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Rapid Report

Voltage-dependent Ca^{2+} channels in the plasma membrane and the vacuolar membrane of *Arabidopsis thaliana*

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Voltage-dependent Ca^{2+} channels in the plasma membrane and the vacuolar membrane of *Arabidopsis thaliana* have been studied at the single-channel level using the patch-clamp technique. The Ca^{2+} channel in the plasma membrane opened for extracellular Ca^{2+} influx. The Ca^{2+} channel in the vacuolar membrane opened for cytoplasmic Ca^{2+} influx.

In animal systems, Ca^{2+} channels have been shown to play an essential role in a wide range of cellular functions. Multiple voltage-dependent Ca^{2+} channels (T, N, and L type) have been identified that can be distinguished by their biophysical and pharmacological properties [1,2]. In plant cells, both the plasma and the vacuolar membranes are available for patch clamp studies and the existence of Ca^{2+} channels seems quite certain in these membranes [3–5]. However, voltage-dependent Ca^{2+} channels have not been investigated at the single-channel level in the plasma membranes of plant cells. Recently, voltage-dependent Ca^{2+} channels were discovered in plant vacuoles [6–8], but these channels showed different properties which might be due to their measurement conditions. The roles of these channels in cellular functions remained unclear. In this paper we present single-channel recordings of voltage-dependent Ca^{2+} channels in the plasma and the vacuolar membranes of *Arabidopsis thaliana*. The possible coordination of the Ca^{2+} channels in the two different membranes of the same plant cell has been discussed.

Arabidopsis thaliana (Columbia wild type) were grown in a green house at 25°C under natural light. Protoplasts and vacuoles were prepared basically as previously described [8]. Briefly, leaves of 1 to 2 month-old were treated with 1% Cellulase Onozuka RS (Yakult Honsha, Tokyo), 0.1% Pectolyase Y-23 (Seishin, Tokyo) in 700 mM mannitol and 10 mM Mes-Tris (pH 5.8) for 1 h at 28°C to prepare protoplasts. The protoplast suspension was added to 100 volumes of bath solutions (310 mosmol/l; ionic composition was indicated in each case). Patch-clamp technique was applied to protoplasts and vacuoles obtained from mature mesophyll. The osmolarity of both bath and pipette solutions used in patch clamp experiments were adjusted to about 310 mosmol/l with sorbitol.

Gigaohm resistance pipette-to-membrane seals were formed with 3–5 megaohm resistance pipettes made from 75-μl disposable glass micropipettes (Duramont, PA, USA) by a two-stage pulling and fire-polishing process. Inside-out patches were produced by drawing back the pipette from the protoplast or vacuole after formation of a tight seal, and exposing the resulting small vesicle at the tip to air for a short period. The membrane portion facing the air was immediately disintegrated, whereas the membrane patch inside the pipette remained intact. Single-channel currents were measured with a patch/whole-cell clamp amplifier (CEZ-2300, Nihon Kohden, Tokyo). Data were stored in a digital audio tape recorder (DTC 55 ES, Sony, Tokyo) and processed with the QP-120J program (Nihon Kohden, Tokyo) in a PC 9801 computer (NEC, Tokyo) for subsequent analysis. All signals were digitized at 2 kHz after filtering at 1 kHz (6-pole, Bessel filter).

Sign conventions throughout the study used the reversed pipette voltage to represent plasma membrane potential or vacuolar membrane potential. For the plasma membrane, the extracellular side was treated as the reference; for the vacuolar membrane, the cytoplasmic side was the reference.

The patch clamp measurements of Ca^{2+} channel activity in the plasma membrane of *Arabidopsis* were

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carried out under the following conditions: inside-out configuration, 0 to −60 mV membrane potential holding, 50 mM $BaCl_2$ in the pipette and 100 mM K-gluconate in the bath. Under these conditions, the reversal potential for K^+ and Cl^- were −118 mV and −83 mV (K^+ in the pipette was assumed to be 1 mM to the maximum, Cl^- in the bath was 4 mM), respectively, while that for Ba^{2+} was 51 mV. Thus, the outward K^+ and Cl^- currents, if they existed, could be distinguished from the inward Ba^{2+} current by their opposite direction; and the inward K^+ and Cl^- currents were prevented by the holding potentials more positive than their reversal potentials.

