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Two Voltage-Gated, Calcium Release Channels Coreside in the Vacuolar Membrane of Broad Bean Guard Cells

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Voltage-gated, Ca^{2+} release channels have been characterized at the vacuolar membrane of broad bean guard cells using patch clamps of excised, inside-out membrane patches. The most prevalent Ca^{2+} release channel had a conductance of 27 pS over voltages negative of the reversal potential (E_{rev}) (cytosol referenced to vacuole), with 5, 10, or 20 mM Ca^{2+} as the charge carrier on the vacuolar side and 50 mM K^+ on the cytosolic side. The single-channel current saturated at ~2.6 pA. The relative permeability of the channel was in the range of a $P_{Ca^{2+}}:P_{K^+}$ ratio of 6:1. Divalent cations could act as charge carriers on the vacuolar side with a conductance series of $Ba^{2+} > Mg^{2+} > Sr^{2+} > Ca^{2+}$ and a selectivity sequence of $Ca^{2+} \approx Ba^{2+} \approx Sr^{2+} > Mg^{2+}$. The channel was gated open by cytosol-negative (physiological) transmembrane voltages, increases in vacuolar Ca^{2+} concentration, and increases in the vacuolar pH. The channel was potently inhibited by the Ca^{2+} channel blockers Gd^{3+} (half-maximal inhibition at 10.3 μM) and nifedipine (half-maximal inhibition at 77 μM). The stilbene derivative 4,4′-diisothiocyano-2,2′-stilbene disulfonate was also inhibitory (half-maximal inhibition for a 4-min incubation period at 6.3 μM). The 27-pS channel coresides in individual guard cell vacuoles with a less frequently observed 14-pS Ca^{2+} release channel that had similar, although not identical, voltage dependence and gating characteristics and a lower selectivity for Ca^{2+} over K^+. The requirement for two channels with a similar function at the vacuolar membrane of guard cells is discussed.

INTRODUCTION

Elevation of cytosolic-free Ca^{2+} ($[Ca^{2+}]_{cyt}$) is central to stimulus–response coupling in plant cells (Bush, 1993). Because guard cells exhibit rapid responses to a defined array of stimuli, they have become a widely used model in which the nature and control of the Ca^{2+} signal has been studied.

Stomatal closure can be induced when $(Ca^{2+})_{cyt}$ is artificially elevated following photolysis of microinjected, caged Ca^{2+} (Gilroy et al., 1990) or when $(Ca^{2+})_{cyt}$ is raised following exposure to abscisic acid (ABA) (Gilroy et al., 1991; McAinsh et al., 1992). Alternatively, elevation of $(Ca^{2+})_{cyt}$ can precede stomatal opening induced by indoleacetic acid, cytokinins, or fusicoccin (Irving et al., 1992).

During stomatal closure, the elevation of $(Ca^{2+})_{cyt}$ has marked effects on the activities of several plasma membrane ion channels. The inward-rectifying K^+ channel responsible for K^+ accumulation is inhibited (Blatt et al., 1990; Lemtiri-Chlieh and MacRobbie, 1994), probably indirectly by way of a dephosphorylation mechanism involving activation of a Ca^{2+}-dependent phosphatase akin to protein phosphatase 2B (calcineurin) (Luan et al., 1993). In addition, anion efflux channels are activated by a rise in $(Ca^{2+})_{cyt}$ (Schroeder and Hagiwara, 1989). The net effect of these changes is K^+ efflux from the cell and stomatal closure.

The origin of the Ca^{2+}, which raises $(Ca^{2+})_{cyt}$, is uncertain but is likely to be diverse and possibly stimulus specific. Calcium can enter guard cells across the plasma membrane by way of stretch-activated Ca^{2+}-selective channels (Cosgrove and Hedrich, 1991), by way of a limited permeability of the K^+-inward rectifier to Ca^{2+} (Fairley-Grenot and Assmann, 1992), or by way of nonselective Ca^{2+}-permeable channels (Schroeder and Hagiwara, 1990). Indeed, Ca^{2+} influx by way of the nonselective Ca^{2+}-permeable channels has been demonstrated to be responsible for fluctuating increases in $(Ca^{2+})_{cyt}$ following exposure to ABA (Schroeder and Hagiwara, 1990).

It is clear that internal pools of Ca^{2+} also act as a source for the Ca^{2+} signal. Fluorescence ratio imaging indicates elevation of $(Ca^{2+})_{cyt}$ can occur in some guard cells in response to ABA in the absence of external Ca^{2+} (Gilroy et al., 1991). Furthermore, guard cell protoplasts will shrink in response to ABA in the absence of external Ca^{2+} or in the presence of the Ca^{2+} channel blocker lanthanum (La^{3+}) (Smith and Willmer, 1988). It has also been demonstrated that Ca^{2+} from internal sources can inhibit the K^+-inward rectifier in the absence of external Ca^{2+} (Lemtiri-Chlieh and MacRobbie, 1994). Imaging studies suggest that the vacuole acts as the major internal Ca^{2+} source (Gilroy et al., 1991). Release of Ca^{2+} from internal pools could involve a phosphoinositide signaling pathway, because artificial elevation of inositol 1,4,5-trisphosphate

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($InsP_3$) in the cytoplasm results in Ca^{2+} mobilization and stomatal closure (Blatt et al., 1990; Gilroy et al., 1990).

The diversity of signal transduction pathways in which elevation of $(Ca^{2+})_{cyt}$ is an integral step suggests that there must be a high degree of control of the amplitude, duration, and spatial arrangement of the Ca^{2+} signal in the cytosol in order for stimulus-specific responses to result. This implies the requirement for multiple pathways for the release of Ca^{2+} from internal pools. In addition to $InsP_3$-gated Ca^{2+} release channels (Alexandre et al., 1990; Alexandre and Lassalles, 1992), vacuoles from beet taproots contain voltage-gated Ca^{2+}-selective channels with gating characteristics that suggest a role in vacuolar Ca^{2+} release (Johannes et al., 1992a, 1992b; Gelli and Blumwald, 1993). Therefore, at least two possible convergence points for discrete primary signals exist, of which activation of either or both would lead to the elevation of $(Ca^{2+})_{cyt}$.

