$ for InsP_3-elicited Ca^{2+} release (540 nM: Brosnan & Sanders 1990). This disparity can be at least partially reconciled by the finding that ATP (which is present in the Ca^{2+} release assays, but not in the binding assays) binds to the receptor, albeit with low affinity ($K_d = 980$ μM).

As might be anticipated from the Ca^{2+} release assays, other inositol phosphates also have low affinities for the InsP_3-specific binding site (K_ds for InsP_2, InsP_4 and InsP_6 all in the supra-micromolar range). Furthermore, low molecular mass heparin binds with a $K_d = 320$ nM ($= 1.6$ μg ml^{-1}), which once again is in excellent agreement with values reported for the InsP_3 receptor from cerebellum (1.8–5.0 μg ml^{-1}: Challis *et al.* 1991; Worley *et al.* 1987).

These studies on the ligand binding to the beet InsP_3 receptor reinforce the conclusions from the Ca^{2+} release assays: the vacuolar InsP_3-gated Ca^{2+} channel from plants bears a striking resemblance to its counterpart in animal cells, despite the obvious difference in membrane location.

3. A NOVEL PATHWAY FOR Ca^{2+} MOBILIZATION FROM THE VACUOLE

(*a*) *General properties*

In a previous report (Johannes *et al.* 1992), we described the properties of a novel Ca^{2+}-permeable channel in vacuolar membranes from the storage root of beet. This channel is voltage-gated and potentially provides an alternative mode for elevation of $[Ca^{2+}]_c$ during signal transduction. Ca^{2+} efflux through this channel was monitored radiometrically in membrane vesicles and patch-clamp methodology, applied to intact isolated vacuoles, was used to confirm location of the channel and to characterize its functional properties. Both approaches showed that the voltage-sensitive Ca^{2+} channel has distinctive features which allow a clear differentiation from the InsP_3-gated channel in the same membrane (Johannes *et al.* 1992). Thus, voltage-sensitive Ca^{2+} release is neither affected by InsP_3 nor by its antagonist heparin, but can be effectively inhibited by Zn^{2+} and Gd^{3+}, to which the InsP_3-gated channel is considerably less sensitive (Johannes *et al.* 1992). Furthermore, voltage-sensitive Ca^{2+} release is not significantly influenced by variation of free calcium on the cytosolic side of the membrane, and we failed to detect effects of ryanodine.

Patch clamp experiments with isolated vacuoles revealed that the voltage-sensitive pathway consists of a moderately Ca^{2+} selective channel whose open state probability increases when the trans-tonoplast potential difference is shifted to the physiological range of positive vacuolar membrane potentials. The properties of the single channel outlined below differ from those of other Ca^{2+} permeable channels so far described, although the mechanism by which Ca^{2+} selectivity is achieved bears some similarity to L-type Ca^{2+} channels in the plasma membranes of animal cells.

(*b*) *Selectivity and permeation*

Current–voltage relationships for the single channel in inside-out patches under bi-ionic conditions (K^+ as a charge carrier on the cytoplasmic side, Ca^{2+} as charge carrier on the vacuolar side) reveal a large conductance for K^+ influx (200 pS with 50 mM K^+) and a small conductance for Ca^{2+} efflux (12 pS with 10 mM Ca^{2+}, figure 2). The value of the zero current potential, however, indicates a 15–20 times higher selectivity for Ca^{2+} over K^+. This type of behaviour suggests that permeant ions do not move through the channel independently and that, as for L-type Ca^{2+} channels, ionic competition for an intrapore binding site is involved in the process of selectivity (Tsien *et al.* 1987). Interaction of ions with the binding site is also reflected in the shape of the current–voltage characteristic in the range of positive currents (efflux): the K^+ current increases linearly with voltage, whereas the Ca^{2+} current saturates at high positive voltages to a maximum current of 0.6 pA (figure 2).