With 50 mM Ba^{2+} as a charge carrier, a 23-pS channel was activated by plasma membrane potentials from 0 to −60 mV in the inside-out patch (Fig. 1A). Opening of multiple channels were increased with polarization of membrane potentials. When Ca^{2+} concentration on the cytoplasmic side was increased from 1 to 25 mM, the channel activity was markedly inhibited (Fig. 1B). Meanwhile, the reversal potential of the channel shifted from 53 mV to 16 mV, which corresponds to the calculated Ca^{2+} Nernst potentials (Fig. 1C). The open probability of the channel showed a voltage-dependency (Fig. 1D). These results indicate the existence of a voltage-dependent Ca^{2+} channel in

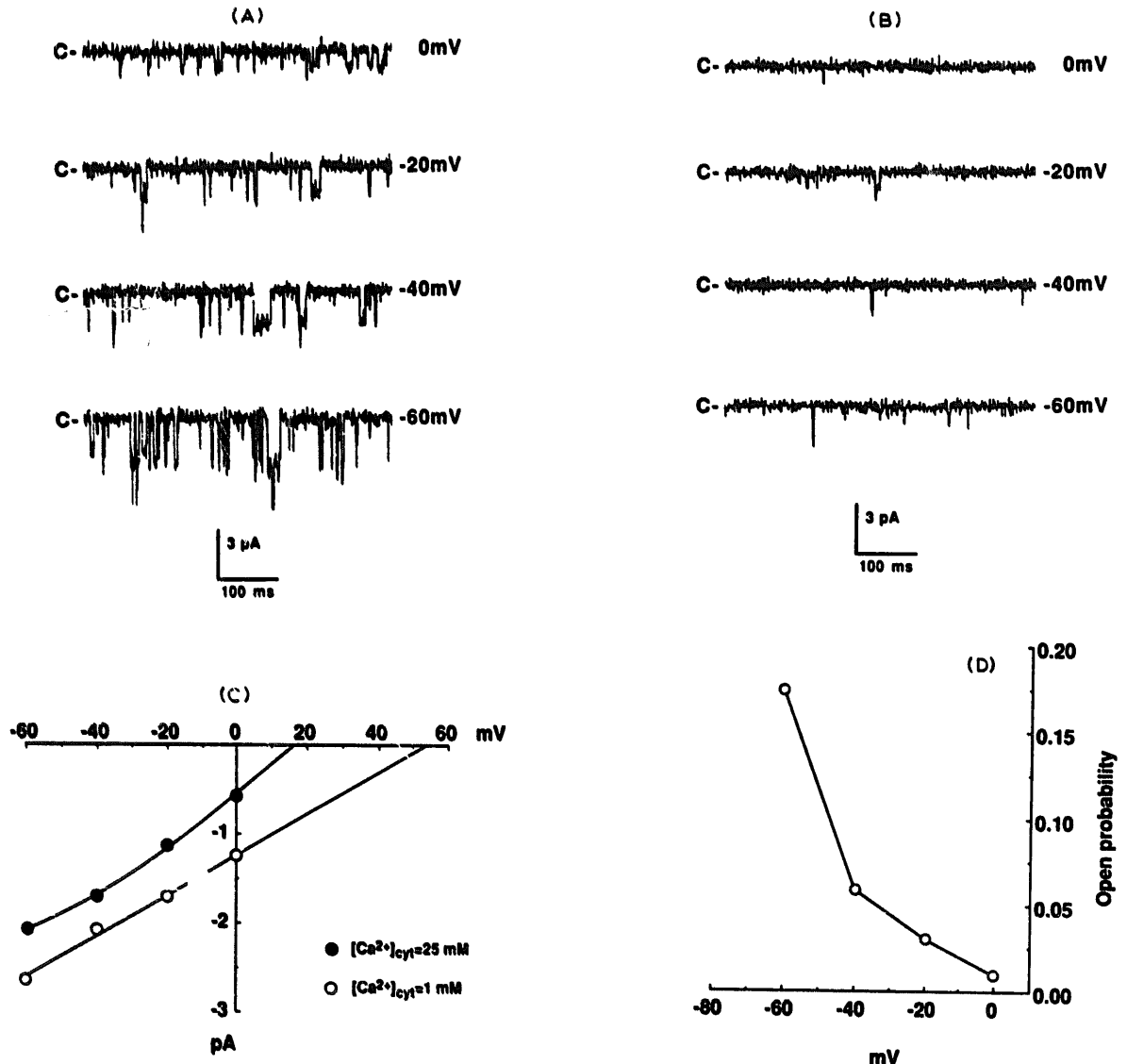

Fig. 1. Single Ca^{2+} channel recordings from an inside-out patch of *Arabidopsis* plasma membrane exposed to constant gluconate but different Ca^{2+} concentrations. (A) Pipette medium contained 50 mM $BaCl_2$, 2 mM $MgCl_2$ and 5 mM Tris-Mes (pH 7.5). Bath medium contained 100 mM K-gluconate, 2 mM $MgCl_2$, 1 mM Ca-gluconate and 5 mM Mes-Tris (pH 5.8). 'C' to the left of each trace indicates the closed state of the channel. (B) The bath solution was changed to 25 mM Ca-gluconate plus 52 mM K-gluconate, other ions in pipette and bath media were the same as in A. (C) Current–voltage relationship of the single Ca^{2+} channel recordings. ○, from A; ●, from B. Every voltage step lasted for 30 s, about 1 min was necessary for changing the bath medium. (D) Voltage dependence of channel opening probability P_o in the inside-out patch of the plasma membrane. P_o was calculated as the ratio of the open time to the total recording time of 30 s at different voltages as shown in A.

