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

Igor Pottosin [a], Tim Wherrett [b], Sergey Shabala [b,*]

[a] Centro de Investigaciones Biomédicas, Universidad de Colima, Mexico
[b] School of Agricultural Science, University of Tasmania, College Rd, Private Bag 54, Hobart, Tas 7001, Australia

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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

* Corresponding author. Fax: +61 3 62622642.
E-mail address: Sergey.Shabala@utas.edu.au (S. Shabala).

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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

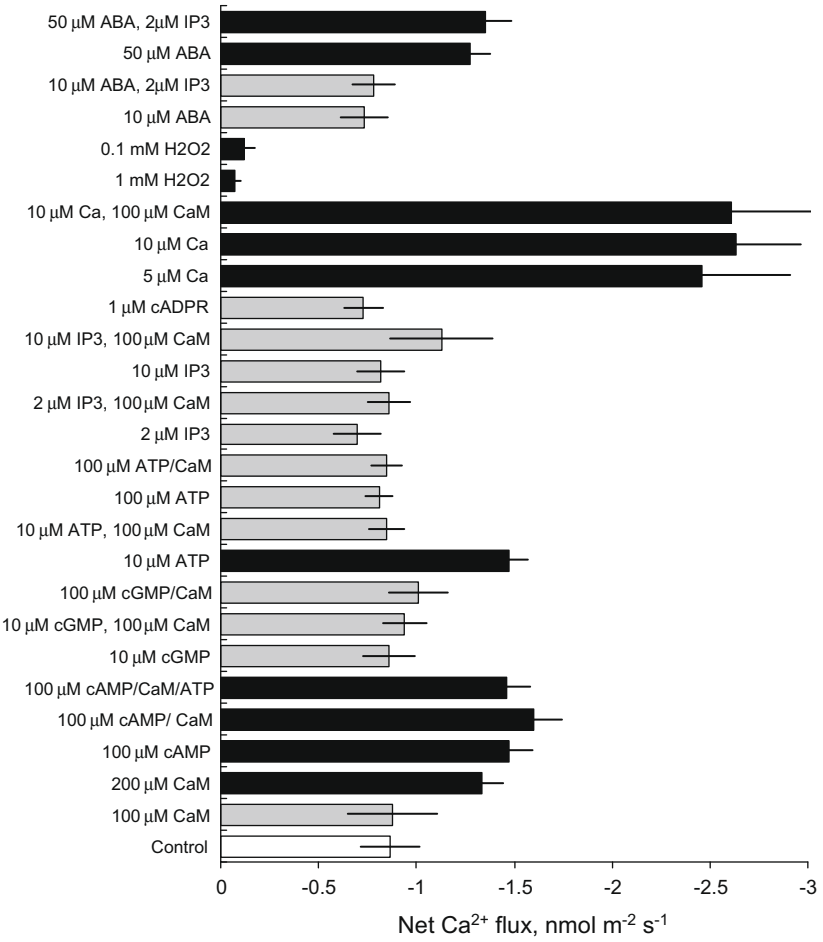

Fig. 1. Modulation of the vacuolar Ca^{2+} release by cytosolic factors. Net Ca^{2+} fluxes were measured from beet root vacuoles in control conditions and after additions of different chemicals (and their combinations). Mean ± S.E. (n = 6–14). Negative flux implies vacuolar Ca^{2+} release. Black bars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