The voltage-gated Ca^{2+} release channel in the vacuolar membrane of sugar beet resides in two activity states

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Received 17 February 1995; revised version received 30 March 1995

Abstract The voltage-gated Ca^{2+} release channel in the vacuolar membrane of sugar beet tap roots resides in two states which show similar reversal potentials in biionic conditions but differ dramatically in their single channel current and open frequency. State I has a unitary Ca^{2+} conductance of 3.9 ± 0.5 pS and channel openings are rare at vacuolar resting potentials. After spontaneous and reversible transition into State II the unitary Ca^{2+} conductance increases three-fold, accompanied by a concomitant rise in channel activity over the physiological voltage range. The 15- to 19-fold increase in total Ca^{2+} current after the State I to State II transition could play an essential role in channel activation during signal transduction.

Key words: Calcium channel; Signal transduction; Patch clamp; Vacuole; *Beta vulgaris*

1. Introduction

Stimulus-evoked changes in cytosolic free calcium ($[Ca^{2+}]_{cyt}$) mediate a large variety of physiological responses in plant cells [1]. Rapid elevation of $[Ca^{2+}]_{cyt}$ can be achieved by opening of Ca^{2+}-permeable channels in the plasma and vacuolar membranes which allow dissipative Ca^{2+} fluxes into the cytosol [2,3]. Previous reports have suggested that Ca^{2+} release from the vacuole, the principal Ca^{2+} pool in mature plant cells, can make a major contribution in increasing $[Ca^{2+}]_{cyt}$ in response to primary stimuli [4,5]. The importance of Ca^{2+} mobilisation from the vacuolar pool in plant signal transduction is furthermore reflected in the increasing number of different Ca^{2+} release channels which have been detected in the vacuolar membrane. Thus Ca^{2+} can be mobilised from the vacuole through ligand-gated Ca^{2+} channels which are activated in the presence of inositol 1,4,5-trisphosphate [6,7] and also through several classes of voltage-operated channels which differ in their voltage- and calcium-sensitivity [8–13]. Two such classes of Ca^{2+} release channel open over the physiological range of cytosol negative voltages. One is activated by vacuolar Ca^{2+} and insensitive towards cytosolic Ca^{2+} [8–11] whereas the other deactivates when $[Ca^{2+}]_{cyt}$ reaches 1 μM [12]. A role in vacuolar Ca^{2+} mobilisation has also been proposed for the abundant Ca^{2+}-permeable slowly activated (SV) channel which is activated by cytosolic Ca^{2+} and opens at cytosol positive voltages [13].

The plant vacuole contains millimolar Ca^{2+} concentrations [14] and, unlike the relatively small intracellular Ca^{2+} pools in animal cells, accounts for 70–90% of the volume of a mature cell. Thus activation of Ca^{2+}-permeable channels can evoke long term as well as short term elevation of $[Ca^{2+}]_{cyt}$. Conversely, the potentially lethal amounts of Ca^{2+} contained within plant vacuoles require that Ca^{2+} release is acutely controlled. The fast activated voltage-gated Ca^{2+} release channel in the vacuolar membrane of sugar beet tap roots described by us earlier [8,9,15] potentially provides a pathway for a sustained rise in $[Ca^{2+}]_{cyt}$. The open state probability of this channel increases massively over the physiological voltage range (−10 to −50 mV) and is unaffected by a rise in $[Ca^{2+}]_{cyt}$ which has been found to shut other Ca^{2+} release channels which are active over the same voltage range [12]. Although vacuolar Ca^{2+} and pH are potent modulators of channel activity [8,9,15], further control by cytosolic factors can be expected. In this report we demonstrate that this voltage-gated Ca^{2+} release channel is able to convert reversibly from a low activity state with a low unitary conductance and a severely reduced open state probability to the high activity state whose permeation and gating properties have been characterised by us in previous studies [8,9,15]. The single channel characteristics of the two channel states are compared and the physiological significance of this conversion is discussed.

