

Calcium-induced calcium release mediated by a voltage-activated cation channel in vacuolar vesicles from red beet

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Abstract Little is known about the mechanisms underlying calcium-induced Ca^{2+} release (CICR) in plants. The slow-activating vacuolar (SV) channel is both permeable to, and activated by Ca^{2+} , and is therefore a prime candidate for a role in CICR. Cytosol-side-out vacuolar membrane vesicles loaded with $^{45}\text{Ca}^{2+}$ showed voltage- and Ca^{2+} -dependent Ca^{2+} release, which was sensitive to the SV channel modulators DIDS, protein phosphatase 2B and calmodulin. Significantly, voltage-dependent Ca^{2+} release strongly depended on cytoplasmic Ca^{2+} concentrations. The results support the notion that CICR occurs in plant cells and that the process can be catalysed by the SV channel on the vacuolar membrane.

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Key words: Calcium signaling; Calcium release; Slow vacuolar channel; Membrane vesicle

1. Introduction

Elevation of cytosolic free Ca^{2+} ($[\text{Ca}^{2+}]_c$) is a central element in many stimulus-response pathways in plants [1]. In mature plant cells, the dominant intracellular Ca^{2+} store is the vacuole, which typically occupies 85–90% of the intracellular volume and contains Ca^{2+} at millimolar concentrations [2]. In accord with the notion that the vacuole represents a source of Ca^{2+} for cellular signaling, a number of Ca^{2+} -permeable release channels has been identified at the vacuolar membrane [2]. Thus Ca^{2+} is mobilised both by inositol 1,4,5-trisphosphate (InsP_3) and by cyclic ADP-ribose (cADPR) through discrete channel types which bear many similarities to animal InsP_3 and ryanodine receptors, respectively [3–8]. In animal cells, these two receptors are localised on the endoplasmic reticulum and are involved in the generation of stimulus-specific Ca^{2+} signals [9]. Both classes of receptor exhibit Ca^{2+} -dependent activation above threshold levels of $[\text{Ca}^{2+}]_c$, thereby providing intrinsic amplification of the Ca^{2+} signal [10]. This process is known as Ca^{2+} -induced Ca^{2+} release (CICR).

In plants there is growing evidence for CICR in cell signaling. Calcium waves, elicited in pollen tubes by localised elevation of $[\text{Ca}^{2+}]_c$, might be mediated through Ca^{2+} -dependent phospholipase C (PLC), which leads to production of InsP_3 [11]. In guard cells, intracellular release of caged Ca^{2+} can lead either to a biphasic increase in $[\text{Ca}^{2+}]_c$, with the second phase indicative of CICR [12], or to oscillations in $[\text{Ca}^{2+}]_c$, which typify CICR events in animal cells [13]. Furthermore,

abscisic acid-induced stomatal closure appears to involve voltage-dependent Ca^{2+} entry across the plasma membrane, which in turn triggers intracellular Ca^{2+} release [14]. Rhizoids of the marine alga *Fucus* respond to hypo-osmotic shock also by mobilisation of Ca^{2+} from intracellular stores after an initial influx of Ca^{2+} across the plasma membrane [15]. In the freshwater alga *Eremosphaera viridis*, internally applied Sr^{2+} , acting as a Ca^{2+} analogue, induces $[\text{Ca}^{2+}]_c$ spiking [16].

Paradoxically however, neither the InsP_3 -gated channel nor the ryanodine receptor-like Ca^{2+} release pathway of vacuoles is activated by elevation of $[\text{Ca}^{2+}]_c$ [17,18]. A hyperpolarisation-activated Ca^{2+} -permeable channel in the same membrane is also insensitive to $[\text{Ca}^{2+}]_c$ [19]. Nevertheless, a potential mechanism for CICR in plants is via the so-called slow-activating vacuolar (SV) channel [20], which is ubiquitously distributed in plant cells [21]. The SV channel is both Ca^{2+} -permeable [20,22–24] and activated by $[\text{Ca}^{2+}]_c$ at approximately 300 nM and above [23,25–28]. In one report [29] it has been claimed that the voltage dependence of the SV channel is such that at membrane potentials negative of the equilibrium potential for Ca^{2+} the open probability (P_o) is negligible. From this finding it was concluded that SV channels can mediate only efflux of Ca^{2+} from the cytosol.

In this study, we have assessed the ability of vacuolar membrane vesicles to release Ca^{2+} in a Ca^{2+} -dependent fashion. We show that, with respect to voltage dependence, inhibitor profile and Ca^{2+} dependence, CICR from vacuolar vesicles has the characteristics of mediation by SV channels.

