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SV channels dominate the vacuolar Ca^{2+} release during intracellular signaling

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ABSTRACT

Vacuoles have long been suggested to mediate a rise in the cytosolic free Ca^{2+} during environmental signal transduction. This study addresses the issue of the control of vacuolar calcium release by some of the known signaling molecules such as IP_3, cADPR, ABA, ATP, cAMP, cGMP, H_2O_2 and CaM. Over 30 concentrations and/or combinations of these signaling compounds were studied in a series of electrophysiological experiments involving non-invasive ion flux measurements (the MIFE) and patch-clamp techniques. Our results suggest that calcium, calmodulin and nucleotides cause calcium release via SV channels.

1. Introduction

Environmental signal transduction in plants is always associated with the rapid elevation in the cytosolic free Ca^{2+} [1,2]. Both internal (such as ER or vacuole) and external (plasma membrane channels) Ca^{2+} sources have been implemented, encoding the specificity of the above signaling.

With the vacuole being the largest intracellular pool of free Ca^{2+}, Ca^{2+} release from the vacuole was reported to be involved in the cellular responses to a variety of environmental stimuli such as cold shock, salinity, drought, and Al toxicity [1,3,4]. The precise signaling pathways by which a particular environmental stimulus may trigger a vacuolar Ca^{2+} release require further elucidation. A plethora of signaling molecules have been suggested including ABA [5], ROS [6], IP_3 and cADPR [7], cyclic mononucleotides [8], CaM [9] and ATP [10]. The specific identity of the tonoplast Ca^{2+} permeable channels potentially mediating the stress-induced Ca^{2+} release from the vacuole remains elusive. Although four different vacuolar Ca^{2+} channels – two voltage-dependent and two ligand (IP_3, cADPR) -gated [1,2] – have been postulated in the literature, only depolarization-activated slow vacuolar (SV) channels have been unequivocally documented so far [11]. In *Arabidopsis* the SV channel is a product of *Atpc1*, encoding the unique double-pore Ca^{2+} channel [12]. This channel is ubiquitous and abundant in all higher plant tissues. Ward and Schroeder [13], who were first to discover the Ca^{2+} permeability of the SV channel, on the basis of the SV channel activation by cytosolic Ca^{2+} proposed that these channels should mediate a Ca^{2+}-induced Ca^{2+} release. Yet the potency of the SV channels to make a substantial contribution to a vacuolar Ca^{2+} release is a matter of debates [14–18].

The purpose of this study was to examine the role of SV channels in vacuolar Ca^{2+} release and to identify second messengers capable of eliciting such release during environmental stress signaling.

2. Materials and methods

Fresh beet taproots were purchased from the local market. Vacuoles were released by incubating root slices in the standard bath solution for five minutes before dissecting needles were used to tear the slices apart. Solution composition was: 100 mM KCl; 1 mM HEPES, pH 7.4 (adjusted with KOH); osmolality 500–650 mOsm (depending on root age) adjusted with sorbitol. Under the microscope 10–20 vacuoles were selected in 5–6 μL of the isolating medium and transferred to the experimental chamber

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containing ~1 mL of fresh bath solution. Vacuoles of a diameter 40–60 μm were selected for measurements.

Net Ca^{2+} fluxes across the tonoplast were measured using the MIFE technique and assuming spherical geometry diffusion [19]. Fabrication and calibration of Ca^{2+} ion-selective microelectrodes were as has been described in our previous publications [19,20] except that they were calibrated in the background of 100 mM K^+ to account for the effect of bath ionic strength. Methodological experiments have revealed a good sensitivity and linearity of the Ca^{2+} LIX in the low micromolar concentration range, starting as low as 0.5 μM (see Supplementary data). For each individual vacuole, the background Ca^{2+} flux was routinely measured and taken into account. For each experiment the flux from 4 to 6 vacuoles was measured before an appropriate second messenger, or their combination, were added to the bath solution, and the measurements were repeated. On average, each vacuole was measured for ~1.5–2 min. The measuring chamber was then emptied, and new preparation was released into the chamber.