2. Material and methods

2.1. Vacuole preparation and media

Vacuoles were isolated from fresh, greenhouse-grown sugar beet tap roots by slicing the tissue with a sharp razor blade and rinsing the cut surface with Medium I (see below) supplemented with 1 mM $MgCl_2$. Isolation and patch clamp media were adjusted with sorbitol to a 10% higher osmolarity than that of the tissue sap (measured with Camlab Mod 200 osmometer). The pipette solution for lumenal-side-out patches comprised 40 mM KOH, 10 mM KCl, 1 mM DTT adjusted to pH 7.3 with 20 mM dimethylglutaric acid and 10 mM HEPES (Medium I). Deviations from this composition and details of the bath solutions are specified in the figure legends. Two peristaltic pumps (Gilson Minipuls 2) allowed exchange of test solutions. Liquid junction potentials between pipette and bath solutions were calculated [16] and subtracted from the holding potential (values are indicated in the figure legends).

2.2. Patch clamp experiments

Patch clamp experiments were performed as described in [17]. The reference Ag/AgCl halfcell was filled with Medium I (without DTT) and connected to the bath via an agar bridge (3% agar in Medium I). Clamp voltages were controlled with an EPC-7 patch clamp amplifier (List Electronics, Darmstadt, Germany). Data were recorded on digital audiotape (DTC-1000 ES; Sony Corporation, Tokyo, Japan). For further analysis with the CED (Cambridge Electronic Design, Cambridge, UK) software package the data were low pass filtered (8-pole Bessel filter) at 100 Hz to 300 Hz and digitized at 1.5 kHz (1401 A/D converter, Cambridge Electronic Design).

Pipettes with a typical resistance of 20 MΩ in Medium I were pulled from borosilicate glass capillaries (Kimax, thinwalled) and coated with Sylgard. The seal resistance was > 20 GΩ.

2.3. Sign convention and presentation of data

Vacuolar membrane potentials and currents are referenced to the

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SSDI 0014-5793(95)00424-6

vacuolar lumen according to the convention for measurements on endomembranes [18] in which inward currents are those that flow into the cytosol. This convention is also adopted when we refer to other studies.

If not otherwise indicated figures show representative measurements obtained on a single patch from at least four independent recordings.

3. Results

Lumenal-side-out patches excised from sugar beet tap root vacuoles show two varieties of unitary Ca^{2+} current which differ dramatically in their gating behaviour and single channel current (Figs. 1 and 2). These two current types were never found to be simultaneously active in one patch. Furthermore we observed frequent spontaneous transitions from one type to the other. Both these observations suggest that the two current types represent different states of the same channel population. In the typical case (n = 16) the conversion is unidirectional from a low activity mode often accompanied by subconductance states (State I) to a high activity mode which displays a larger unitary conductance and uniform current levels (State II) (Figs. 1 and 2). Occasionally (n = 2), however, as illustrated in Fig. 1, the transition reverses spontaneously within the first 30 s. In cases where the channels already resided in State II at the start of an experiment (n = 37) we never observed a conversion back to State I. Current traces and amplitude histograms recorded after the State I to State II transition can not be distinguished from those in which only State II currents were observed. Subtraction or addition of the smaller State I current, however,

