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Calcium-Activated K^+ Channels and Calcium-Induced Calcium Release by Slow Vacuolar Ion Channels in Guard Cell Vacuoles Implicated in the Control of Stomatal Closure

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Stomatal closing requires the efflux of K^+ from the large vacuolar organelle into the cytosol and across the plasma membrane of guard cells. More than 90% of the K^+ released from guard cells during stomatal closure originates from the guard cell vacuole. However, the corresponding molecular mechanisms for the release of K^+ from guard cell vacuoles have remained unknown. Rises in the cytoplasmic Ca^{2+} concentration have been shown to trigger ion efflux from guard cells, resulting in stomatal closure. Here, we report a novel type of largely voltage-independent K^+-selective ion channel in the vacuolar membrane of guard cells that is activated by physiological increases in the cytoplasmic Ca^{2+} concentration. These vacuolar K^+ (VK) channels had a single channel conductance of 70 pS with 100 mM KCl on both sides of the membrane and were highly selective for K^+ over NH_4^+ and Rb^+. Na^+, Li^+, and Cs^+ were not measurably permeant. The Ca^{2+}, voltage, and pH dependences, high selectivity for K^+, and high density of VK channels in the vacuolar membrane of guard cells suggest a central role for these K^+ channels in the initiation and control of K^+ release from the vacuole to the cytoplasm required for stomatal closure. The activation of K^+-selective VK channels can shift the vacuolar membrane to more positive potentials on the cytoplasmic side, sufficient to activate previously described slow vacuolar cation channels (SV-type). Analysis of the ionic selectivity of SV channels demonstrated a Ca^{2+} over K^+ selectivity (permeability ratio for Ca^{2+} to K^+ of ~3:1) of these channels in broad bean guard cells and red beet vacuoles, suggesting that SV channels play an important role in Ca^{2+}-induced Ca^{2+} release from the vacuole during stomatal closure. A model is presented suggesting that the interaction of VK and SV channel activities is crucial in regulating vacuolar K^+ and Ca^{2+} release during stomatal closure. Furthermore, the possibility that the ubiquitous SV channels may represent a general mechanism for Ca^{2+}-induced Ca^{2+} release from higher plant vacuoles is discussed.

INTRODUCTION

Stomatal pores in the leaf epidermis control gas exchange between leaves and the atmosphere including CO_2 uptake, necessary for photosynthesis, and H_2O loss by transpiration. Stomatal apertures are regulated in response to environmental and physiological stimuli, such as light, humidity, temperature, CO_2, and the hormone abscisic acid (ABA). Stomatal opening is osmotically driven and depends on the uptake of K^+ and anions into guard cells and the production of malate (for reviews, see Outlaw, 1983; Mansfield et al., 1990). Greater than 90% of the K^+ taken up by guard cells during stomatal opening is subsequently sequestered in guard cell vacuoles (Humble and Raschke, 1971). Furthermore, the volume of the guard cell vacuole increases during stomatal opening and decreases during closing, closely paralleling the volume changes measured for the entire guard cell (Fricker and White, 1990).

Stomatal closing is driven by a decrease in guard cell turgor as a result of an efflux of K^+ and anions from the guard cell vacuole into the cytoplasm and across the plasma membrane to the extracellular space. Ion channels in the plasma membrane of guard cells that mediate K^+ and anion efflux during the closing of stomata have been identified and characterized (Schroeder et al., 1987; Keller et al., 1989; Schroeder and Hagiwara, 1989). However, the molecular mechanisms that control and mediate efflux of K^+ from guard cell vacuoles, which are of central importance for stomatal closing, have remained largely unknown.