Patch-clamp technique was used to examine effect of selected second messengers (identified to be active in the MIFE experiments) on the SV channel activity in small (C = 1–10 pF) cytosolic side out-oriented tonoplast vesicles. Fabrication of patch pipettes, patch-clamp electronics, data acquisition protocols and non-linear regression fitting of the data were essentially as described in our previous publications [21]. The pipette solution was 120 mM KCl, 62 mM NaCl, 8 mM $MgCl_2$, 0.2 mM $CaCl_2$; 10 mM MES/KOH, pH 6.0; osmolality 640 mOsm (sorbitol). The basic bath solution was 100 mM KCl, 0.66 mM free Mg^{2+}, 7.5 μM free Ca^{2+}; 10 mM HEPES adjusted to pH 7.4 with KOH and 600 mOsm with sorbitol. The free divalent cation concentrations were calculated using WinMAXC[32] v.2.50 (Chris Patton, Stanford University) software. Chelating potency of ATP on free Ca^{2+} and Mg^{2+} concentrations was accounted for.

3. Results

The effect of IP_3, ABA, ATP, cAMP, cGMP, cADPR, H_2O_2, CaM, and Ca^{2+} known to be involved in various intracellular signaling pathways were studied in MIFE experiments. These were used in various concentrations and/or combinations (>30 combinations in total). Some of the results are summarized in Fig. 1. In general, a significant ($P < 0.01$) increase in Ca^{2+} release was observed in response to ABA, ATP, cAMP and Ca^{2+} treatment, while vacuolar Ca^{2+} efflux was strongly suppressed by H_2O_2. No significant ($P < 0.05$) effects of cGMP (10–100 μM), IP_3 (2 μM) or cADPR (1 μM) were found (Fig. 1).

The observed stimulatory effects of signaling compounds on Ca^{2+} efflux were dependent on concentrations. In case of ABA, 50 μM concentration was stimulatory while 10 μM ABA concentration was not effective (Fig. 1). Increased Ca^{2+} efflux was observed in response to 10 μM ATP, while 100 μM concentration was not effective. Interestingly, adding 100 μM CaM to 10 μM ATP eliminated the stimulatory effect on Ca^{2+} efflux (Fig. 1). At the same time, by itself 100 μM CaM was not efficient, while 200 μM concentration