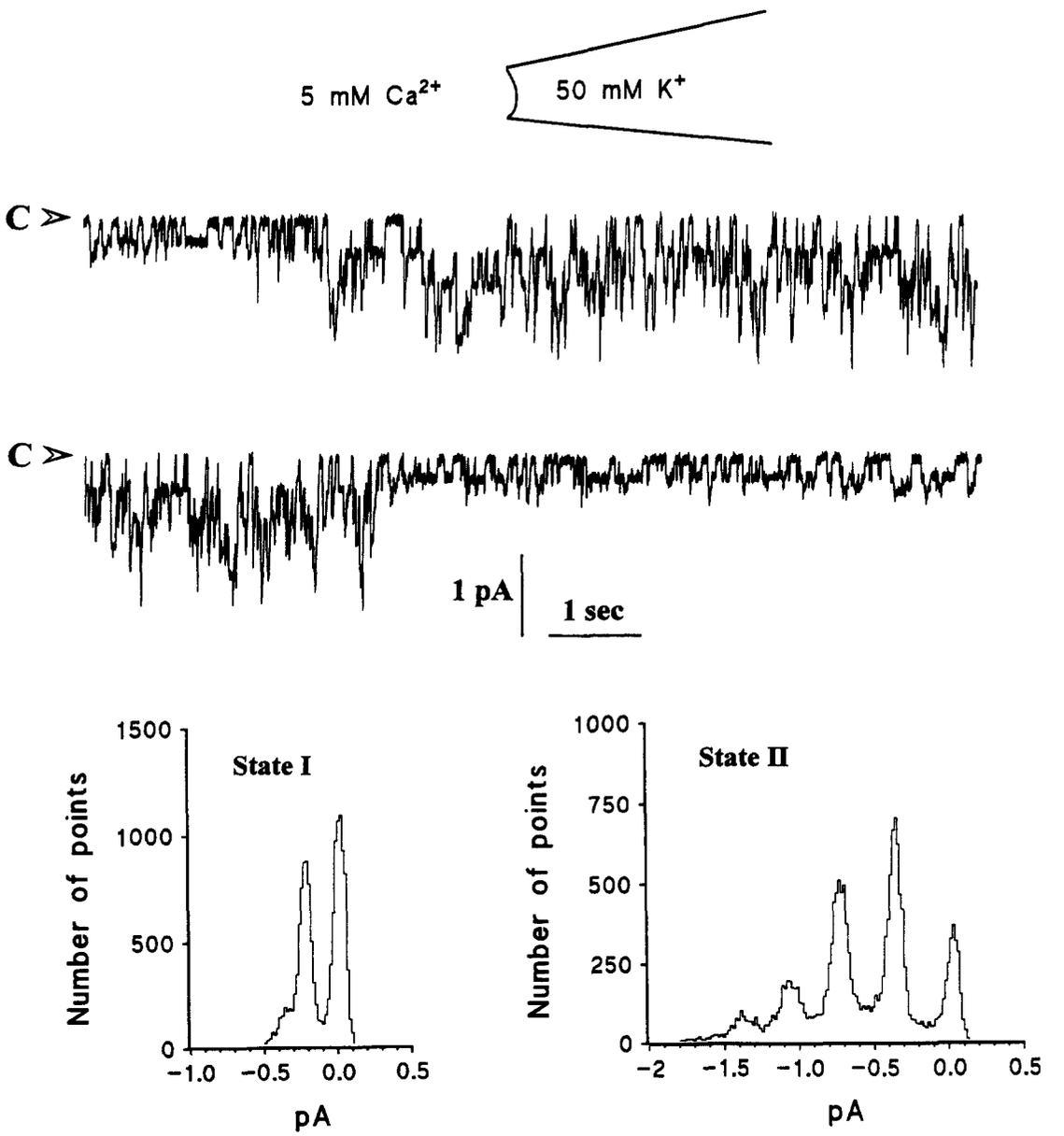

Fig. 1. Single channel traces and amplitude histograms demonstrating spontaneous transitions between the two activity states of the voltage-gated Ca^{2+} release channel in sugar beet tap roots. Lumenal-side-out patch held at −40 mV. Pipette solution: 50 mM KOH, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 3 mM DL-dithiothreitol (DTT), 21 mM dimethylglutaric acid + HEPES, pH 7.3; bath solution: 5 mM $Ca(OH)_2$, 2.5 mM dimethylglutaric acid + HEPES, pH 7.3; liquid junction potential: 15 mV. Data were low pass filtered at 100 Hz. Amplitude histograms were created from 12 s recordings in either state, bin width: 0.05 pA. C▷ marks the zero current level where all channels are closed.