Preliminary electrophysiological studies of guard cell vacuoles have been undertaken previously (Hedrich et al., 1988; Raschke et al., 1988). The presence of an H^+-ATPase and slow vacuolar (SV) channels were reported, although neither transport system was characterized in detail. In this paper, we report that two classes of voltage-gated Ca^{2+} release channels coreside in the vacuolar membrane of isolated broad bean guard cell protoplasts.

RESULTS

Reference and Sign of Voltages and Currents

In accord with a recent proposal (Bertl et al., 1992), transvacuolar membrane potentials in this study are referenced to the extra-cytosolic medium (i.e., the vacuolar lumen). As the two primary pumps at the vacuolar membrane pass current (positive charge) out of the cytosol (Rea and Sanders, 1987), negative potentials correspond to the physiological situation (cytosol negative, vacuolar lumen positive). A negative current represents cations moving from the lumen to the cytosol (from bath to pipette in an inside-out vacuolar membrane patch). This is the opposite of the convention adopted in some previous studies concerning vacuolar Ca^{2+} release channels (Alexandre et al., 1990; Johannes et al., 1992a, 1992b).

Characteristics of Single Ca^{2+} Release Channels in Inside-Out Membrane Patches

High-resistance seals between the patch pipette and guard cell vacuolar membrane were readily formed on the application of gentle suction to the pipette. Of all attempted sealings, 53% formed a vacuole-attached patch which, when pulled away from the vacuole surface, resulted in an inside-out patch that was stable for 2 min or longer. Of these patches, 55% (61 of 111 patches) contained channels that gated open at negative potentials.

The single-channel current–voltage (I/V) relationship for the most frequently observed type of channel is shown in Figure 1. The inset shows a plot of $\log_{10}(Ca^{2+})_v$ versus reversal potential (E_{rev}), which indicates a linear shift in E_{rev} as $\log_{10}(Ca^{2+})_v$ increased. The gradient of this plot is 28.4 mV [$\log_{10}(Ca^{2+})_v]^{-1}$, which is a Nernstian shift in E_{rev} as the $(Ca^{2+})_v$ increased. This demonstrates that at potentials more negative than the E_{rev}, the current was solely carried by Ca^{2+} under these biionic conditions. The permeability ratios for the values of E_{rev} shown in Figure 1 were calculated from the derivation of the Goldman-Hodgkin-Katz current equation presented in Methods (Equation 1). Permeability ratios ($P_{Ca^{2+}}$:P_{K^+}) of 6:1 were obtained.

The Ca^{2+} inward current dominates the I/V relationship at potentials negative of the E_{rev}. The conductance of the linear portion of this curve is 41 ± 0.75 pS (n = 15) (range of 36 to 43 pS) and is virtually unaffected by $(Ca^{2+})_v$ in the range of 5 to 20 mM. However, the mean conductance negative of the E_{rev} is 27 ± 2.4 pS. At potentials more negative than −40 mV, the single-channel current tends toward a Ca^{2+}-independent saturation at a value of ~2.6 pA. It can be seen from the traces in Figure 1A that the channel is gated open at negative potentials, and openings at positive potentials are rare. At potentials positive of E_{rev}, the current was carried by K^+ and had a conductance of 120 to 170 pS. The rarity of these openings meant that most of the points positive of the E_{rev} were obtained by observing the reversed current through the closing channel. To do this, the potential was rapidly stepped from a negative holding potential to positive potentials. This enabled the current to reverse through the open channels before they closed. The ability to measure the reversal of current through the open channels confirmed that the currents at positive potentials were being carried by the same channels.

The conductance and single-channel current of this channel are both significantly larger than those of other Ca^{2+} release channels previously reported from beet vacuolar membranes in similar ionic conditions (Johannes et al., 1992a, 1992b; Gelli and Blumwald, 1993).

Evidence that the 27-pS channel can function as a vacuolar Ca^{2+} release channel is shown in Figure 2 by the increase in open-state probability (P_o) at physiological vacuolar membrane potentials that are thought to lie in the region −20 to −50 mV (Rea and Sanders, 1987). The channel was inwardly rectifying. Thus, with a $(Ca^{2+})_v$ of 5 mM, the channel was gated open as the potential shifted negative from −20 mV to ~−70 mV, although at more negative potentials P_o fell. The channel was also gated open by $(Ca^{2+})_v$. Thus, when $(Ca^{2+})_v$ was 10 mM, the P_o was higher at any given potential than when $(Ca^{2+})_v$ was 5 mM (Figure 2B). The effect arises largely through a tendency for the channel to gate open at potentials negative of −30 mV at the higher value of $(Ca^{2+})_v$. A stimulatory effect of negative membrane potential and of $(Ca^{2+})_v$ has also been reported for the vacuolar Ca^{2+} release channel of beet (Johannes et al., 1992a). This P_o profile strongly contrasts with that of the weakly cation-selective SV channel of vacuolar membranes, which opens dominantly at nonphysio-