2. Materials and methods

2.1. Preparation of vacuolar membrane vesicles

Vacuole-enriched vesicles from the storage root of *Beta vulgaris* L. were isolated by sucrose density gradient centrifugation as described previously [30] with the following modifications: in the homogenisation buffer, nupercaine was replaced by 1 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor, 1 $\mu\text{g}/\text{ml}$ leupeptin, 0.5 mM PMSF (phenylmethylsulphonyl fluoride) and 5 mM benzamidinium HCl. Soybean trypsin inhibitor (1 $\mu\text{g}/\text{ml}$) and leupeptin (1 $\mu\text{g}/\text{ml}$) were also added to the suspension medium. The final vacuolar membrane pellet was resuspended in calcium transport medium (CTM; 400 mM glycerol, 5 mM Bis-Tris propane (BTP)-MES (pH 7.4), 25 mM K^+ gluconate, 3 mM MgSO_4 , 1 mM dithiothreitol and 0.3 mM NaN_3) and frozen at -80°C before use.

2.2. Protein assay

Protein concentration was determined using a Bio-Rad assay kit based on a modification of the dye binding method [31]. Bovine serum albumin was used as a standard.

2.3. Ca^{2+} transport assay

Vacuole-enriched membrane vesicles (60 μg) were diluted into CTM and loaded with $^{45}\text{Ca}^{2+}$ via an ATP-generated proton motive force [32] as previously described [7] with the following modifications: 10 μM CaCl_2 contained 5.92 kBq $^{45}\text{CaCl}_2$ (specific activity 74 MBq/

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ml), and uptake was inhibited with carbonyl cyanide *p*-(trifluoromethoxy)phenyl-hydrazone (FCCP) after 8 min when the vesicles were loaded to about half-maximal capacity. Vacuum filtration was carried out using a multi-port filtration manifold (Model VFM-I Amicon, Lexington, MA, USA). Radioactivity remaining on the filters after the addition of the Ca^{2+} ionophore A23187 was defined as non-accumulated Ca^{2+} and was subtracted from all data. Typically, non-accumulated Ca^{2+} amounted to about 30% of total uptake.

2.4. Vesicle membrane potentials

Vesicles containing 25 mM K^+ gluconate were diluted into CTM which contained one of a range of K^+ gluconate concentrations (2.5, 5, 12.5 or 25 mM). Once loading of Ca^{2+} had been terminated, a membrane potential was imposed in the form of a K^+ diffusion potential by the addition of the potassium ionophore valinomycin (10 μM). Membrane potentials were calculated from the Nernst equation and assumed to be equal to the equilibrium potential for K^+ . Potentials are expressed relative to the luminal side of the vacuolar membrane. Vacuolar membrane vesicles are known to be relatively Cl^- -permeant [33] so Cl^- -free media were used throughout to prevent attenuation of the membrane potential.

2.5. Calcium calculations

Free calcium levels were calculated with the aid of CALCIUM [34]. Binding constants for EGTA and cations were taken from a previous report [35].

2.6. Chemicals

$^{45}\text{CaCl}_2$ was from NEN-DuPont (Boston, MA, USA); all other reagents were from Sigma Chemical (Poole, Dorset, UK).

3. Results

SV channels are activated by cytosol-positive membrane potentials. To obtain a preliminary indication of whether SV channels might participate in vacuolar Ca^{2+} release, we loaded vacuolar vesicles with $^{45}\text{Ca}^{2+}$ by initiating $\text{Ca}^{2+}/\text{H}^+$ antiport driven by the vacuolar H^+ -ATPase [5], then applied a range of cytosol-positive K^+ diffusion potentials. External ($\equiv$ cytosolic) K^+ concentrations of 25, 12.5, 5 and 2.5 mM

Table 1
Pharmacology of voltage-induced Ca^{2+} release

Addition	Ca^{2+} release at $\Delta\psi=60$ mV (%)
None	18.96 ± 4.1
DIDS (10 μM)	-2.2 ± 4.0
CaM (100 U/ml)	17.0 ± 7.8
PP2B (2 U/ml)	8.98 ± 4.3
CaM plus PP2B	-1.98 ± 6.0
EGTA (150 μM)	0.65 ± 3.2