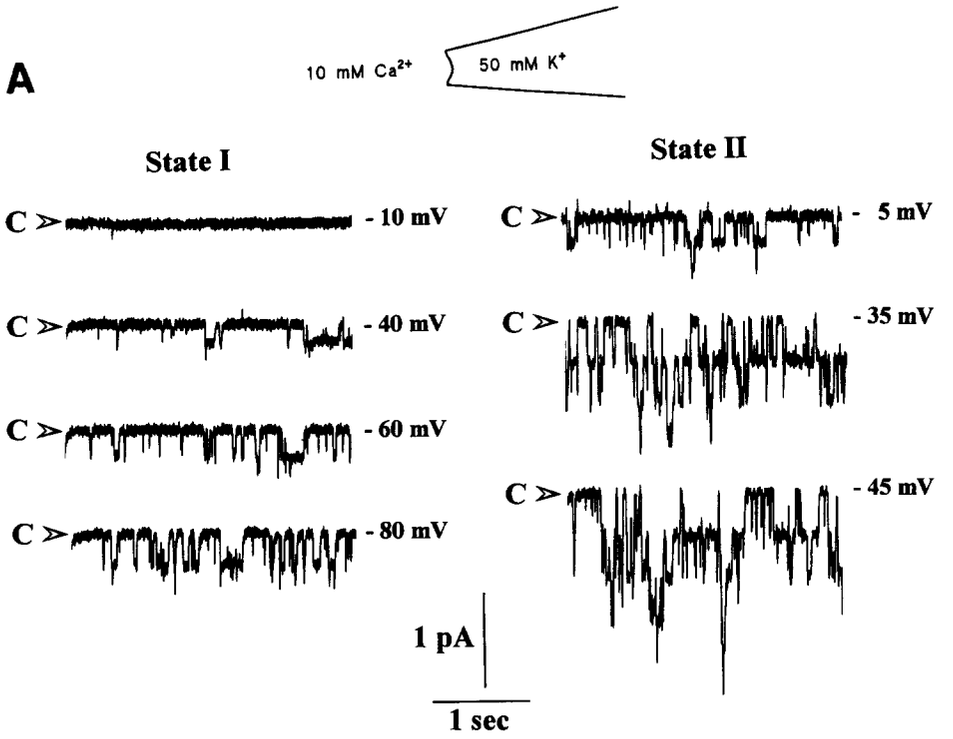

Fig. 2A.