Dissociation constants for ion binding can be obtained by investigating ionic conductance as a function of ion activity and by studying inhibition of monovalent ion current by divalent ions. In L-type channels the binding constants for Ca^{2+} estimated with these approaches differ by several orders of magnitude, indicating the presence of two Ca^{2+} binding sites inside the channel pore (Tsien *et al.* 1987). Monovalent cation flux through these channels is blocked by micromolar Ca^{2+} concentrations which ensures an almost perfect Ca^{2+} selectivity. Occupation of the second, low affinity binding site with Ca^{2+} leads to ion repulsion and helps to dissociate Ca^{2+} from its

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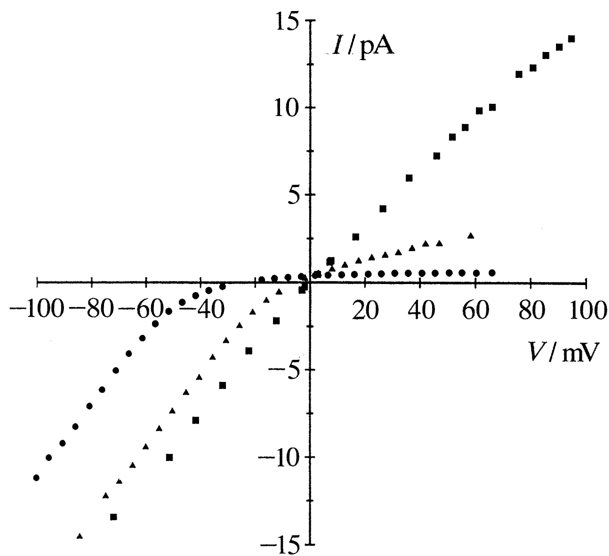

Figure 2. Single channel current–voltage characteristics of a Ca^{2+} permeable channel in the tonoplast of sugar beet – effect of different charge carriers on the vacuolar side. Excised inside-out patches with 50 mM K^+ on the cytoplasmic side (pipette solution) were successively bathed in media containing either K^+ (■, ▲) or Ca^{2+} (○) as the main charge carrier. All solutions were adjusted to pH 7.3 with dimethylglutaric acid and HEPES. With 50 mM K^+ (+30 μM Ca^{2+} to activate the channel) on the vacuolar side unitary current increases linearly with voltage with a conductance of 200 pS (■). Addition of 1 mM Ca^{2+} to the vacuolar side causes a dramatic decrease in the K^+ efflux current (▲). When 10 mM Ca^{2+} is used as a charge carrier the zero current potential is shifted to the negative range and the Ca^{2+} efflux current saturates at positive vacuolar potentials to a maximum level of about 0.6 pA (●).

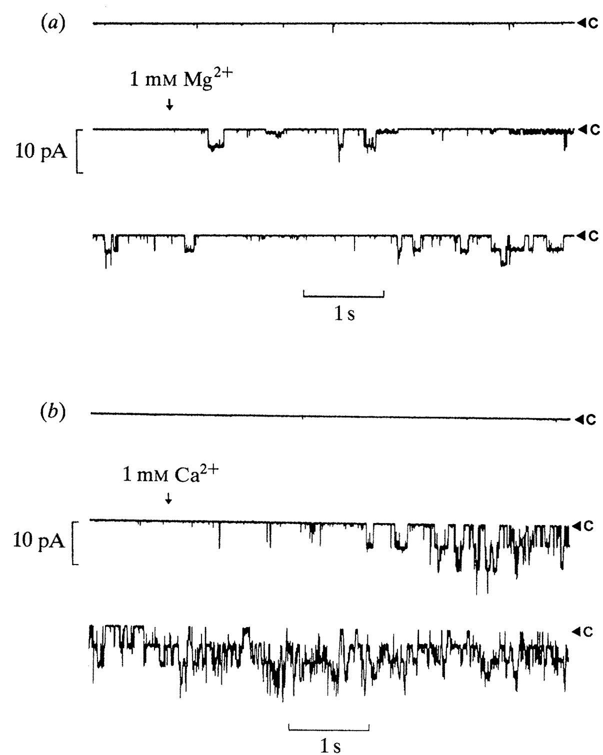

Figure 3. Activation of cation efflux from the vacuole by divalent cations on the vacuolar side. Inside-out patch with symmetrical 50 mM K^+. The membrane potential was clamped at +40 mV (vacuolar side positive). C marks the closed state. Channel openings are shown as downward deflections. In the absence of divalent cations, the channels are closed ((*a*) and (*b*), upper traces). Addition of 1 mM Mg^{2+} (*a*) or 1 mM Ca^{2+} (*b*) to the bath/vacuolar side causes activation of channel openings. Both conditions were tested on the same patch under continuous perfusion. Ca^{2+} has a stronger effect than Mg^{2+}.

high affinity binding site, so that a high flux can be achieved (Tsien *et al.* 1987).