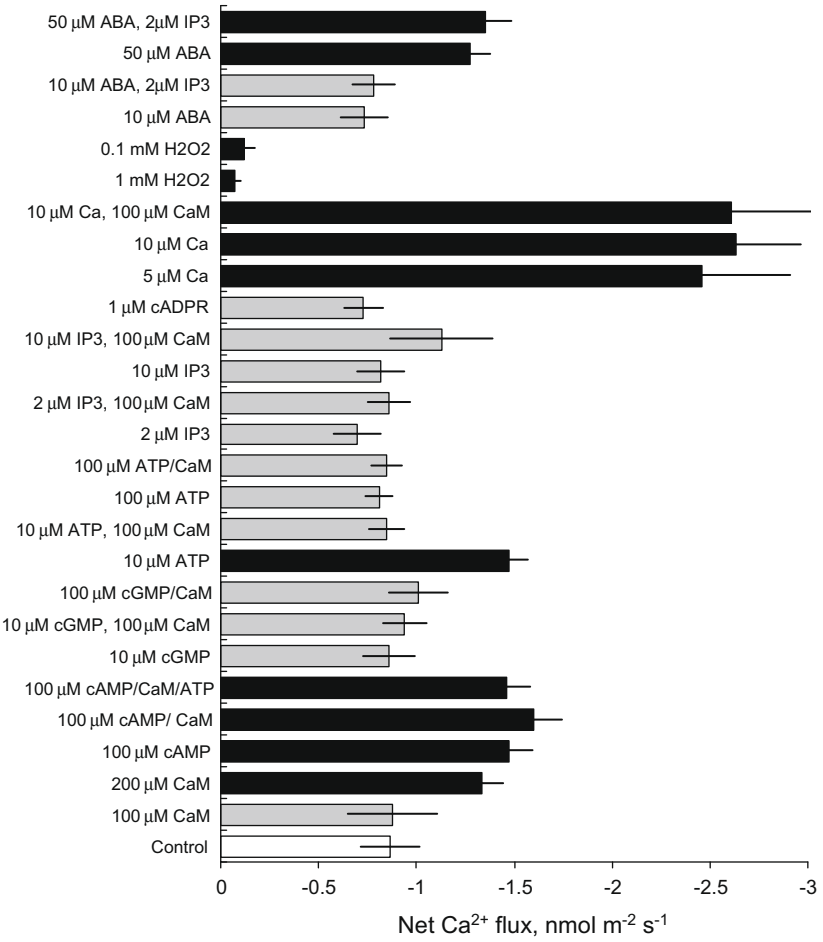

Fig. 1. Modulation of the vacuolar Ca^{2+} release by cytosolic factors. Net Ca^{2+} fluxes were measured from beet root vacuoles in control conditions and after additions of different chemicals (and their combinations). Mean ± S.E. (n = 6–14). Negative flux implies vacuolar Ca^{2+} release. Black bars – significant to control at $P < 0.05$; grey bars – not significant.