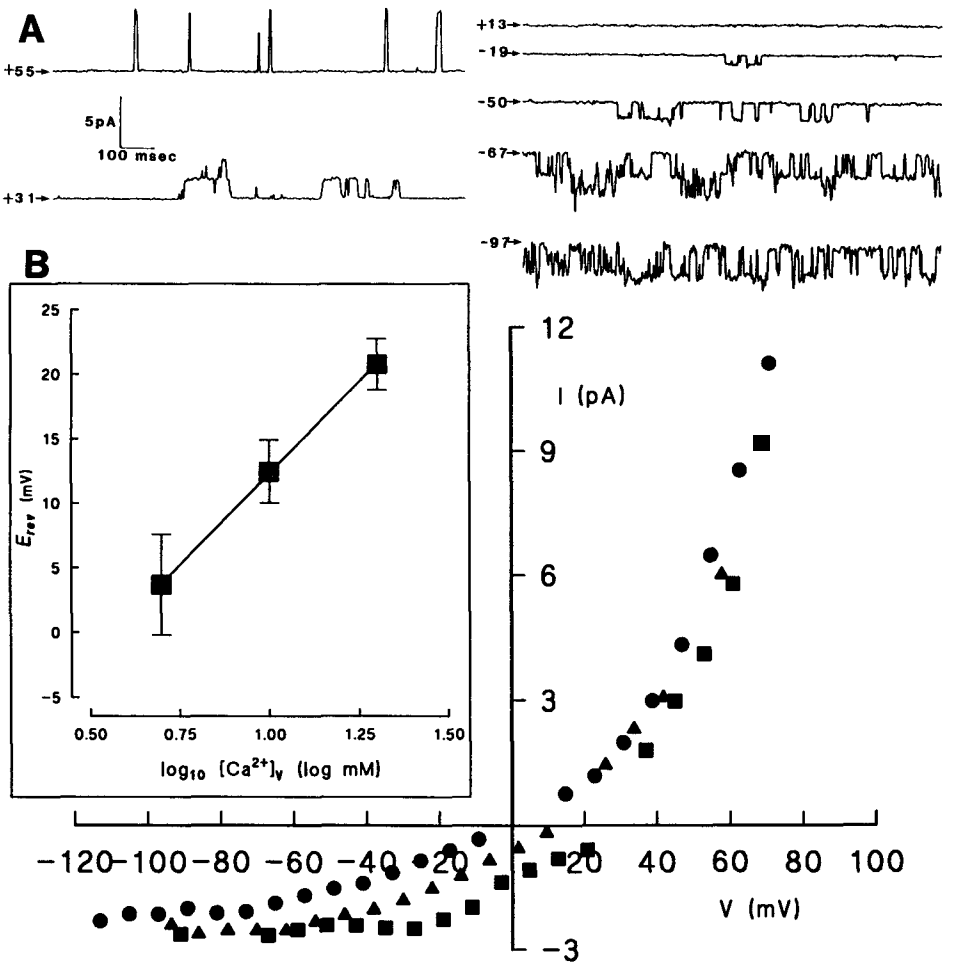

Figure 1. Characteristics of the 27-pS Ca^{2+} Release Channel.

(A) Single-channel current trace obtained from an inside-out patch pulled from an isolated vacuole of a broad bean guard cell. The vacuolar (bath) medium contained 5 mM $CaCl_2$, 3 mM Hepes-Tris, pH 7.3, 0.5 mM DTT, and sorbitol to an osmotic pressure of 450 mosmol/kg. The pipette (cytosolic) medium contained 10 mM KCl, 40 mM potassium gluconate, 3 mM Hepes-Tris, pH 7.3, 0.5 mM DTT, and sorbitol to an osmotic pressure of 450 mosmol/kg. Numbers denote the holding potential corrected for the junction potential and arrows mark the closed state. The traces are from a representative patch.

(B) Unitary current–voltage relationship of the open 27-pS channel. The vacuolar-side medium contained 5 mM (●), 10 mM (▲), or 20 mM (■) Ca^{2+}. Inset: E_{rev} versus $\log_{10}$ $(Ca^{2+})_v$. Data are the mean ±SE of five separate patches.

logical positive values of membrane potential (Hedrich et al., 1988).

Divalent Cation Permeation of the 27-pS Channel

Interaction of the 27-pS Ca^{2+} channel with other divalent cations was also explored under biionic conditions, with K^+ on the cytosolic side and substitution of Ca^{2+} by other divalent ions on the luminal side. The resultant unitary I/V relationships give insight into both the relative conductances and permeability of the ions.

Figure 3 shows the single-channel I/V relationships of the 27-pS channel with 5 mM Ca^{2+}, Ba^{2+}, Sr^{2+}, or Mg^{2+} as the charge carrier on the vacuolar side. The conductance sequence (an indication of the rate at which ions pass through the open channel, as indicated from the linear portion of each I/V curve) was $Ba^{2+} > Mg^{2+} > Sr^{2+} > Ca^{2+}$. The single-channel current did not saturate with Ba^{2+} or Mg^{2+}. The permeability sequence (a measure of the selectivity of the channel for each ion, given by the E_{rev} and application of Equation 1) was $Ca^{2+} \approx Ba^{2+} \approx Sr^{2+} > Mg^{2+}$. The outward current carried by K^+ was not significantly affected by the luminal divalent cation. The shift in E_{rev} in the presence of Mg^{2+} is further evidence that Cl^- is not contributing to the current. Exchange

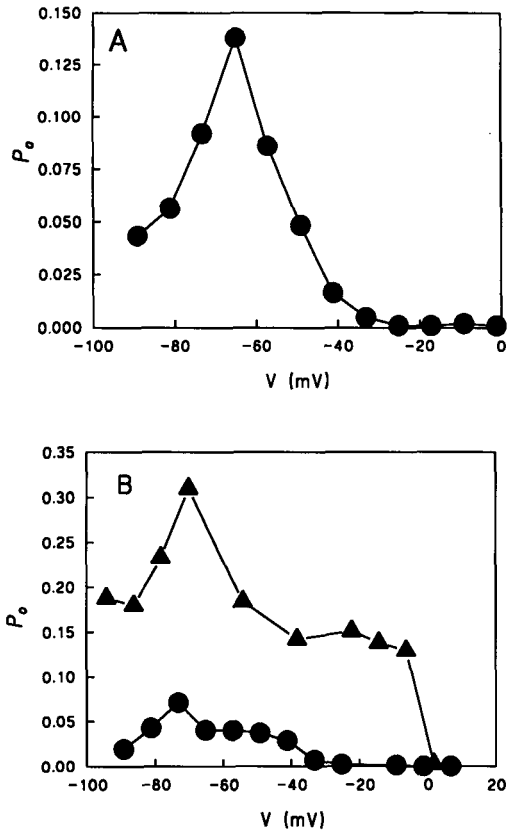

Figure 2. Voltage Dependence of P_o for the 27-pS Channel.

(A) P_o as a function of the vacuolar membrane potential. The vacuolar medium contained 5 mM (●) Ca^{2+}. All other conditions are as given in Figure 1A.
(B) P_o as a function of the vacuolar membrane potential at two $(Ca^{2+})_v$ concentrations. The vacuolar medium contained 5 mM (●) or 10 mM (▲) Ca^{2+}. All other conditions are as given in Figure 1A.
Recordings in both **(A)** and **(B)** were for 15 sec at each potential and were made from active patches containing at least seven open-state levels.

of Ca^{2+} for Mg^{2+} did not change the Cl^- gradient and yet a change in E_{rev} was observed, indicating the divalent cation is the major contributor to the current.

