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Modulation of calcium and potassium permeation in plant TPC channels

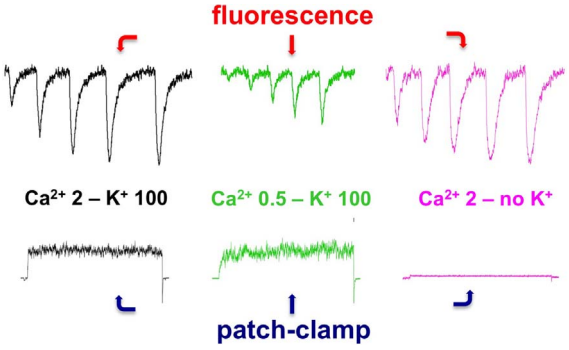
Armando Carpaneto[a,b,*], Antonella Gradogna[b]

[a] Department of Earth, Environment and Life Sciences (DISTAV), University of Genoa, Viale Benedetto XV 5, 16132 Genova, Italy
[b] Institute of Biophysics, CNR, Via De Marini 6, 16149 Genova, Italy

HIGHLIGHTS

- By using patch-clamp and fluorescence, we quantify the flow of calcium and potassium through plant TPC channels;
- changes in calcium and cytosolic potassium are strongly reflected in the permeation of these ions across the channel;
- currents alone should be taken with caution in interpreting the movements of individual ion species in cation channels.

GRAPHICAL ABSTRACT



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ABSTRACT

Plant two-pore channels (TPCs) are non-selective cation channels permeable both to monovalent potassium and divalent calcium. We previously developed a technique that allowed the simultaneous determination of the fluxes of these two ions across the channel by a combined use of patch-clamp and fluorescence. In this paper we studied how potassium and calcium fluxes were influenced by modification of cytosolic concentrations of K^+ and Ca^{2+}. A decrease in cytosolic calcium from 2 to 0.5 mM led to a shift of the activation curve of about +60 mV; although at positive potentials currents were very similar, calcium ion permeation was significantly reduced and the ratio between the total and calcium-mediated current increased about two-fold. Upon removal of cytosolic potassium, in the presence of 2 mM cytosolic calcium, the voltage-dependent activation curve was not modified but a dramatic reduction of the currents at positive voltages was apparent. However, calcium permeation did not change significantly in this condition. This work demonstrated that the electrophysiological measurements alone were not capable to predict the extent of the flow of different ions through cation channels. The parallel use of calcium detection by fluorescent dyes proved to be a valuable tool for the correct quantification of the permeation mechanisms in non-selective ion channels.

1. Introduction

TPCs are intracellular channels in animals and plants [1]. Human cells have two isoforms: TPC1 and TPC2, located respectively in endosomes and lysosomes. Their functional properties have been studied by using different techniques including functional reconstitution in planar lipid bilayer [2], use of enlarged endo-lysosomes [3–6] and expression in heterologous systems [7–10]. Human TPCs are sodium selective ion channels, with marginal potassium permeability. Calcium permeability is still a controversial point; it should be lower than the sodium one but it is not clear whether it is sufficient to drive a significant cytosolic calcium increase [11]. The model plant, *Arabidopsis*

* Corresponding author.
E-mail address: armando.carpaneto@unige.it (A. Carpaneto).

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thaliana, has a single tpc gene coding for a non-selective cation channel residing in the vacuolar membrane and permeable to both potassium and calcium [12]. The physiological role of the plant TPC is elusive: the channel is open at positive potentials far from the resting voltage of the vacuole (about −20 mV) and needs non-physiological high concentrations of calcium (up to 1 mM) to be active [13]. From the functional point of view, plant TPC was one of the first plant channels to be characterized and it has been found in all species so far investigated, even in sweet-water pond [14] or marine [15] plants. Because of its slow time of activation (hundreds of ms), the current was named SV (Slow Vacuolar) [16]. In 2005 Peiter et al. [12] clearly demonstrated that, in *Arabidopsis*, SV channels were encoded by the tpc1 gene. Recently, AtTPC1 has been crystallized by two different groups [17,18]; the protein has 12 transmembrane domains associated in a dimer to form a functional channel. Each monomer is composed by two similar shaker domains so that the structure looks like a tetramer. Interestingly, the link between the two shaker domains has two EF-hands facing the cytosolic side, which give to the plant channel a very strong dependence on cytosolic calcium [19]. Human counterparts do not possess EF-hands domains, even if the activation of human TPC1 can be facilitated by intracellular calcium, albeit this effect is not as pronounced as in the plant channel [9]. Plant TPC1 has been studied intensively. The channel is modulated by a variety of parameters such as oxidizing and reducing agents [20–24], the voltage protocol [25,26], magnesium [27], divalent ions [28,29], polyamines [30], antibiotics [31], ruthenium red [32], polyunsaturated fatty acids [33]. Interestingly, plant TPC has a cytosolic pH optimum for its activity [21], similar to other animal channels [34]. Nicotinic acid adenine dinucleotide phosphate (NAADP), a very powerful Ca^{2+} mobilizing messengers [35], and phosphatidylinositol-3,5-bisphosphate, $PI(3,5)P_2$, a low-abundance signaling lipid associated with endo-lysosomal and vacuolar membranes in eukaryotic cells [36] recently discovered to inhibit the plant vacuolar anion/H^+ exchanger AtCLC-a [37], were both able to activate human TPCs [3,4,9,38–40] but were not effective on plant TPCs [38]. Vacuolar calcium is also a strong modulator of plant TPC functionality: an increase in its concentration decreases the activity of the channel by shifting the open probability to positive voltages [13]; the molecular bases of this effect were elucidated [41]. Human TPC1 has a similar modulation although with different molecular determinants, still object of investigation [9]. The physiological function of the plant TPC1 has not yet been clarified; recent works suggest an involvement in salinity stress by the modulation of the associated intracellular calcium waves [42] and in plant pathogen interaction [43]. Potassium and calcium permeation has been studied by typical electrophysiological approaches, i.e. by measuring the reversal voltage and current intensity upon significant changes in the concentration of the two ions [13,44,45]. However, since the physiological concentration of potassium in the cytosol of a plant cell and inside the vacuole (about 100 mM) is much higher than the concentration of calcium (from 100 to 1000 nM in the cytosol and up to 1 mM in the vacuole), potassium permeation is largely facilitated and it is difficult to quantify the real flow of calcium through the channel. To address the single contribution of potassium and calcium to the total current flowing in the plant TPC channel, in a previous work, we combined the patch-clamp technique with calcium detection by the fluorescent calcium sensitive dye fura-2 [46]. We loaded the dye in the recording pipette, used the cytosolic side-out recording mode and focused the photomultiplier on the tip of the recording pipette. This configuration had many advantages: no delay in loading the fluorophore, no interference by internal calcium chelators/buffers, absence in photobleaching due to the quasi-infinite dye content inside the pipette and to the optimal solution exchange between the tip and the back of the pipette. With this technique (FLEP, fluorescence combined with excised patch), we could directly access to the flux of calcium in plant TPC channel [46].