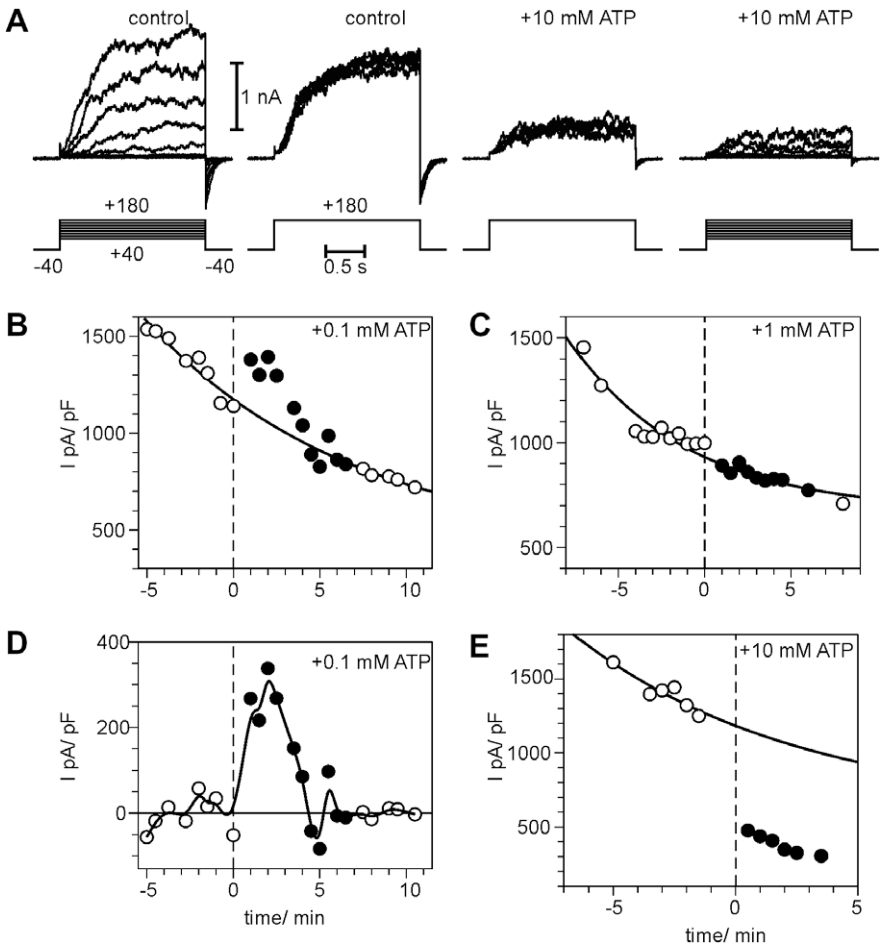

Fig. 2. Cytosolic ATP effects on the SV current in *Beta vulgaris* vacuoles. (A) High (10 mM) ATP inhibits the SV current. A stabilization of the SV current in control conditions was verified by a comparison of the magnitude of responses for a series of 8 equal steps to +180 mV delivered with a 30 s a pause between pulses. The same pulse series was repeated immediately after the ATP addition. (B–E) Effects of different ATP concentrations on the steady-state SV current at +180 mV. Open and closed circles represent control/washout and ATP treatment, respectively. Zero time corresponds to the moment of the ATP addition. Panel D shows a transient increase of the SV current upon the application of 0.1 mM ATP after the observed current rundown was subtracted from experimental data.

was stimulatory. Both 5 and 10 μM Ca^{2+} stimulated Ca^{2+} efflux from vacuoles, while 0.1 and 1 mM H_2O_2 caused a very strong inhibition (Fig. 1). Ca^{2+} fluxes were also inhibited by ~80% by 100 μM zinc (data not shown). Together with a strong stimulation of Ca^{2+} efflux by micromolar Ca^{2+} these data suggest that measured flux is mainly through Ca^{2+}-activated Zn^{2+}-sensitive SV channels [22,23].

Selected signaling compounds with pronounced effect on vacuolar Ca^{2+} fluxes were tested in patch-clamp experiments. These experiments were complicated by a well-known phenomenon of the SV channel rundown [24]. To deal with this issue, SV currents were measured in a large number (>200) of samples at various times from the vacuoles isolation into the artificial bath solution. These experiments have suggested that, despite some possible variations in the absolute current densities, the current decay due to the rundown can be described by exponential curve with a time constant $\tau \sim 20$ min (see Fig. 4 for an example). Therefore, a relative efficiency of a particular chemical might be estimated by comparing the measured SV channel currents with the "trendline current" at any particular time.

The above approach was used to study effect of various concentration of ATP on the magnitude of SV current in sugar beet vacuoles. 0.1 mM ATP applied to the bath solution has caused a transient (peaking ~2 min after application) increase in the SV current (Fig. 2B). The transient "bump" becomes obvious once the trendline is subtracted from the records (Fig. 2D). No effect of 1 mM ATP was observed (Fig. 2C), and 10 mM ATP added to the bath has caused a very substantial decrease in SV current (Fig. 2E). The representative examples of original records for control and 10 mM ATP are shown in Fig. 2A, and the dose-dependency of ATP effects on SV channels is illustrated in Fig. 3. Statistically significant stimulation of SV currents was observed for 10 and

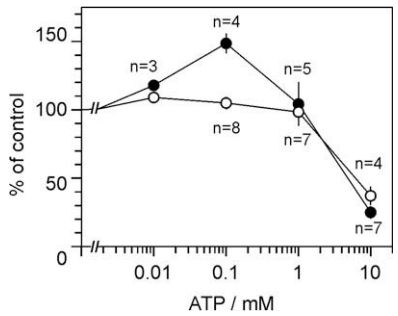

Fig. 3. Transient (closed symbols) and steady-state (open symbols) ATP effects on the SV current at +180 mV. Transient ATP-induced current increase was evaluated as shown in Fig. 2D by subtracting the mono-exponential rundown trend and measuring the peak amplitude of the relative current. Data are presented as mean ± S.E. (*n* = number of vacuoles tested).