Inhibition of the 27-pS Channel

Figure 4 shows that the 27-pS channel was inhibited by nifedipine. The half-maximal concentration for inhibition ($K_{1/2}$) was 77 μM, and inhibition was reversible. Nifedipine also inhibits a 6-pS Ca^{2+} release channel in beet (Gelli and Blumwald, 1993). Furthermore, the 27-pS channel was inhibited by Gd^{3+} with a $K_{1/2}$ of 10.3 μM (Figure 4). Inhibition by Gd^{3+} was similar to inhibition of a 12-pS Ca^{2+} release channel in beet (Johannes et al., 1992a), reducing the open-state probability but not affecting the single-channel current (data not shown).

The data in Figure 5 demonstrate that the 27-pS channel was also inhibited by the stilbene derivative 4,4′-diisothiocyano-2,2′-stilbene disulfonate (DIDS). As might be anticipated for a covalently modifying inhibitor, inhibition by DIDS was irreversible, and the I_{50} for inhibition after a 4-min application was 6.3 μM.

DIDS is usually considered to be an inhibitor of anion transport systems (Cabantchik and Rothstein, 1974; Greger, 1990), where it acts by covalent modification of ε-amino groups of lysine residues. However, lysine residues can occur in a range of channel types, and so DIDS cannot be predicted to act solely on anion channels. Indeed, DIDS inhibits the weakly cation-selective SV channel of plant vacuoles by binding to a site on the cytosolic surface of the vacuolar membrane (Hedrich and Kurkdjian, 1988). Here, however, DIDS is acting from the vacuolar surface of the membrane.

Control of Channel Activity

The preceding experiments were performed in the presence of a luminal pH of 7.3, which contrasts with the in vivo pH of the vacuolar lumen of 5.5 or lower (Rea and Sanders, 1987).

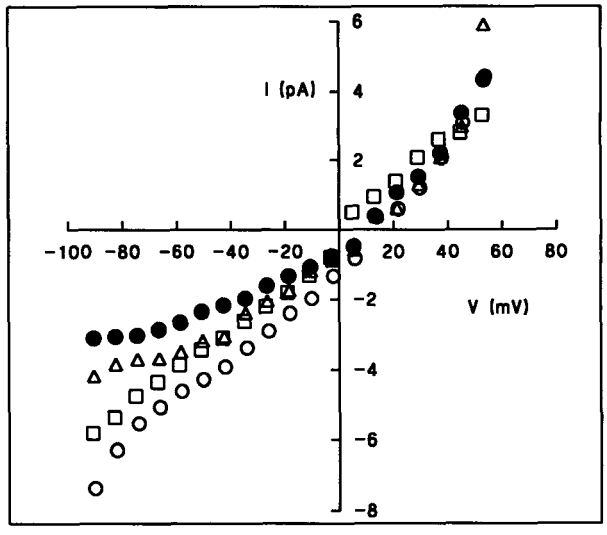

Figure 3. Divalent Ion Permeation of the 27-pS Channel.

Unitary current–voltage relationship of the 27-pS channel with 5 mM Ca^{2+} (●), Ba^{2+} (○), Sr^{2+} (△), or Mg^{2+} (□) as the charge carrier on the vacuolar side is shown. All other conditions are as given in Figure 1A. The conductances of the linear portions of the curves are Ca^{2+} = 38 ± 3 pS, Ba^{2+} = 63 ± 3 pS, Sr^{2+} = 48 ± 3 pS, and Mg^{2+} = 56 ± 0.5 pS. The E_{rev} values are Ca^{2+} = 8.7 ± 1.9 mV, Ba^{2+} = 11.0 ± 3.0 mV, Sr^{2+} = 11.1 ± 2.7 mV, and Mg^{2+} = 3.7 ± 5.2 mV. Data are the means from five separate patches.

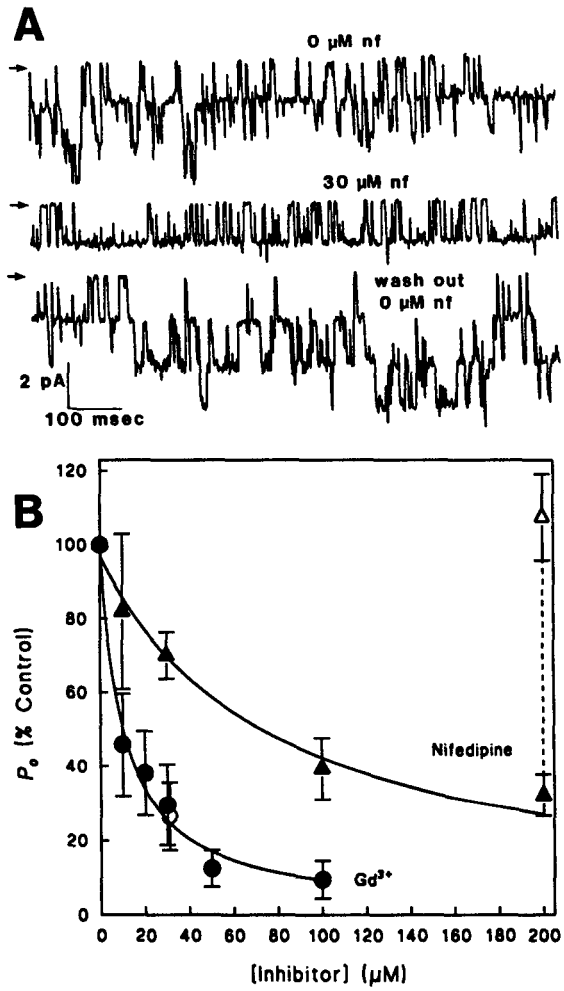

Figure 4. Inhibition of the 27-pS Channel by Calcium Channel Inhibitors.