The hormone ABA can induce stomatal closure in response to drought stress, thereby reducing transpirational H_2O loss of plants. ABA application results in a net efflux of K^+ from the vacuole of guard cells as measured in $^{86}Rb^+$ flux studies (MacRobbie, 1990). These data underline the important role of vacuolar K^+ transporters during ABA-induced signaling. ABA induces an increase in guard cell cytoplasmic Ca^{2+} which precedes stomatal closure (McAinsh et al., 1990; Schroeder and Hagiwara, 1990). There is accumulating evidence that increases in the cytoplasmic Ca^{2+} concentration

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of guard cells represent a regulatory mechanism controlling stomatal closing. Additional studies suggest that ABA-induced stomatal closure can proceed through Ca^{2+}-independent signaling pathways (Gilroy et al., 1991; see MacRobbie, 1992). However, the release of Ca^{2+} in the cytoplasm of guard cells by photolysis of either caged calcium or caged inositol trisphosphate is a sufficient signal to induce stomatal closure (Gilroy et al., 1990). Regulation of K^+ transport at the vacuolar membrane of guard cells by Ca^{2+} and other second messengers has remained an open and important question. Large-conductance voltage-dependent slow-vacuolar (SV) ion channels have been identified in guard cells (Hedrich et al., 1988; Amodeo et al., 1994). Several physiological functions for these ubiquitous Ca^{2+}-activated vacuolar channels have been proposed in various higher plant cells and remain a matter of debate (Hedrich et al., 1986; Colombo et al., 1988; Hedrich and Kurkdjian, 1988; Sanders et al., 1990; Pantoja et al., 1992). In addition, Ca^{2+}-permeable ion channels with the opposite voltage dependence and a smaller single channel conductance than SV channels were reported in sugar beet vacuoles (Johannes et al., 1992).

In this study, we report the characterization of a novel class of highly selective K^+ channels in the vacuolar membrane of broad bean guard cells; these channels are regulated by physiological changes in both cytoplasmic Ca^{2+} and cytoplasmic pH. Vacuolar K^+ channels may provide a regulated mechanism for initiation and control of K^+ efflux from guard cell vacuoles during stomatal closing and provide a mechanism for SV channel activation. The Ca^{2+} permeability of guard cell SV channels reported here indicates that SV channel activation and the resulting Ca^{2+} efflux from the vacuole may contribute to rises in cytoplasmic Ca^{2+} during guard cell signaling events leading to stomatal closure.

RESULTS

The hormone ABA, which triggers stomatal closure in response to drought stress, causes increases in the cytoplasmic Ca^{2+} concentration (McAinsh et al., 1990; Schroeder and Hagiwara, 1990) and pH (Irving et al., 1992; Blatt and Armstrong, 1993). The guard cell vacuole, a storage organelle comprising 90 to 95% of the cell volume (Humble and Raschke, 1971; Fricker and White, 1990), plays an important role in osmotic adjustment during turgor-driven guard cell movements (for review, see Outlaw, 1983). To investigate whether ion transport mechanisms in the vacuole of guard cells are regulated by cytosolic Ca^{2+}, we applied the patch clamp technique to guard cell vacuoles (recordings on n = 78 vacuoles).

Calcium Activation of Single Channels in the Guard Cell Vacuolar Membrane

To directly analyze the regulatory effects of the cytoplasmic Ca^{2+} concentration on the activity of vacuolar membrane ion channels, single channel currents were recorded and the cytosolic Ca^{2+} concentration was modified within the physiological range. In all experiments, membrane potentials are specified as the potential on the cytoplasmic side of the membrane relative to the vacuolar side (Bertl et al., 1992a). Under this convention, the physiological resting potential of the vacuolar membrane is negative on the cytoplasmic side due to vacuolar proton pumps that transport positive charges into vacuoles (for review, see Sze et al., 1992). In all experiments, the cytoplasmic side of the membrane was exposed to the bath solution, allowing efficient changes in cytosolic Ca^{2+} concentrations.