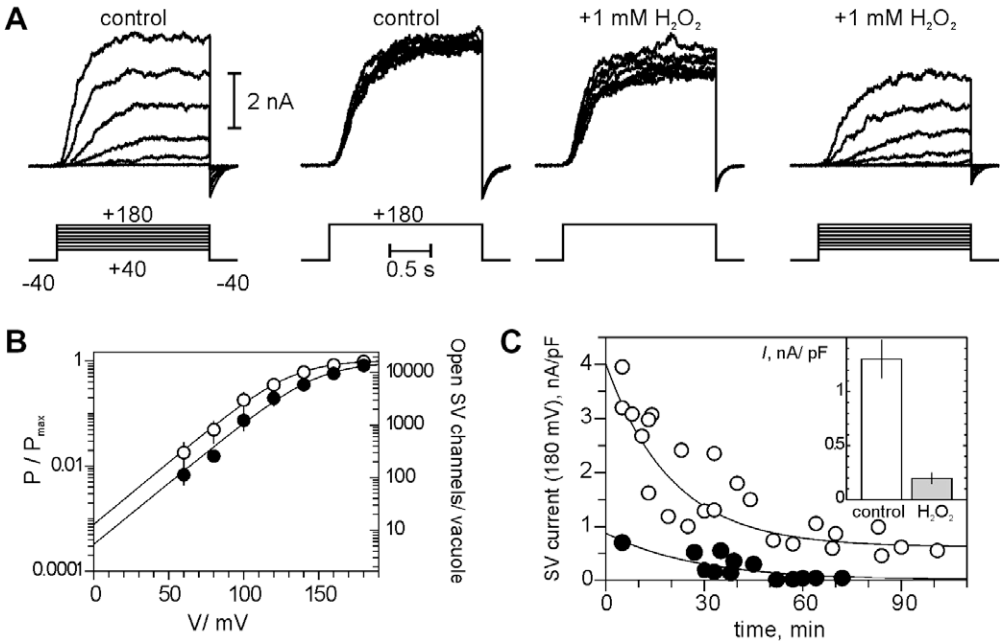

Fig. 4. Summary of the peroxide effects on the SV current. A direct application of H_2O_2 (1 mM) to the bath caused only a moderate decrease of the SV current (A) but increased a voltage threshold for the SV channels activation (B). Open symbols – control; closed symbols – H_2O_2. Mean ± S.E. (n = 3 separate vacuoles). (C) Pre-treatment with 1 mM H_2O_2 caused a dramatic decrease of the SV current density. Each point represents a separate vacuole. Inset shows the mean SV current density at +180 mV in control (open bar) and in the presence of 1 mM H_2O_2 (closed bar).

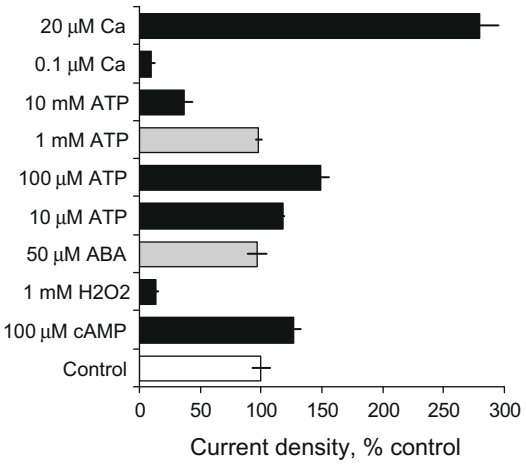

Fig. 5. Modulation of the steady-state SV current density by some second messengers added from the cytosolic side. Mean ± S.E. (n = 5–8 vacuoles). Black bars – significant to control at $P < 0.05$; grey bars – not significant.

100 μM ATP, although in both cases it was only transient. No difference in the steady-state SV currents was found for any ATP concentrations except 10 mM, which had strong inhibitory effect on SV currents (Fig. 3). At the same time, 10 mM ATP did not cause a significant effect on the SV channel activation curve (the voltage dependence of the channel open probability, result not shown).