(A) The effect of nifedipine on single-channel properties: single-channel current traces with 0 or 30 μM nifedipine (nf) or after an 8-min wash with nifedipine-free medium following exposure to the entire concentration series of nifedipine (wash out). $(Ca^{2+})_v$ was 10 mM, the holding potential was −30 mV, and all other conditions are as given in Figure 1A. **(B)** Reduction in P_o by increasing nifedipine (▲) or Gd^{3+} (●) concentration on the vacuolar side with a $(Ca^{2+})_v$ of 10 mM. All other conditions are as given in Figure 1A. The curves were fitted to the equation $P_o - P_o'/[1 + (I/K_{1/2})]$, where P_o' is the open-state probability in the absence of inhibitor. The derived $K_{1/2}$ values were 10.3 μM for Gd^{3+} and 77.0 μM for nifedipine. Spontaneous channel rundown was negligible, as demonstrated by the absence of activity loss at 30 μM Gd^{3+} after a 4-min pause with no solution exchange (○) or by the complete reversibility of nifedipine inhibition following an 8-min wash with nifedipine-free solution (△) at the end of the experiment. Recordings were made for 1 min at a holding potential of −30 mV. Control P_o was 0.22 ± 0.06 and 0.21 ± 0.03 for the Gd^{3+} and nifedipine data sets, respectively. Data are the mean ±SE of five separate patches for both inhibitors.

Figure 6 indicates that lowering the vacuolar pH to more physiological values reduced channel activity markedly. Although the decline in P_o over the range 7.3 to 6.1 was observed in all experiments, lowering the pH to 5.5 occasionally caused a restoration of activity to levels similar to those at pH 7.3 (data not shown). Nevertheless, the results indicated that channel activity is acutely sensitive to luminal pH.

Occasionally, the 27-pS channel exhibited a lower activity state upon patch formation than that shown in Figure 1. This lower activity state also has a conductance of 27 pS, but the single-channel current was saturated with voltage at a value lower than 2.6 pA, and the P_o was also lower (data not shown). This low-activity state could spontaneously switch to a high-activity state, with characteristics as shown in Figure 1. The activity change may be due to loss of an in vivo regulator that controls activity at resting potentials. Switches from the high- to the low-activity state were never observed. Spontaneous changes in activity have also been reported for the 12-pS Ca^{2+} release channel of beet taproot vacuolar membrane (Johannes et al., 1992b).

Two Classes of Ca^{2+} Release Channel Coreside in the Same Vacuole

The presence of the 27-pS channel at the vacuolar membrane of guard cells was supplementary to that of a Ca^{2+} release channel that more closely resembled the low-conductance voltage-gated vacuolar Ca^{2+} channels of beet (Johannes et al., 1992a; Gelli and Blumwald, 1993). Figure 7 shows I/V relationships for a single inside-out patch containing the 27-pS channel and a smaller channel with a conductance of 14 pS. The 14-pS channel was observed in 8% (9 of 111) of all patches but was often difficult to analyze because it was masked by the more active 27-pS channel and passed no outward K^+ current. This latter property required that E_{rev} be determined by extrapolation of the I/V relationship. Despite this, the 14-pS channel clearly had a less positive E_{rev} than the 27-pS channel. The mean E_{rev} values ($[Ca^{2+}]_v$ = 5 mM) from four inside-out patches where the two channels could clearly be distinguished were −2.2 ± 0.8 and 9.0 ± 2.2 mV for the 14- and 27-pS channels, respectively. These values give mean permeability ratios of $P_{Ca^{2+}}:P_{K^+}$ of 4.4:1 and 8.7:1, respectively. Figure 7C shows that the voltage sensitivity of the open-state probability of this smaller channel was similar to that of the 27-pS channel, indicating that the lower conductance channel also functions as a Ca^{2+} release channel.

The voltage-gating and ionic selectivities of the 14- and 27-pS guard cell channels reinforce the conclusion that they are involved in Ca^{2+} release. Both channels are gated open at physiologically relevant values of membrane potential. These values are further from the equilibrium potential for Ca^{2+} (E_{Ca}) (∼+130 mV for a 5mM:200nM vacuole-to-cytosol gradient) than they are from the equilibrium potential for K^+ (E_K) (∼+28 mV for a 300:100mM vacuole-to-cytosol gradient). Thus, even in the presence of a five- to 10-fold molar excess of K^+ over Ca^{2+},

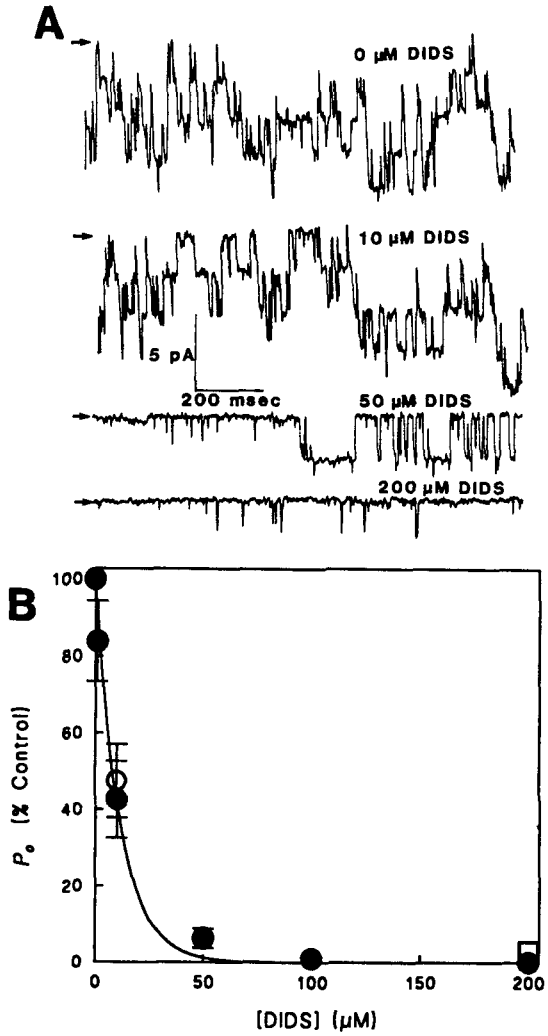

Figure 5. Inhibition of the 27-pS Channel by DIDS.