Accumulation of $^{45}\text{Ca}^{2+}$ proceeded for 8 min in a reaction medium containing 2.5 mM K^+ , after which further accumulation was prevented by addition of 10 μM FCCP. After a further 30 s, the following additions were made in individual experiments (final concentrations in parentheses): 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS: 10 μM) or EGTA (150 μM). Spinach calmodulin (final concentration 100 U/ml) and protein phosphatase 2B (PP2B: final concentration 2 U/ml), when applied, were present from the start of the accumulation period, together with sodium orthovanadate (100 μM) to prevent vacuole-located Ca^{2+} -ATPase activity. In these cases, valinomycin was added 30 s after termination of Ca^{2+} loading with FCCP. In all experiments, four samples were taken during the period 30–120 s after valinomycin addition, a mean vesicular Ca^{2+} accumulation determined, and the mean change in intravesicular Ca^{2+} calculated with reference to the sample taken just prior to addition of valinomycin. Absolute levels of Ca^{2+} accumulation prior to valinomycin addition were (nmol/mg) control: 6.3 ± 0.25 , EGTA: 9.3 ± 0.61 , DIDS: 4.5 ± 1.1 , PP2B: 3.6 ± 0.28 , calmodulin: 2.8 ± 0.3 and PP2B with calmodulin: 1.7 ± 0.17 . Each value represents the mean $\pm$ S.E.M. of three or six different trials from one or two membrane preparations.

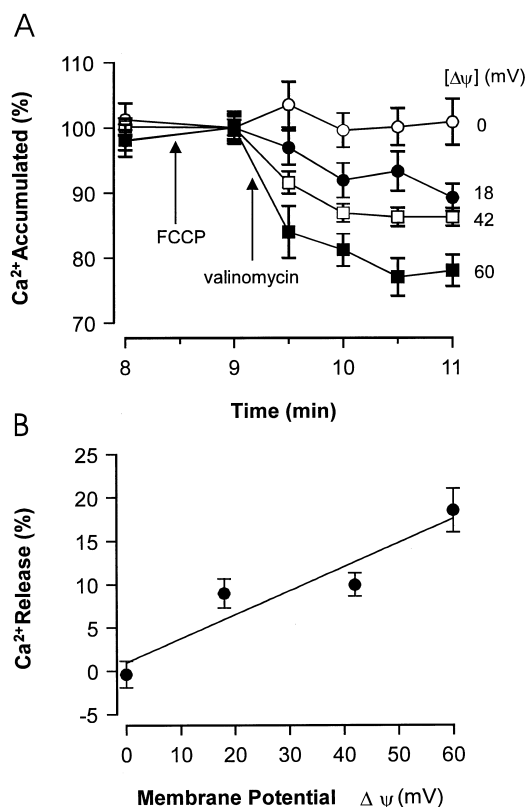


Fig. 1. Voltage-dependent Ca^{2+} release from vacuolar vesicles. Red beet vacuolar vesicles containing 25 mM potassium gluconate were loaded for 8 min with $^{45}\text{Ca}^{2+}$ as described in Section 2. The extravesicular K^+ concentration was 25, 12.5, 5 or 2.5 mM respectively. Loading was stopped by addition of 10 μM FCCP and the membrane potential ($\Delta\psi$) was clamped to either 0 ($\circ$), 18 ($\bullet$), 42 ($\square$) or 60 ($\blacksquare$) mV by the addition of 10 μM valinomycin as shown. A: Vesicular Ca^{2+} content as a function of time at different clamp potentials. Accumulated Ca^{2+} was normalised prior to addition of valinomycin (100% = 5.3 ± 0.31 nmol Ca^{2+} /mg). Each point is the mean $\pm$ S.E.M. for 12 independent determinations from four different preparations. B: Calcium release from vesicles averaged over the period 90–180 s after imposition of the K^+ diffusion potential and expressed as % of the total amount of accumulated Ca^{2+} . Data from A.

were used to generate potentials of 0, 18, 42 and 60 mV, respectively.

Fig. 1A demonstrates that imposition of a cytosol-positive K^+ diffusion potential resulted in release of Ca^{2+} . Calcium release was nearly complete after 30 s, and the extent of Ca^{2+} release exhibited a dependence on the magnitude of the imposed voltage. The apparent voltage dependence of steady-state Ca^{2+} release depicted in Fig. 1B is in accord with electrophysiological data obtained on the SV channel which show progressive channel opening over the range of potentials positive of zero [25]. It should be noted that the voltage dependence of Ca^{2+} release from membrane vesicles must represent a channel gating phenomenon, since the electrochemical driving force for Ca^{2+} release from the vesicles actually decreases with the imposition of increasingly positive membrane potentials.