As MIFE data showed, the application of 1 mM H_2O_2 severely (by >90%) reduced the vacuolar Ca^{2+} release. However, a direct application of 1 mM H_2O_2 to patch samples caused only a moderate (<30%) decrease of the SV current magnitude. At the same time, when vacuoles were pre-treated in H_2O_2 solution for long enough (e.g. >30 min), a very strong (>80%) suppression of the SV current was found in the presence of 1 mM H_2O_2 in the bath (Fig. 4C). A difference with a direct effect may be caused by a fact that under patch-pipette perfusion a much lower concentration of H_2O_2 is present at the vacuolar side of the tonoplast patch. Alternatively/additionally some vacuolar factors, washed out by a perfusion with a patch-pipette, could mediate the effect of H_2O_2 on the SV channels. Overall, changes in the SV current density for most substances tested (Fig. 5) were consistent with changes in net Ca^{2+} efflux measured by the MIFE technique (Fig. 1).

4. Discussion

Due to the limitation of the MIFE technique, all Ca^{2+} flux measurements were performed in non-buffered media containing ~2 μM Ca^{2+} [25]. Thus, some caution is needed in extrapolating these results to *in planta* situation. Nonetheless, in most cases a good agreement between MIFE and patch-clamp results was observed (Figs. 1 and 5). A pronounced increase of the vacuolar Ca^{2+} release caused by an increase in cytosolic Ca^{2+} was consistent with the Ca^{2+}-activation of the SV current in patch-clamp studies [14,15,22,25] indicating that SV channels mediated a substantial portion of Ca^{2+} flux through the tonoplast. At 2 μM Ca^{2+} the SV channel activity was approximately a half of that observed at 20 μM and the dose-dependency revealed a Hill coefficient ~1 for Ca^{2+} binding [22,25]. Therefore, the SV channel open probability increases ~5 times upon the change from the resting (~0.2 μM) cytosolic Ca to 2 μM. However, due to a concomitant decrease of the Ca^{2+} gradient upon the cytosolic Ca^{2+} increase from 0.2 to 2 μM, the actual increase in the net SV channels' mediated vacuolar Ca^{2+} release will be smaller than a respective increase of the channel's open probability, about threefold instead of fivefold. The higher activity of SV channels under control conditions in MIFE experiments may, therefore, preclude the detection of the effects induced by some of the chemicals tested such as IP_3, ABA and calmodulin. Assuming this was the case, however, such responses, if any, should be relatively small.

Apart from the requirement for using non-buffered media, the conditions of usage of non-invasive microelectrode ion flux measuring technique are less artificial then those for patch-clamp measurements, as the vacuolar composition remains intact. Addi-

tionally, the MIFE method reports net Ca^{2+} fluxes, whereas patch-clamp records a total channel-mediated net ionic current, which in the case of SV channels is mainly carried by monovalent cations under physiological ionic conditions [11]. This approach, therefore, was used to screen a large number of second messengers known to be capable to elicit elevated $[Ca^{2+}]_{cyt}$.

No effects alone or in combination with any other chemicals were observed for IP_3 or cADPR. Several possible explanations may be given. First, the effect may be below the MIFE detection limit. However, as the latter is <1 pA per typical vacuole, the physiological contribution of the ligand-gated channels should be really small, at least under our conditions. More likely, the reported ability of this second messenger to affect vacuolar Ca^{2+} release [7] is either indirect, or is tissue specific, implying that ligand – gated Ca^{2+}-release channels are not expressed in the tonoplast of *Beta*. Finally, the expression of such channels may be induced by environmental factors as suggested in [26], which was not the case in our study.