(A) The effect of DIDS on single-channel properties. DIDS was added to the vacuolar side of an inside-out patch pulled from an isolated vacuole of a broad bean guard cell. $(Ca^{2+})_{cyt}$ was 10 mM, the holding potential was −30 mV, and all other conditions are as given in Figure 1A.
(B) Reduction in P_o (●) by increasing the concentration of DIDS on the vacuolar side of an inside-out patch pulled from an isolated vacuole of a broad bean guard cell. The curve was fitted to the equation $P_o = P_o'(\exp[-K_{obs}[DIDS])$, where P_o' is the open-state probability in the absence of DIDS and K_{obs} is an effective inhibition constant (K_{obs} = 0.088 μM^{-1}). The derived concentration for effective half-maximal inhibition during the 4-min incubation period was 6.3 μM. Inhibition was complete during the 4-min solution exchange as demonstrated by the absence of reduction in P_o at 10 μM DIDS after an additional 4-min pause with no solution exchange (○). Inhibition was irreversible because there was no recovery of activity following an 8-min wash with DIDS-free medium at the end of the experiment (□). Recordings were made for 1 min. Control P_o = 0.24 ± 0.06. Data in **(B)** are the mean ±SE of six separate patches.

the extremely positive value of E_{Ca} compared to E_K indicates that a large proportion of the current through these relatively nonselective channels will be carried by Ca^{2+}.

DISCUSSION

Classes of Ca^{2+}-Permeable Channels in Plant Vacuoles

Using the patch clamp technique and inside-out patches, we have demonstrated the presence of voltage-gated, Ca^{2+}

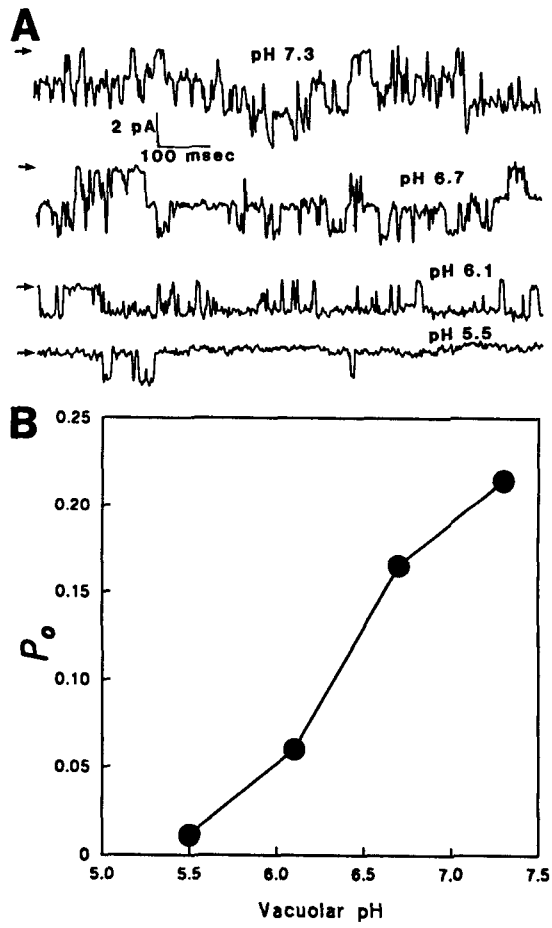

Figure 6. Effect of Vacuolar pH on the Activity of the 27-pS Channel.

(A) Single-channel current trace at various vacuolar lumen pH values: current trace obtained with 10 mM $(Ca^{2+})_v$ at vacuolar lumen pH values of 7.3, 6.7, 6.1, and 5.5. All other conditions are as given Figure 1A. The holding potential was −30 mV.
(B) The effect of varying vacuolar lumen pH on P_o of the 27-pS channel.
Recordings were made for 1 min. Data are from a single representative patch (the same patch as shown in **[A]**). Similar results were obtained from four other patches.

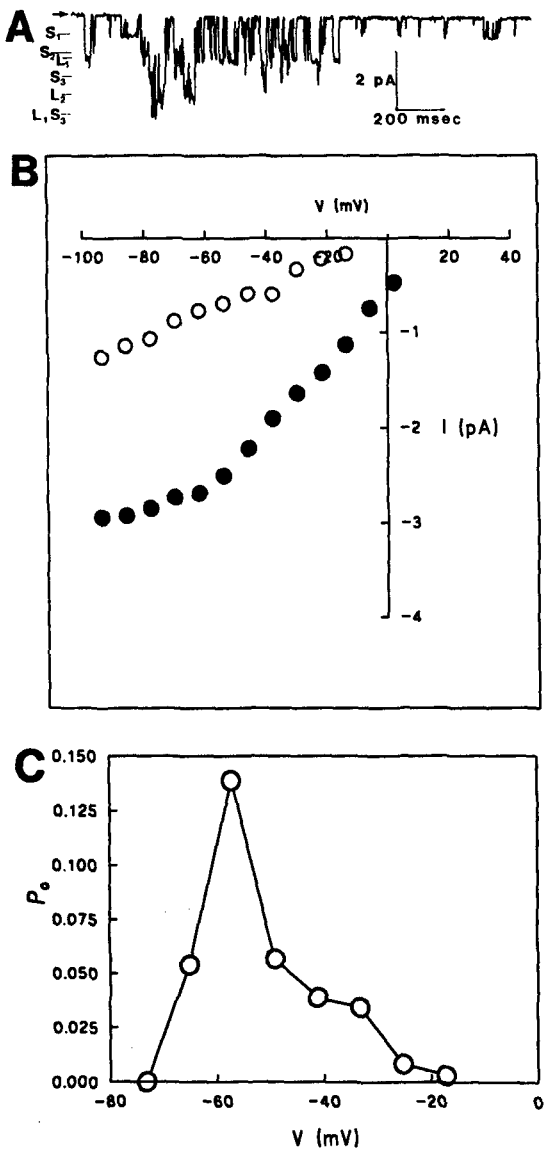

Figure 7. Two Ca^{2+} Release Channels Observed in the Same Patch.