In this work we extend that type of measurements by changing cytosolic potassium and calcium.

2. Materials and methods

2.1. Vacuole isolation and electrophysiology

Experiments were performed on vacuoles isolated from *Daucus carota* taproot parenchymal tissue.

After peeling the carrot, thin slices were cut and gently set down into the recording chamber containing the bath solution. Mechanical cutting could casually break the cell wall and plasma membrane, consequently allowing vacuole release.

Patch-clamp recordings were performed in cytosolic side-out excised patch configuration [47].

The standard bath solution was (in mM): 100 KCl, 2 or 0.5 $CaCl_2$, 2 DTT, 20 HEPES, pH 7.0 (with 5 KOH). For measurements in the absence of K^+, bath solution contained (in mM): 20 HEPES, 2 $CaCl_2$, 2 DTT, pH 7.0 (with 5 KOH). Standard pipette (vacuolar) solution was (in mM): 100 KCl, 0.1 fura-2, 20 HEPES, pH 7.0 (with 5 KOH). Free calcium in this solution (considering a Ca^{2+} contamination of 2.5 μM measured by atomic absorption spectroscopy) was estimated to be 5 nM (http://maxchelator.stanford.edu). The osmolarity of all solutions was adjusted to 420 mOsm by addition of D-sorbitol. During the experiment, bath solution was exchanged by a gravity-driven perfusion system.

2.2. Fluorescence

The recording chamber was placed on the stage of an inverted microscope (IM35 Zeiss, Germany) with a ×100 Nikon Fluor objective. Fura-2 was excited by a xenon lamp (PTI, Brunswick, USA) via a light fiber illuminating the tip of the recording pipette. The light beam crossed a rotating wheel supplied with two interference filters, centered at 340 and 380 nm respectively. The control of the rotor speed (20 rotation per second) and acquisition of emission light was performed by a computer-driven spectrophotometer system (Cairn Research, UK) connected to a photomultiplier (ThornEMI, UK). The final recording frequency was 20 Hz. Traces in the figures were filtered off-line at 1 Hz. Patch-clamp recordings and detection of the fluorescence signals were synchronized by the A/D board of the computer controlling the spectrophotometer.

2.3. Data analysis

Positive currents correspond to cations flowing from the cytoplasmic side to the lumen of the vacuole or to anions moving in the opposite direction. Unless otherwise indicated, data are reported as mean ± sem. Quantification of the fluorescence signals was performed as in [46]. Data analysis and figure preparation were done with IgorPro software (Wavemetrics, Lake Oswego, OR, USA).

3. Results and discussion

In Fig. 1 we recorded currents from carrot vacuoles by using the patch-clamp technique in the cytosolic side-out configuration. The high expression of endogenous TPC channels present on the tonoplast allowed us to have macroscopic signals. We performed experiments by decreasing cytosolic calcium from 2 to 0.5 mM. In Fig. 1a, the positive currents recorded at +80 mV showed a slower time of activation and a slight larger stationary current at 0.5 mM (green trace) compared to 2 mM (black trace) cytosolic Ca^{2+}. The deactivation currents at −80 mV are also different at 0.5 and 2 mM Ca^{2+}, decaying with a slower time constant at the lower concentration. Steady-state currents as a function of the applied voltage are shown in Fig. 1b: while currents in 2 mM Ca^{2+} started to be active at about −70 mV, the threshold of activation was more positive (at around 0 mV) in 0.5 mM calcium. For voltages larger of +50 mV the currents in 0.5 mM were slightly larger than those at 2 mM. The normalized conductance as a function of the applied potential is shown in Fig. 1c: data were fitted by a Boltzmann