In the case of the tonoplast Ca^{2+} channel, which is much less Ca^{2+} selective, our recent results suggest that binding constants obtained by saturation and block experiments are likely to fall within a similar range. With K^+ as a charge carrier, addition of increasing Ca^{2+} concentrations to the vacuolar side results in a progressive decrease in unitary K^+ current (figure 2) with half maximal inhibition at around 0.3 mM Ca^{2+} (E. Johannes and D. Sanders, unpublished results). With Ca^{2+} as a charge carrier, the single channel conductance is saturated at 5 mM vacuolar Ca^{2+} (Johannes *et al.* 1992). In this case a half saturation constant has not been determined since suitable impermeant cations to substitute for Ca^{2+} have yet to be identified. [Bis-tris-propane, *N*-methyl glucamine and choline all have direct effects on the unitary current.] The saturation of unitary conductance at relatively low, physiologically relevant Ca^{2+} concentrations is quite exceptional and a more detailed analysis concerning the numbers of binding sites and their location within the membrane dielectric field is currently being undertaken.

(*c*) *Gating*

As reported previously, Ca^{2+} efflux through this channel is strongly voltage-sensitive and is additionally affected by vacuolar Ca^{2+} (Johannes *et al.* 1992). In the negative, non-physiological range of tonoplast membrane potentials channel openings are rare, whereas in the positive range the open state probability increases e-fold per 18 mV depolarisation (with Ca^{2+} as a charge carrier). Elevation of vacuolar Ca^{2+} over the range 5–20 mM shifts the threshold for voltage activation to less positive vacuolar potentials and leads to an additional increase in the open state probability over the physiological range of membrane potentials (Johannes *et al.* 1992).

Recent tests with K^+ as a charge carrier reveal an absolute requirement for divalent ions (on the vacuolar side) for channel opening. As depicted in figure 3, Ca^{2+} has a much stronger effect on channel activation than Mg^{2+}, so a simple charge screening effect can be ruled out. In the presence of low, sub-physiological levels of vacuolar Ca^{2+} (30 μM), channel activation is slow and openings can only be observed at high positive vacuolar potentials. Stepwise increase of added Ca^{2+} (in a background of 50 mM K^+) shifts the threshold for voltage activation progressively to the negative range and also causes fast activation. Analogous experiments with Mg^{2+} show that a considerably higher concentration is required to activate

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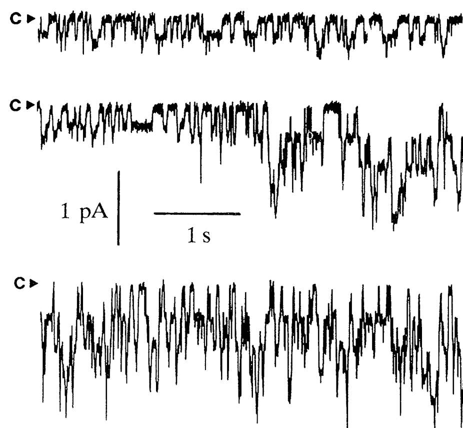

Figure 4. Spontaneous transition in a continuous recording from the low (upper trace) to the high conductance state (lower trace) of the voltage-sensitive Ca^{2+} channel in the tonoplast (inside-out patch). The membrane potential was clamped at +40 mV. C marks the closed state. Channel openings (Ca^{2+} efflux) are shown as downward deflection of the current trace. The pipette solution (cytoplasmic side) comprised 50 mM K^+ and the bath solution/vacuolar side contained 5 mM Ca^{2+}.

cation efflux, and channel openings in the negative range (K^+ influx) are barely detectable. Preliminary tests have also demonstrated that channel gating is affected by divalent cations on the cytoplasmic side. In this case they cause inhibition of cation influx by strongly decreasing the open probability at negative vacuolar membrane potentials. The complex behaviour of divalent cations on channel gating will be analysed further in forthcoming studies.