(A) Channel current trace obtained from an inside-out patch pulled from an isolated vacuole of a broad bean guard cell. Holding potential was −38 mV; all other conditions are as given in Figure 1A. Two different open levels can be resolved, one labeled L (large) and the other S (small). The subscripts denote the number of open levels; the arrow marks the closed state.

(B) Unitary I/V relationships of the channels observed in **(A)**. The large channel is the 27-pS Ca^{2+} release channel (●). The small channel is a 14-pS channel (○) with a slightly negative E_{rev}. Data are from a single patch; the same channels were found to coreside in at least five other patches.

(C) P_o of the 14-pS channel as a function of the vacuolar membrane potential. The vacuolar medium contained 5 mM (○) Ca^{2+}. All other conditions are as given in Figure 1A. The data are from a different patch than the data in **(A)** and **(B)**. The patch contained the 14-pS channel only.

release channels at the vacuolar membrane of broad bean guard cells. The gating characteristics and selectivities of these channels suggest a role in vacuolar Ca^{2+} release. We identified an active, 27-pS channel in the vacuolar membrane that coresides in individual guard cell vacuoles with a smaller 14-pS Ca^{2+} release channel.

The 27-pS channel showed similarities to the beet Ca^{2+} release channel identified by Johannes et al. (1992a, 1992b) in the following respects: an outward current can be carried by K^+; the single-channel current saturates with Ca^{2+} as the charge carrier; the activity is not sensitive to $(Ca^{2+})_{cyt}$ (data not shown); the channel is inhibited by micromolar concentrations of Gd^{3+}; and the channel is gated open by voltage. However, the single-channel conductance was at least threefold greater than that of the beet channel and the selectivity fourfold lower in identical ionic conditions.

With respect to single-channel conductance, the 14-pS channel exhibited greater similarity than that of the 27-pS channel to the 12- and 6-pS Ca^{2+} release channels identified by Johannes et al. (1992a, 1992b) and Gelli and Blumwald (1993), respectively. However, unlike the 12-pS beet channel and the 27-pS broad bean guard cell channel, the single-channel current did not saturate, and no outward K^+ current was observed. These characteristics are similar to those of the 6-pS beet channel (Gelli and Blumwald, 1993), although the selectivity of the guard cell channel was lower.

A range of Ca^{2+}-selective channels has been characterized at the vacuolar membrane of plant cells to date. These channels fall into three categories. The first category comprises the inward-rectifying, voltage-gated channels identified in this study and others (Johannes et al., 1992a, 1992b; Gelli and Blumwald, 1993). The properties of these channels suggest they release Ca^{2+} from the vacuolar pool into the cytosol. This proposed role has justifiably been questioned on the basis that if the observed high level of channel activity were maintained in vivo, transvacuolar membrane Ca^{2+} gradients would rapidly dissipate (Bush, 1993). It is therefore of physiological relevance that the activity of the 27-pS channel in the present study was markedly reduced at a luminal pH of 5.5, which prevails in vivo. It was therefore to facilitate identification of the channel with reasonable frequency that the rather unphysiological luminal pH of 7.3 was selected.

The second group of Ca^{2+} channels is made up of ligand-gated Ca^{2+} release channels. These are gated open by increases in the cytosolic concentration of the second messenger $InsP_3$ and have a high conductance of 30 to 50 pS (Alexandre et al., 1990; Alexandre and Lassalles, 1992). Their existence has been independently confirmed by both Ca^{2+} release from vacuolar membranes in response to $InsP_3$ and radioligand binding studies (Ranjeva et al., 1988; Brosnan and Sanders, 1993).

The third class is comprised of outward-rectifying Ca^{2+} channels that have been identified with nonphysiological $(Ca^{2+})_{cyt}$ in the millimolar range (Pantoja et al., 1992; Ping et al., 1992). These channels are hypothesized to conduct Ca^{2+} from the cytosol into the vacuole following a rise in $(Ca^{2+})_{cyt}$

in response to $InsP_3$-induced Ca^{2+} release. However, this hypothetical role has been criticized (Maathuis and Sanders, 1992; Bush, 1993) because the required elevation of $(Ca^{2+})_{cyt}$ would be unlikely to occur in vivo and because the opening of an $InsP_3$-gated Ca^{2+} channel could not, for thermodynamic reasons, induce a swing in vacuolar membrane potential to values positive of the equilibrium potential for Ca^{2+} at which this outward-rectifying channel would conduct Ca^{2+} into the vacuolar lumen (Sanders et al., 1990).

Role of Vacuolar Voltage-Gated, Ca^{2+} Release Channels

Evidence is growing that internal Ca^{2+} pools are important in acting as a Ca^{2+} source for the elevation of $(Ca^{2+})_{cyt}$ prior to stomatal movements (Gilroy et al., 1991; Lemtiri-Chlieh and MacRobbie, 1994). Although the capacity of $InsP_3$ to mobilize intracellular Ca^{2+} has been demonstrated (Blatt et al., 1990; Gilroy et al., 1990), the particular stimulus–response pathways mediated by $InsP_3$ remain to be elucidated. The channels we report here could provide alternative and/or additional pathways for intracellular Ca^{2+} mobilization.

The question of why individual vacuoles should contain two voltage-gated, Ca^{2+} release channels that would perform essentially the same function in vivo must be addressed. Two distinct Ca^{2+} release channels might be required if they are part of signal transduction pathways arising from different primary signals that converge on the same terminal response, namely the elevation of $(Ca^{2+})_{cyt}$. If this is the case, differences in the regulatory properties of the channels might relate to the incoming signal. Although those regulatory factors that have emerged to date (voltage, luminal Ca^{2+}, and luminal pH) have very similar effects on the two channel types, it remains possible that the two channels operate under the influence of some as-yet-unidentified factor. Alternatively, the two channel types may be sensitive to the same or similar primary signals, and the dual presence of the channels might relate to the diverse roles of Ca^{2+} in intracellular signaling, endowing the cell with the capacity to vary the amplitude and/or duration of a Ca^{2+} signal. The presence of the 27-pS channel in guard cell vacuoles and not in beet taproots reinforces this argument in the context of the high profile of Ca^{2+}-mediated signaling in guard cell biology.