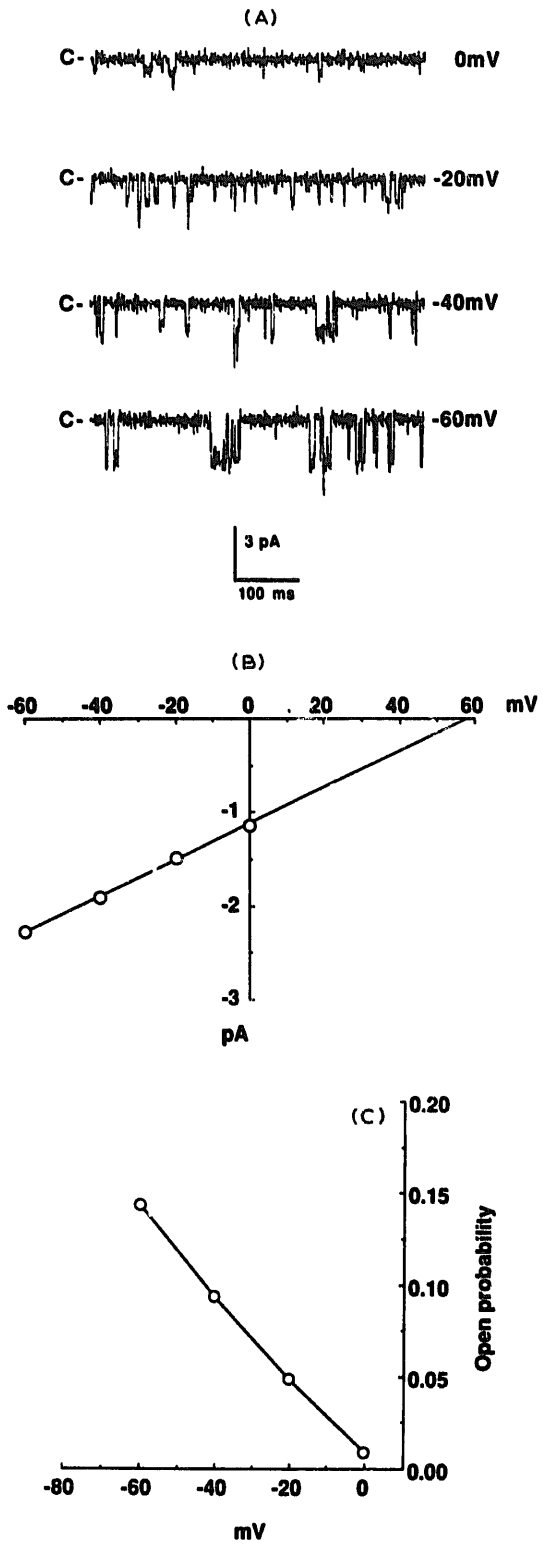

Fig. 2. Single Ca^{2+} channel recordings from an inside-out patch of *Arabidopsis* vacuolar membrane. (A) Pipette and bath media were the same as in Fig. 1A, the channel opened when Ba^{2+} (used as a Ca^{2+} analog) concentration on cytoplasmic side was higher than physiological condition. 'C' to the left of each trace indicates the closed state of the channel. (B) Current–voltage relationship of the single Ca^{2+} channel recordings from A. (C) Voltage dependence of channel opening probability in the inside-out patch of the vacuolar membrane. P_o was calculated as the ratio of the open time to the total recording time of 30 s at different voltages as shown in A.

the plasma membrane of higher plant cell, and that the extracellular Ca^{2+} influx through this channel could be markedly inhibited by high concentration of cytoplasmic Ca^{2+}. Cell-attached configuration was also applied to Ca^{2+} channel recording in the plasma membrane. The 23-pS Ca^{2+} channel opened occasionally ($P_o <$ 0.01). This suggests that channel activity is well controlled by cytoplasmic factors.

With the similar methods, we also recorded a Ca^{2+} channel in the vacuolar membrane of the same plant cells (Fig. 2A). Opening of multiple channels appeared occasionally. The channel was for cytoplasmic Ca^{2+} flux into the vacuole. The channel had a conductance of 20 pS with 50 mM Ba^{2+} as a charge carrier. The reversal potential of the channels currents was 56 mV corresponding to the calculated Ca^{2+} Nernst potential (Fig. 2B). The open probability of the channel was dependent on voltage (Fig. 2C). The channel showed a density of 2–3 channels/μm^2 with high activity. These characteristics are very similar to the vacuolar Ca^{2+} channels in tobacco and red beet cells reported previously [8].

According to Goldman-Hodgkin-Katz equation and the values of reversal potential, the Ca^{2+} channels in both the plasma and the vacuolar membranes show a permeability ratio of $P_{Ba^{2+}}/P_{K^+} > 30$. The P_o of the Ca^{2+} channel in the vacuolar membrane showed a lower voltage dependency than in the plasma membrane as observed in 7 of 10 experiments. Activity of both Ca^{2+} channels run down within several min, leading to difficulties in further pharmacological studies.