To clarify further the identity of the voltage-activated Ca^{2+} mobilisation pathway, we explored the sensitivity of Ca^{2+} release to the stilbene derivative DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid), an SV channel antagonist

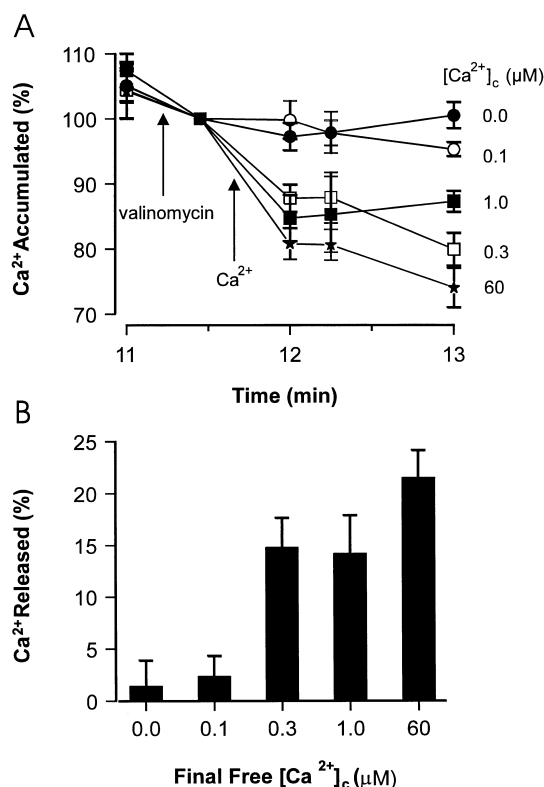


Fig. 2. Voltage-dependent Ca^{2+} release is dependent on cytosolic free Ca^{2+} . Vacuolar vesicles were loaded (starting time 0) with $^{45}Ca^{2+}$ in a medium containing 2.5 mM K^+ and loading was terminated by addition of FCCP. After a further 30 s, free Ca^{2+} in the loading medium was reduced to 5 nM by addition of 150 μ M EGTA. This resulted in negligible Ca^{2+} loss ($2 \pm 1.5\%$ drop of total accumulated Ca^{2+}). Subsequently, valinomycin was added 30 s after the addition of EGTA and after a further 30 s the free $[Ca^{2+}]_c$ in the loading medium was adjusted by adding either Ca^{2+} free buffer (●), or $CaCl_2$ to create free $[Ca^{2+}]_c$ of 0.1 μ M (○), 0.3 μ M (□), 1 μ M (■), 60 μ M (★). Accumulated Ca^{2+} has been normalised to the sample taken prior to the addition of Ca^{2+} (100%). The absolute values (in nmol Ca^{2+} /mg) were respectively for 0 $[Ca^{2+}]_c$: 4.7 ± 0.29 , 0.1 μ M $[Ca^{2+}]_c$: 4.1 ± 0.28 , 0.3 μ M $[Ca^{2+}]_c$: 5.6 ± 0.29 , 1 μ M $[Ca^{2+}]_c$: 4.1 ± 0.28 and for 60 μ M $[Ca^{2+}]_c$: 5.3 ± 0.31 . Each point is the mean $\pm$ S.E.M. for 6–9 independent determinations from two or three different preparations. B: Calcium released from the vesicles averaged from separate time points over the displayed time course, and expressed as % of the total accumulated Ca^{2+} , after titration of Ca^{2+} into the reaction medium. Data from A.

[36]. When applied at 10 μ M the covalently-binding inhibitor DIDS completely antagonised Ca^{2+} release from vesicles when triggered by a 60 mV voltage clamp (Table 1). Since DIDS has been reported to inhibit more than one vacuolar channel type [37] a more specific study was undertaken.

Phosphorylation of the SV channel has been mooted as a mechanism for regulating its activity [26,38] and indeed SV channel currents are potently inhibited by protein phosphatase 2B (PP2B) in the presence of its activator calmodulin (CaM) [22]. Table 1 shows that, when applied in the absence of CaM, PP2B was moderately effective in inhibiting voltage-dependent Ca^{2+} release from the vesicles, while CaM alone had no effect upon the release. However, jointly, PP2B and CaM completely blocked voltage-dependent Ca^{2+} release.