METHODS

Isolation of Guard Cell Protoplasts and Release of Vacuoles

Guard cell protoplasts were isolated from 2- to 4-week-old broad bean plants (*Vicia faba* cv Long Pod) by the method of Raschke and Hedrich (1989). Briefly, epidermal strips were removed from the adaxial surface of 10 to 15 fully expanded leaves and floated on 100 mL of ice-cold 10 mM sodium ascorbate, pH 6.4, 1 mM $CaCl_2$ for 15 min. The strips were removed and incubated for 2 hr at 26°C in 5 mL of 400 mM sorbitol, 10 mM sodium ascorbate, pH 6.4, 1 mM $CaCl_2$, 2% (w/v) cellulase (Onozuka RS; Yakult Pharmaceutical Ltd., Tokyo, Japan), and 0.1% (w/v) pectolyase Y-23. The digest was passed through a 100-μm nylon mesh, and the filtrate was then passed through a 20-μm mesh and rinsed through with a solution containing 500 mM sorbitol, 10 mM sodium ascorbate, pH 6.4, and 1 mM $CaCl_2$. The protoplasts were pelleted at 100*g* for 6 min in a bench-top centrifuge, resuspended in 500 mM sorbitol, 10 mM sodium ascorbate, pH 6.4, and 1 mM $CaCl_2$, and stored on ice.

Protoplasts were commonly of 98% guard cell origin as determined by the presence and morphology of chloroplasts. A 200-μL sample was placed into a patch clamp chamber, and the protoplasts were allowed to adhere to the glass bottom. They were then washed with a solution containing 100 mM KCl, 10 mM Hepes-KOH, pH 8.0, and 2 mM EGTA (200 mosmol/kg). This caused the protoplasts to rupture and release vacuoles. The appropriate bath solution for patch clamping was then perfused into the chamber. Only those vacuoles that could be positively identified as coming from guard cells (by having the remnants of the small, grey-green guard cell chloroplasts attached) were selected for seal formation.

Patch Clamp Media and Protocols

The standard bath (vacuolar) medium contained 5 mM $CaCl_2$, 3 mM Hepes-Tris, pH 7.3, and 0.5 mM DTT, and sorbitol to an osmotic pressure of 450 mosmol/kg. Increases in Ca^{2+} were achieved by supplementing with calcium gluconate. The pipette (cytosolic) media contained 10 mM KCl, 40 mM potassium gluconate, 3 mM Hepes-Tris, pH 7.3, and 0.5 mM DTT and sorbitol to an osmotic pressure of 450 mosmol/kg.

Patch pipettes were pulled from thin-walled borosilicate glass capillaries (Kimax, Vineland, NJ), coated with Sylgard (Dow Corning, Seneffe, Belgium), and fire polished. Pipette resistance in the experimental solutions was commonly 20 to 30 MΩ. Inside-out membrane patches were formed by pulling the pipette from the vacuole surface after pipette-vacuolar membrane seals of 5 GΩ or greater were achieved.

Single-channel current–voltage (I/V) relationships were determined by clamping the membrane potential using a bipolar pulse protocol of 8-mV steps from a holding potential of 0 mV. Open-state probability was determined from 15-sec or 1-min recordings, depending on channel activity, by the method of Johannes et al. (1992a). Open probability was determined as the ratio of mean open time to the total recording time divided by the number of channels that could clearly be resolved in the patch when the holding potential was −60 mV. This method assumes that all channels open independently of each other.

The inhibitors 4,4′-diisothiocyano-2,2′-stilbene disulfonate (DIDS), nifedipine, and Gd^{3+} were added to bath media (vacuolar side). Nifedipine was dissolved in 50% (v/v) ethanol, resulting in a final concentration of 1% (v/v) ethanol, which was maintained throughout this series of experiments. Solution changes were achieved via two low-noise peristaltic pumps (Gilson Minipuls 3; Gilson Medical Electronics, Middleton, WI), with the exchange being complete after 4 min. To check for spontaneous channel rundown, patches were left for 4 min partway through a concentration sequence before an identical recording was made. Patches showing significant rundown were not used for further analysis.

Patch Clamp Apparatus and Analysis

Patch clamp recordings were made as described by Hamill et al. (1981) with one Ag/AgCl half cell connected via a 3% agar bridge in pipette medium to the bath. Pipette potentials (V_p) were controlled with an

EPC-7 patch clamp amplifier (List Electronic, Darmstadt, Germany). Data were digitized at 2 kHz (1401 A/D converter; Cambridge Electronic Design, Cambridge, UK) and recorded on digital audiotape (DTC-1000 ES; Sony Corporation, Tokyo, Japan). For analysis, data were read into software packages (Cambridge Electronic Design) after low-pass filtering at 150 to 300 Hz. The actual potential across the membrane (V_m) was calculated by correcting V_p for the liquid junction potential (E_L), such that $V_m = V_p - E_L$. The value of E_L was calculated using a relationship that accounts for the presence of polyvalent ions (Equation 12 in Barry and Lynch, 1991). The values of E_L obtained for $(Ca^{2+})_v$ of 5, 10, and 20 mM were 17.2, 14.2, and 11.1 mV, respectively. For the solutions containing 5 mM $BaCl_2$, $SrCl_2$, and $MgCl_2$, the values of E_L were 16.6, 17.0, and 17.6 mV, respectively.

Values of E_{rev} measured were used to calculate the permeability ratio ($P_{Ca^{2+}}:P_{K^+}$) of the channel using a specific form of the Goldman-Hodgkin-Katz current equation simplified for the biionic conditions used here (Fatt and Ginsborg, 1958). The equation used was:

$$P_{Ca^{2+}}:P_{K^+} = \frac{(-[K]_c \exp^{(EF/RT)})\ (1 - \exp^{(2EF/RT)})}{4\ (Ca_v)\ (1 - \exp^{(EF/RT)})} \quad (1)$$

where v is the internal (vacuolar) ion concentration, c is the external (cytosolic) ion concentration, E is the measured reversal potential (E_{rev}), F is the Faraday constant, R is the gas constant, and T is the absolute temperature.

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