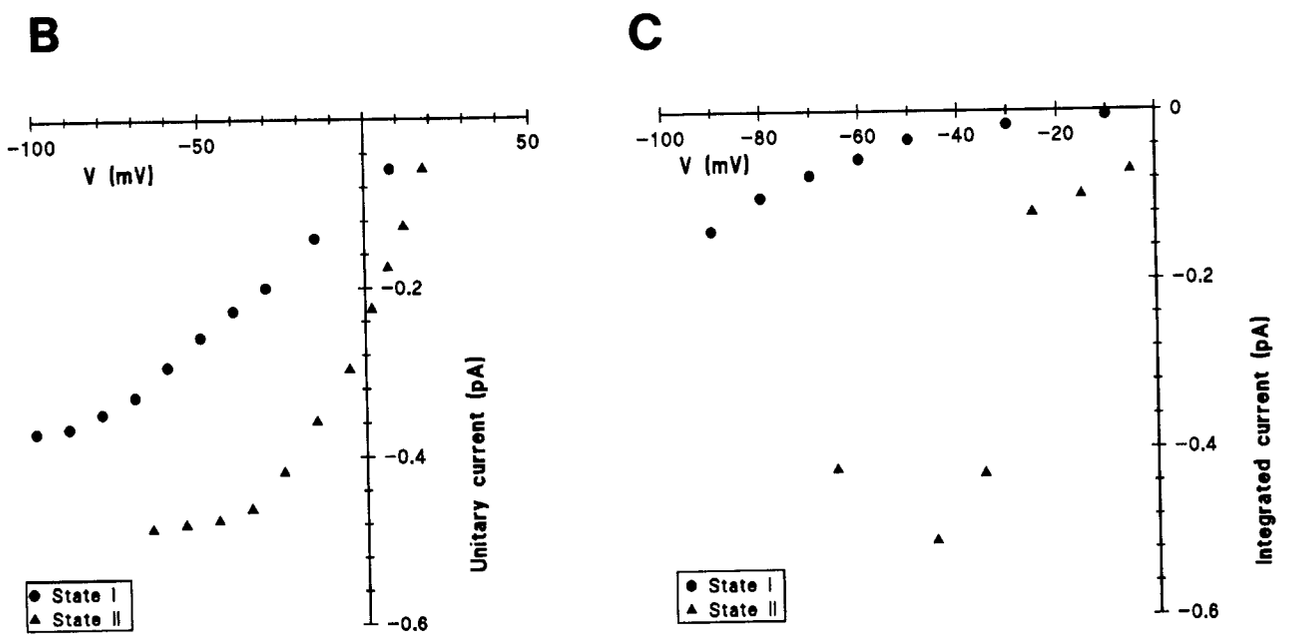

Fig. 2. Single channel traces (A), single channel current voltage relationships (B) and integrated current as a function of voltage (C) recorded in State I and State II. Both recordings were derived from the same patch before and after transition of the channel from State I to State II. Lumenal-side-out patch with Medium I in the pipette and 10 mM $Ca(OH)_2$ adjusted to pH 7.3 with 8.4 mM dimethyl glutaric acid + HEPES in the bath. Liquid junction potential: 12.5 mV. Single channel traces were low pass filtered at 150 Hz. C> marks the zero current level. State I: (●); State II: (▲).

would change the pattern of uniform current levels after transition to State II (to yield clear peaks at 0.24 pA intervals in the State II diagram in Fig. 1) which makes it is unlikely that State I is an independent background channel. Furthermore it should be noted that the observed transitions are not accompanied by changes in the background conductance and that in either state the channels respond rapidly to changes in the bath solution which rules out the possibility that the observed effect represents transient formation of vesicles.

The single channel properties of each state were investigated

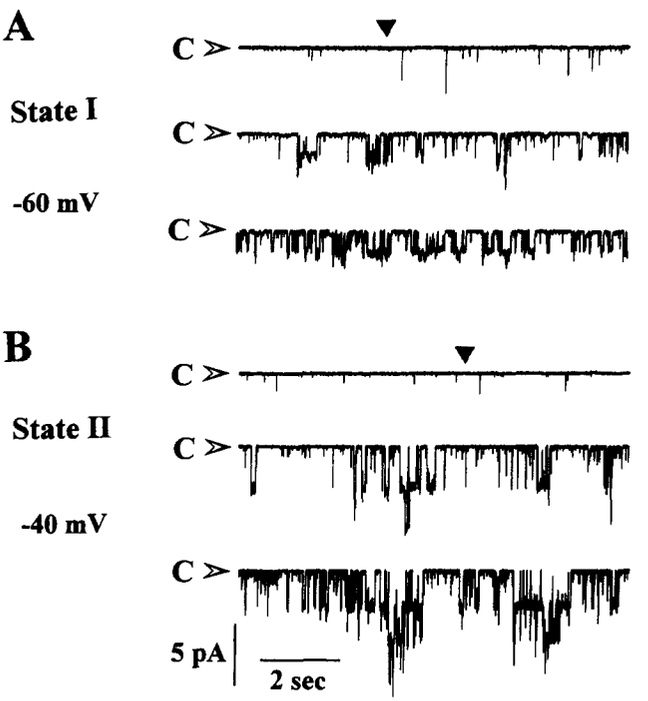

Fig. 3. Channel activation after addition of 1 mM Mg^{2+} to the lumenal side. Lumenal-side-out patch in symmetrical Medium I. The bath solution containing Medium I + 1 mM $MgCl_2$ was continuously perfused into the recording chamber (flow rate 0.2 ml min^{-1}, chamber volume: 120 μl). (A) State I, clamped at −60 mV, (B) State II, clamped at −40 mV and recorded on the same patch 40 min after recording in A. The lag phase in observing the full response is due to the time required for the new solution to reach the membrane. C▷ marks the zero current level. ▼ marks the arrival of the Mg^{2+}-containing solution in the recording chamber.