The effect of ATP on Ca^{2+} fluxes was variable and ranged from transient stimulation at 100 μM to inhibition at 10 mM (Fig. 4). Such transient elevation (lasting for only few minutes: Fig. 2B–D) may explain an apparent absence of 100 μM ATP effects in MIFE experiments, where Ca^{2+} fluxes were measured much later after the peak Ca^{2+} current was observed. As high concentrations of ATP could mask the Ca^{2+} release by chelating Ca^{2+}, concentrations of 1 and 10 mM ATP were not explored in MIFE experiments.

In the presence of 100 μM CaM, no significant effects of ATP on Ca^{2+} flux was observed, suggesting CaM may alter the effect of ATP. Previous reports, based on patch-clamp experiments with barley aleuron protein-storage vacuoles (PSVs), have suggested that at 200 μM ATP, SV channel activity decreases because of phosphorylation, though in the same series of experiments 2 mM ATP activated SV, possibly through enhanced activation of a protein kinase [10]. These authors suggest a complex regulation of the SV channel by protein phosphorylation at two sites. Our data showed here supports this two-phosphorylation site model. However, in our case the direction of effects at low and high ATP levels (Fig. 3) was opposite to that observed for PSVs [10] suggesting that inhibitory and stimulatory phosphorylation sites may be exchanged. Overall, such "switch" from SV channel inhibition to stimulation in the physiologically-relevant range of ATP concentrations [27] is consistent with the potential role of ATP as a signaling compound [28]. Bethke and Jones [10] also observed a stimulatory effect on the SV current of a non-hydrolysable ATP analogue AMP–PNP, which implies that the binding of a nucleotide and not necessarily the ATP hydrolysis is at least in part responsible for the effect. Interestingly, cAMP also significantly increased the vacuolar Ca^{2+} release through SV (Figs. 1 and 5).

Significant Ca^{2+} efflux only occurred only at high levels of ABA indicating a threshold of response (Fig. 1). Therefore, physiologically relevant ABA fluctuations are not likely to induce a release of Ca^{2+} from the vacuole, at least under elevated cytosolic Ca^{2+} levels as in our experiments. Consistent with this notion, ABA did not affect SV currents in barley aleuron vacuoles [29], and no difference in ABA effects on root growth was found between WT and Arabidopsis *tpc1* knockout mutant [18]. However, it cannot be excluded that different ABA signaling networks may exist in different tissues, not that the threshold of ABA sensitivity may differ between species. Thus, the results reported here for storage root vacuoles have to be conducted on some other tissues/species before the role of SV channels as a downstream target of ABA signaling can be ruled out.

Exogenous application of peroxide significantly reduced both Ca^{2+} efflux (Fig. 1) and SV currents (Fig. 4) in our experiments. Kawano et al. [6] have demonstrated that cytosolic Ca^{2+} increase in response to Al^{3+} was mediated by ROS and required the expression of TPC1, a double pore Ca^{2+} channel, whose identity with the SV channel has been proved [12]. Taken together, their results and our data suggest that peroxide applied exogenously to intact cells may stimulate the SV channel-mediated Ca^{2+} release by some downstream messenger rather than directly interacting with the channel itself. It is well established that the SV channels from different preparations are stimulated by reducing conditions [24,30]. It was also proposed that H_2O_2, due to its oxidizing effects on both CaM and calcineurin, may impact the SV channel activity in vivo [31]. Our results suggest that the inhibitory effects of H_2O_2 on the SV channels are conducted, at least in part, from the vacuolar interior. More detailed study of reactive oxygen species interactions with SV channels should be undertaken in a separate investigation.

In conclusion, this work has demonstrated that vacuolar calcium release from *Beta vulgaris* was mediated mainly by tonoplast SV channels under experimental conditions of this study. We have also revealed the efficiency of some of the known intracellular signaling compounds to control the vacuolar Ca^{2+} release. This data should be taken into the account when particular intracellular Ca^{2+} signaling cascades (e.g. environmental stress signaling) are considered.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.02.009.

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