(*d*) *Regulation*

Although the physiological regulators of channel gating (except voltage) are still obscure, there are several indications that channel activity is under both vacuolar and cytoplasmic control. Lowering of vacuolar pH to more physiological values dramatically decreases open state probability and also leads to a marked rundown of channel activity with time. Fast channel rundown has also been observed in excised patches for L-type Ca^{2+} channels, which need cytoplasmic factors to sustain activity (Romanin *et al.* 1991). Furthermore, there is preliminary evidence that the channel exists in two different 'conductance states' with similar ionic specificity, pH-dependence and pharmacology, but different conductance levels and open probabilities (figure 4). Spontaneous transition from the low to the high conductance state has been observed frequently in inside-out patches, and might be explained by dissociation of a regulatory compound which keeps the channel in a rather inactive state in resting conditions.

4. PHYSIOLOGICAL SIGNIFICANCE AND CONCLUSIONS

Plant cells clearly possess two potential mechanisms for mobilization of vacuolar Ca^{2+} during signal transduction. The first is an InsP_3-gated Ca^{2+} channel which, with respect to both its Ca^{2+} release and ligand-binding properties exhibits a striking resemblance to InsP_3-gated Ca^{2+} channels in animal cells. The second is a voltage-gated Ca^{2+} channel which is activated over the physiological, inside-positive range of trans-tonoplast potentials and which exhibits some characteristics suggestive of additional control mechanisms (such as phosphorylation).

The respective roles of both channels in signal transduction remains to be elucidated. Thus, while artificial elevation of InsP_3 levels results in an increase in $[Ca^{2+}]_c$ and the ensuing responses are associated with stomatal closure in guard cells (Blatt *et al.* 1990; Gilroy *et al.* 1990), convincing reports that InsP_3 constitutes a part of the signal transduction chain in response to natural stimuli have not been forthcoming for any plant system. Nevertheless, there are some preliminary indications that elevated InsP_3 levels might play a role in the control of cell turgor in beet (Srivastava *et al.* 1989).

Growing evidence from a variety of animal cell types has indicated the presence of parallel pathways for intracellular Ca^{2+} mobilization (e.g. Ehrlich & Watras 1988; Bezprozvanny *et al.* 1991; Dehlinger-Kremer *et al.* 1991). InsP_3-gated Ca^{2+} channels and caffeine- (or Ca^{2+}-) activated Ca^{2+} channels can often be found in different intracellular pools but they can also coexist in the same organelles, as has been shown in cerebellar Purkinje cells (Walton *et al.* 1991). The joint involvement of these pathways generates considerable flexibility in the temporal characteristics of Ca^{2+} release, as well as the amplitude of the resulting elevation of $[Ca^{2+}]_c$.

Plant cells also have evolved independent methods to release Ca^{2+} from intracellular pools which befits the notion that a widely used second messenger like $[Ca^{2+}]_c$ must be subject to a spectrum of sophisticated and variable controls to endow this signal with stimulus-specificity (Tsien & Tsien 1990). With the exception of its different intracellular location, the InsP_3 channel bears many similarities to its animal counterpart. By contrast, the voltage-sensitive Ca^{2+} channel has distinct features, as far as its pharmacology and single channel properties are concerned, and might well prove to be specific to plants. Both Ca^{2+} channels are localized in the tonoplast and mobilize Ca^{2+} from the central vacuole, a large Ca^{2+} pool which in contrast to the intracellular Ca^{2+} pools in animal cells does not become significantly depleted. At present, however, we cannot be sure that both Ca^{2+} release mechanisms co-reside in the same vacuoles. An alternative possibility to the parallel arrangement of channels suggested above is therefore that expression of the channels is cell-specific. This would imply a corresponding selectivity in the ability of given cells to respond to individual environmental stimuli.

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