in lumenal-side-out patches which displayed a transition in channel activity. With 50 mM K^+ on the cytosolic side and 10 mM Ca^{2+} on the lumenal side the single channel Ca^{2+} current saturates at negative voltages with a maximum current of 0.32 ± 0.05 pA (n = 4) in State I (main conductance state) and 0.49 ± 0.01 pA (n = 5) in State II (Fig. 2A,B). Together with the markedly lower slope conductance of 3.9 ± 0.5 pS (n = 5) in State I compared with 11.2 ± 0.7 pS (n = 5) in State II, this results in a more linear unitary current voltage relationship when the channel resides in State I and saturation occurs at more negative voltages than in State II (Fig. 2B). However, both states have a similar reversal potential in biionic conditions of 22.0 ± 3.1 mV (n = 5; State I) and 23.4 ± 0.8 mV (n = 5; State II). For State II where the permeation properties have been analysed in detail [8,9,15] the positive reversal potential indicates a permeability ratio for Ca^{2+} over K^+ of about 15:1. Preliminary results concerning ion permeation in State I also show that shifts in the reversal potential after increasing lumenal Ca^{2+} follow $E_{Ca^{2+}}$ (data not shown). Thus, as in State II, the positive reversal potential is likely to be due to channel selectivity for Ca^{2+}, rather than anions. However, more information on the ionic selectivity of State I is needed to assess accurately whether the State I to State II transition is accompanied by changes in ion selectivity.

The most striking difference between State I and II is the dramatically enhanced open frequency observed in State II (Figs. 1 and 2A). The open frequency of both states increases with negative voltages but much larger negative potentials are necessary for activation of State I. The Ca^{2+} current through the channels in States I and II was compared by averaging currents conducted through both states in the same membrane patch. Over the physiological range of vacuolar membrane potentials (−10 mV to −50 mV), the integrated Ca^{2+} current (i.e. total charge) which flows through the channel in State II is about 15- to 19-fold higher than that measured in State I (Fig. 2C). The high open frequency and absence of subconductance levels identifies State II closely with the voltage-gated channel characterised previously by us in this tissue [8,9,15].

We previously reported that in State II channel activity requires the presence of divalent cations on the lumenal side, with Ca^{2+} being the most effective activator [9,15]. This is also the

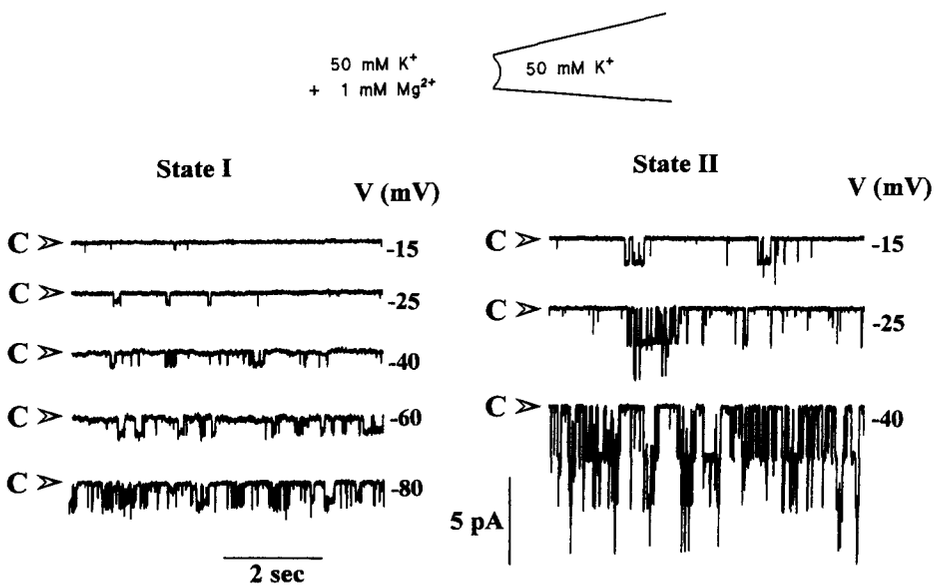

Fig. 4. Single channel traces comparing channel activity in State I and State II recorded on a single patch. Lumenal-side-out patch with Medium I in the pipette and Medium I supplemented with 1 mM $MgCl_2$ in the bath. C▷ marks the zero current level.