This study demonstrates the existence of voltage-dependent Ca^{2+} channels in the plasma and the vacuolar membranes of *Arabidopsis*. The 23-pS Ca^{2+} channel in the plasma membrane may play an important role in triggering cellular responses [9]; the 20-pS Ca^{2+} channel in the vacuolar membrane may serve to remove the local and transient rise in cytoplasmic Ca^{2+} [8]. The similar characteristic inward rectification of these channels suggests that the Ca^{2+} channels in the plasma and the vacuolar membranes might coordinate during cytoplasmic Ca^{2+} oscillations which have been observed in many essential cellular responses [10]. However, since the ionic conditions in the extracellular environment and cytoplasmic and vacuolar spaces are different resulting in the different in the plasma and the vacuolar membrane potentials, to what extent the Ca^{2+} channels in the two membranes coordinate in an intact plant cell may be more complicated than the patch-clamp study could answer. Furthermore, a Ca^{2+}/H^+ antiport system driven by H^+ transporting ATPase has been demonstrated to function in the accumulation of Ca^{2+} into the vacuole [11], while the inward rectificating vacuolar Ca^{2+} channel represents a completely passive Ca^{2+} transport system when cyto-

plasmic Ca^{2+} concentration is high. Thus, it is interesting to investigate how and when the two systems share their own functions during the cytoplasmic Ca^{2+} oscillation.

It should be noted that the experimental conditions used for identification of Ca^{2+} channels was far from physiological conditions because the unitary Ca^{2+} current is so small that it can only be detected under conditions where the activities of other channels are prevented. Thus, once the existence of a Ca^{2+} channel has been demonstrated, the next step would be to isolate K^+ or Cl^- channels in the same patch-clamped membrane and investigate the relationship between different channels.

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