The dependence of Ca^{2+} release on cytosolic free Ca^{2+} was tested by chelating extravesicular Ca^{2+} with EGTA subsequent to loading. After addition of 150 μ M EGTA, the free

Ca^{2+} in the extravesicular medium was calculated as 5 nM and this treatment completely blocked voltage dependent Ca^{2+} release. Fig. 2A,B demonstrates that only in conditions where the extravesicular $[Ca^{2+}]_c$ was 300 nM or higher was Ca^{2+} release observed after imposition of a membrane potential of 60 mV. This suggests that activation of the voltage-sensitive release pathway is associated with elevation of $[Ca^{2+}]_c$ over a physiologically-meaningful range.

4. Discussion

The present results strongly suggest that CICR occurs in *Beta vulgaris* L. tap root cells and that voltage and Ca^{2+} -sensitive Ca^{2+} release is mediated through SV channels. First, activation is observed in the cytosol-positive range of membrane potentials over which the SV channel is known to activate [22,25]. Second, Ca^{2+} release is totally inhibited by DIDS [21] and also by PP2B in the presence of CaM [22], antagonists known to down-regulate SV channel activity strongly. Third, the dependence of Ca^{2+} release on extravesicular Ca^{2+} mirrors the range of free Ca^{2+} over which SV channel-mediated currents are activated [23,25,26]. All these lines of evidence support a role for the SV channel in CICR in plants.

The SV channel behaves as a multi-ion pore which confounds classical electrophysiological analysis of its ionic permeability [28], and hence a detailed understanding of the Ca^{2+} release properties of the channel. Previously, applying the patch clamp technique, researchers have been unable to distinguish between the ionic components which comprise the SV channel mediated currents. Using a radio tracer ($^{45}Ca^{2+}$), it has been possible to follow the movements of one ion upon SV channel activation. The results displayed in Fig. 1 and Table 1 demonstrate that we are able to use the radiometric assay to observe the Ca^{2+} conductance of the SV channel in red beet vacuolar vesicles.

The present data seem to contradict studies of Pottosin et al. [29] who proposed that the open probability (P_o) of SV channels depends on the prevailing Ca^{2+} electrochemical gradient rather than on $[Ca^{2+}]_c$ per se, in such a way that channel activation would exclusively allow Ca^{2+} efflux from the cytoplasm. Thus, SV channels would be prevented from mediating CICR. However, these observations were made in the absence of other divalent ions [29], and notably the presence of mM cytoplasmic Mg^{2+} , as is the case in our experimental system, has now been shown to drastically affect the SV channel P_o by shifting the channel activation potential up to 60 mV negative [39]. Since cytosolic Mg^{2+} concentrations in plant cells are around the millimolar level [40] these data support the notion that SV channels can mediate CICR.

In addition, earlier studies (e.g. [27]) have shown substantial SV currents in conditions where E_{Ca} is negative of the membrane potential. In such conditions Reifarth et al. [27] measured a 4 pA current. Recent studies on the ionic selectivity of SV channels have concluded that in physiological conditions about 4% of the current is carried by Ca^{2+} , with the remainder being a K^+ current [41]. These estimates permit a realistic appraisal of the ability of SV channels to mediate CICR in vivo. For a whole vacuole SV channel current of 4 pA [27], of which 4% is a Ca^{2+} current [41], a cytosol volume of 10^{-15} l and the cytosolic buffer capacity for Ca^{2+} permitting only 2% of released Ca^{2+} to enter the free Ca^{2+} pool [42], we can

calculate that the rate of change of $[Ca^{2+}]_c$ as a result of SV channel activity would be 10 nM/s. This $[Ca^{2+}]_c$ change is of similar magnitude to that reported for whole cells in response to a range of natural and artificial stimuli, including cold, osmotic shock and inositol trisphosphate [11,12,15,43]. The amplification of Ca^{2+} signals by CICR in conjunction with the considerable Ca^{2+} store in the vacuolar lumen and the large cytosol-directed electrochemical potential difference for Ca^{2+} amounting to -22 kJ/mol [2], necessitates that the feed-forward CICR mechanism is countered by a negative feedback control. The SV channel has indeed been postulated to have stringent controls governing its activity. It seems likely that one such mechanism will be inhibition of channel activity by Ca^{2+} -dependent phosphorylation which will result in considerable attenuation of SV channel-mediated Ca^{2+} mobilisation at high $[Ca^{2+}]_c$.

In conclusion, our study shows that the SV channel is capable of eliciting Ca^{2+} release, and that it displays properties indicative of CICR. Additionally, the SV channel has a negative feed back control via PP2B and calmodulin to ensure that Ca^{2+}_c does not rise to levels that will endanger the cell.

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