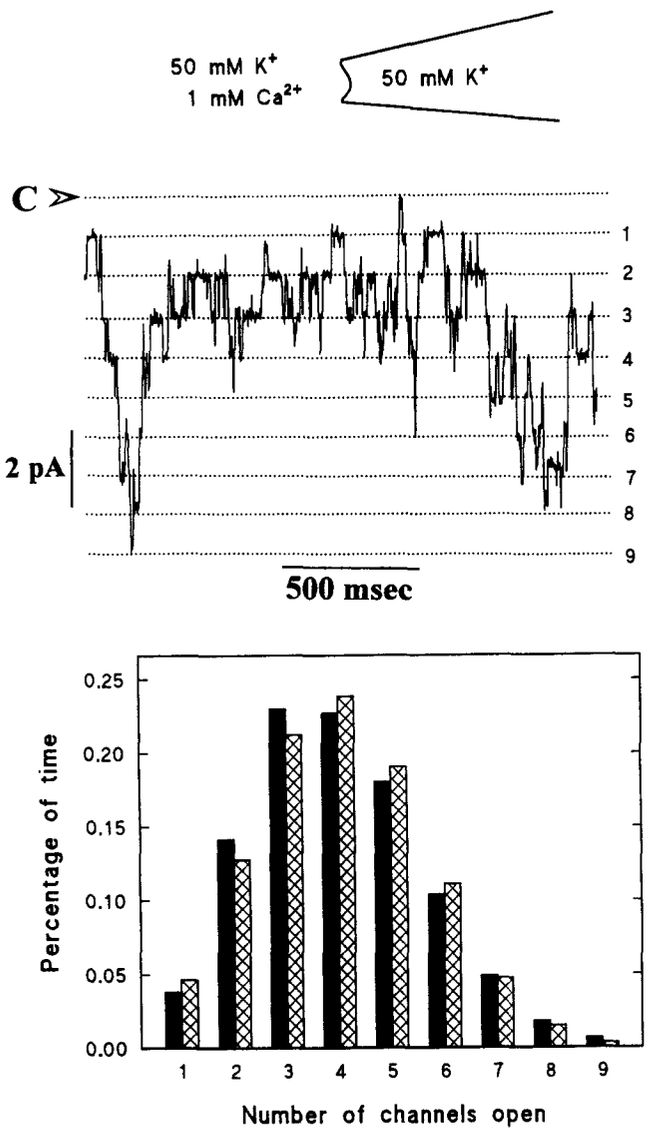

Fig. 5. (A) Single channel traces from a lumenal-side-out patch, clamped at −16 mV with Medium I in the pipette and Medium I + 1 mM $CaCl_2$ in the bath solution. The recording is low pass filtered at 300 Hz. C▷ marks the zero current level. (B) Percentage of time that 1, 2,...,9 channels are open. Filled columns: measured values obtained from a 15 s section of the recording shown above; hatched columns: binomial distribution calculated for 12 identical channels with an open state probability of 0.33.

case when the channels reside in State I. Fig. 3 compares the response of both states (recorded on the same patch) to addition of 1 mM $MgCl_2$ to the bathing medium of a lumenal-side-out patch in symmetrical 50 mM K^+. In both cases we observe channel activation associated with the arrival of the Mg^{2+}-containing solution at the membrane. Similar results were obtained after addition of Ca^{2+} to the lumenal side (data not shown). As in the case for which Ca^{2+} is a charge carrier, there is also a profound difference between the two states with respect to unitary current and open frequency of the channel when the inward current is mainly carried by K^+. Indeed Fig. 4 demonstrates that the inward K^+ current undergoes an even more pronounced increase after conversion from State I to State II than does the inward Ca^{2+} current (cf. Fig. 2A). However, in both states the unitary K^+ inward current is larger than that obtained with Ca^{2+} in spite of the higher permeability ratio for Ca^{2+} over K^+ indicated by the reversal potential in biionic conditions (Figs. 2A and 4). This suggests that selectivity is achieved by ionic competition for binding sites in the channel pore [19]. We recently characterised a Ca^{2+} binding site for State II which is located 9% through the dielectric field of the membrane from the vacuolar side and has an affinity constant of $K^{V=0}_{0.5}$ = 0.29 mM [9,15]. However, the variable conductance levels in conditions when K^+ is the main charge carrier, especially in the presence of submillimolar lumenal Ca^{2+} (data not shown), make a detailed analysis of ion permeation and channel gating in State I difficult. Thus it remains to be determined whether the Ca^{2+} binding properties of the channel change after transition between the two States.

