# 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 $^{45}Ca^{2+}$ 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.**



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

## 1. Introduction

Elevation of cytosolic free $Ca^{2+}$ ($[Ca^{2+}]_c$) 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 $[Ca^{2+}]_c$, 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 $[Ca^{2+}]_c$, 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 $[Ca^{2+}]_c$, with the second phase indicative of CICR [12], or to oscillations in $[Ca^{2+}]_c$, 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 $[Ca^{2+}]_c$ 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 $[Ca^{2+}]_c$ [17,18]. A hyperpolarisation-activated $Ca^{2+}$-permeable channel in the same membrane is also insensitive to $[Ca^{2+}]_c$ [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 $[Ca^{2+}]_c$ 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 μg/ml soybean trypsin inhibitor, 1 μg/ml leupeptin, 0.5 mM PMSF (phenylmethylsulphonyl fluoride) and 5 mM benzamidine HCl. Soybean trypsin inhibitor (1 μg/ml) and leupeptin (1 μg/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 $^{45}Ca^{2+}$ via an ATP-generated proton motive force [32] as previously described [7] with the following modifications: 10 μM $CaCl_2$ contained 5.92 kBq $^{45}CaCl_2$ (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*

$^{45}CaCl_2$ 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 $^{45}Ca^{2+}$ by initiating $Ca^{2+}/H^+$ antiport driven by the vacuolar $H^+$-ATPase [5], then applied a range of cytosol-positive $K^+$ diffusion potentials. External (≡cytosolic) $K^+$ concentrations of 25, 12.5, 5 and 2.5 mM were used to generate potentials of 0, 18, 42 and 60 mV, respectively.

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

| Addition | $Ca^{2+}$ release at $\Delta\psi \equiv 60$ 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 $^{45}Ca^{2+}$ 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 ± S.E.M. of three or six different trials from one or two membrane preparations.

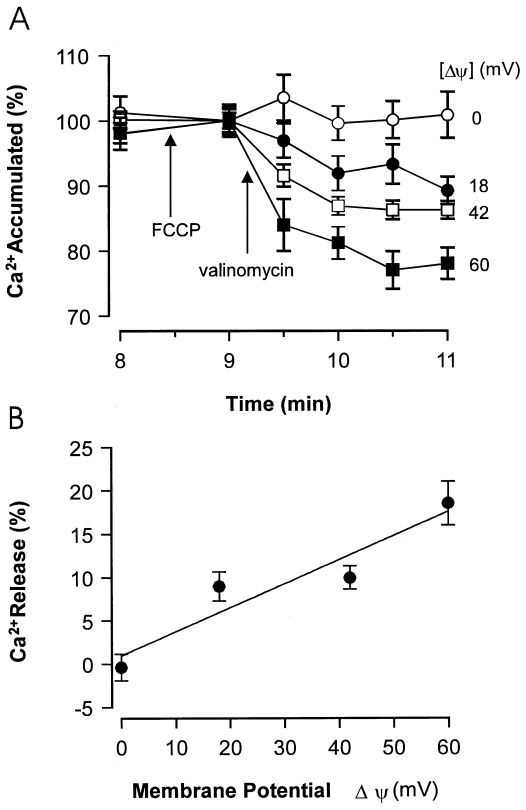


Fig. 1. Voltage-dependent $Ca^{2+}$ release from vacuolar vesicles. Red beet vacuolar vesicles containing 25 mM potassium gluconate were loaded for 8 min with $^{45}Ca^{2+}$ as described in Section 2, The extravesicular $K^+$ concentration was 25, 12.5, 5 or 2.5 mM respectively. Loading was stopped by addition of 10 μM FCCP and the membrane potential ($\Delta\psi$) was clamped to either 0 (○), 18 (●), 42 (□) or 60 (■) 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 ± 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.

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′-disulphonic acid), an SV channel antagonist

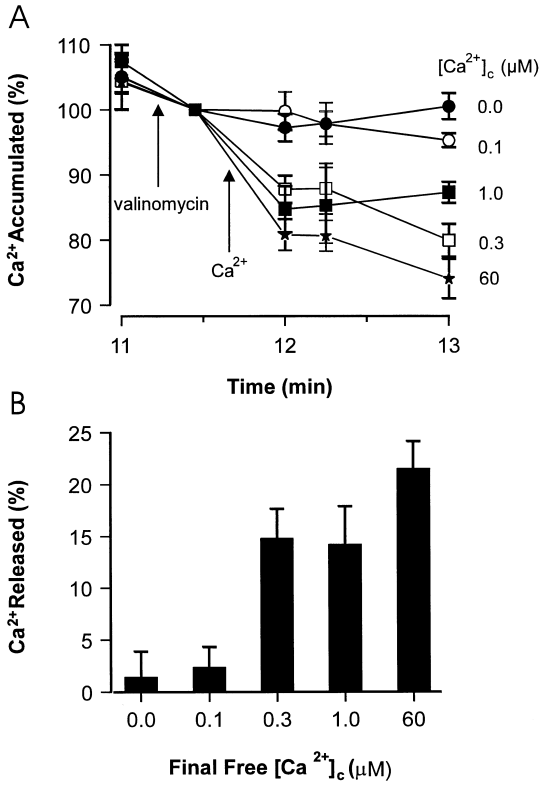


Fig. 2. Voltage-dependent $Ca^{2+}$ release is dependent on cytosolic free $Ca^{2+}$. Vacuolar vesicles were loaded (starting time 0) with $^{45}Ca^{2+}$ in a medium containing 2.5 mM $K^+$ and loading was terminated by addition of FCCP. After a further 30 s, free $Ca^{2+}$ in the loading medium was reduced to 5 nM by addition of 150 μM EGTA. This resulted in negligible $Ca^{2+}$ loss (2 ± 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 μM (○), 0.3 μM (□), 1 μM (■), 60 μM (★). 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 μM $[Ca^{2+}]_c$: 4.1 ± 0.28, 0.3 μM $[Ca^{2+}]_c$: 5.6 ± 0.29, 1 μM $[Ca^{2+}]_c$: 4.1 ± 0.28 and for 60 μM $[Ca^{2+}]_c$: 5.3 ± 0.31. Each point is the mean ± 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 μM 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 μM 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 $[Ca^{2+}]$ 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 feed-back 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 $Ca_c^{2+}$ does not rise to levels that will endanger the cell.

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