4. Discussion

We found that the voltage-gated Ca^{2+} release channel in the vacuolar membrane of sugar beet tap roots resides in two activity states which share many features. These include an increase in the open frequency with negative membrane potentials, rapid voltage activation, a similar reversal potential in biionic conditions, activation by divalent cations on the lumenal side and inhibition by the lanthanide Gd^{3+} (data not shown). However, both states differ dramatically with respect to their unitary conductance and open frequencies and this results in a 15- to 19-fold increase in Ca^{2+} flux into the cytosol after transition from State I to State II.

Therefore it seems likely that the State I to State II transition could play an essential role in channel activation during intracellular signalling. Conversely, the State II to State I transition results in down-regulation of Ca^{2+} release and could prevent cytosolic overload with Ca^{2+} from the large vacuolar Ca^{2+} pool. Hence, it seems possible that the transition in channel activity is triggered by an intermediary factor in signal transduction. However, our efforts to link these changes in the channel properties to altered redox [20] or phosphorylation [21] states of the channel protein have not yet yielded any conclusive results. Sudden increases in channel gating and unitary current have also been observed in Ca^{2+} release channels in guard cells which have similar characteristics to the beet channel [10,11]. Although in guard cells this phenomenon has not been characterised in further detail, it is likely that the switch in channel activity comprises an integral part of this class of Ca^{2+} release channel. Time-dependent conversions between rapid and slow gating modes have also recently been reported for a guard cell plasma membrane anion channel, although there the permeation properties remain unaffected [22]. As in the current study, the underlying mechanism and physiological trigger of the switch remain obscure.

In the beet channel, where dramatic changes occur in the unitary current as well as in channel activity, a rather complex control mechanism has to be anticipated. It is likely that the transition between State I and II is accompanied by an increase in the number of functional channels since multiple openings rarely occur in State I, even at extreme negative voltages (−180 mV), whereas more than 15 conductance levels are regularly observed in single patches at only moderate voltages (ca. −50 mV) when the channel resides in State II. We can

therefore speculate that State II functions as a cluster of identical 'oligochannels' which are simultaneously brought into action by conformational changes of State I, possibly involving reorientation of channel subunits in the membrane. To determine whether the individual channels in the cluster display cooperative or independent gating we compared the measured pattern of channel openings in State II with that predicted by a binomial distribution [23]. As demonstrated in Fig. 5 the measured and calculated values are not significantly different indicating that the channels are gated independently from each other.

The dramatic change in the Ca^{2+} current after transition from State I to State II provides a powerful means to enhance Ca^{2+} release from the vacuole in the presence of an eliciting signal. In State I Ca^{2+} leakage from the vacuole is very low and also rather insensitive towards small changes in the membrane potential. When a rise in $[Ca^{2+}]_{cyt}$ is required in response to an environmental signal the channels are switched into State II by an intermediary element in the signal transduction chain, possibly involving the removal of a cytosolic factor. In this activated state Ca^{2+} release is dramatically enhanced even at physiological vacuolar membrane potentials (about −20 mV, cf. [24]) and the steep voltage dependence of channel gating in State II allows additional modulation of the Ca^{2+} flux via voltage changes across the vacuolar membrane. The organisation of channels in clusters has the additional advantage of enabling the generation of large localised changes in $[Ca^{2+}]_{cyt}$ without flooding the cytosol with potentially lethal amounts of Ca^{2+}. The identification of the eliciting factor(s) which cause the switch in channel activity will be a challenging task for future investigations and will yield major insights in stimulus–response coupling in plants.

Acknowledgements: We thank Ian Jennings for writing and implementing some of the software used in this study and Anna J. Bate for technical assistance. The work was supported by grants from the Biotechnology and Biological Sciences Research Council to E.J. (PDF/14) and D.S. (PG87/529).

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