When the free Ca^{2+} concentration on the cytoplasmic side of the membrane (bath solution) was buffered to <10 nM (referred to as 0 Ca^{2+}), few channel openings were observed between −100 and +100 mV. Figure 1A shows typical recordings where the membrane potential was held constant, within the physiological range, at −50 mV on the cytoplasmic side. When the Ca^{2+} concentration was increased to 5 μM by local perfusion, single channels activated rapidly (Figure 1A, n = 6 membrane patches). These Ca^{2+}-activated ion channels were continuously active in the entire voltage range analyzed (−100 to +100 mV). Currents resulting from the movement of cations from the vacuolar side to the cytoplasmic side of the membrane are plotted as downward deflections (Figures 1A and 1B). Figure 1A shows the high open probability of these single ion channels activated by 5 μM Ca^{2+}. When the cytosolic free Ca^{2+} concentration was returned to submicromolar levels, the vacuolar membrane ion channels rapidly deactivated; therefore, Ca^{2+} activation was reversible (Figure 1B). The Ca^{2+}-activated and largely voltage-independent channels described in Figures 1 through 6 differed from voltage-dependent SV ion channels (Hedrich and Neher, 1987; see below, Figure 7).

Single channel current recordings provided further information regarding the mechanism of Ca^{2+} activation, which is important for understanding the physiological role of these ion channels in guard cells. In all experiments, ATP was absent from the bath solution; therefore, a protein phosphorylation event was not required for channel activation. The rapid activation of these channels when Ca^{2+} was increased (Figures 1A and 1B) as well as channel deactivation when the cytoplasmic Ca^{2+} concentration was again buffered to low levels (Figure 1B) indicated that Ca^{2+} was bound directly to these ion channels or to a closely associated regulatory protein. These data indicated that stimuli triggering increases in the cytoplasmic Ca^{2+} concentration of guard cells, such as ABA, can result in the direct activation of vacuolar membrane ion channels.

Selectivity of Ca^{2+}-Activated Ion Channels

To further elucidate the effects of channel activation on ionic fluxes across the guard cell vacuolar membrane, it was necessary to determine the ionic selectivity of these Ca^{2+}-activated ion channels. The ionic selectivity of the Ca^{2+}-activated

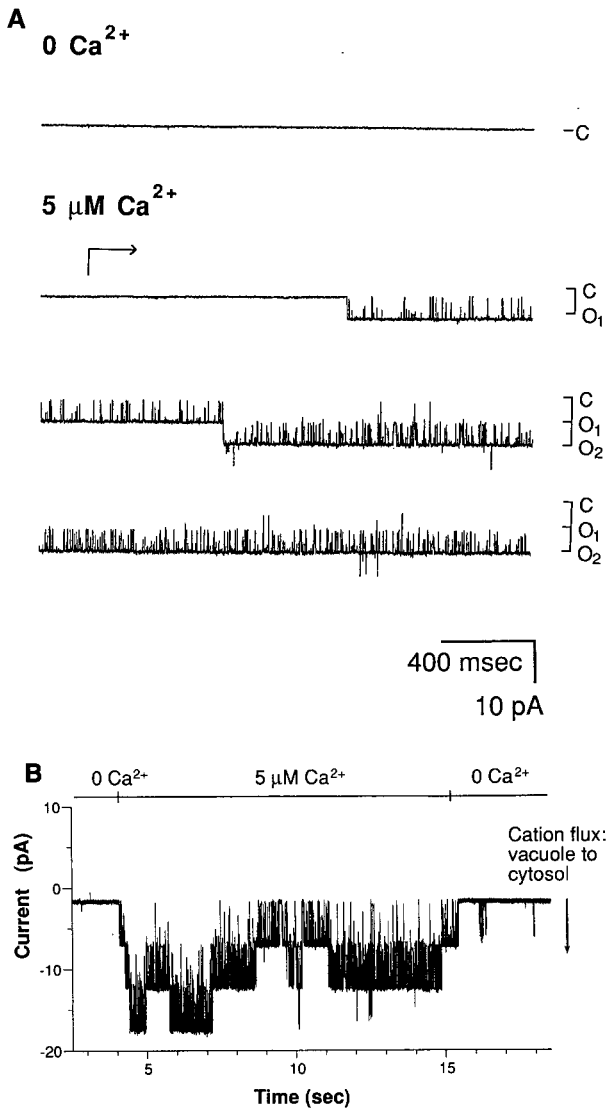

Figure 1. Increasing the Ca^{2+} Concentration at the Cytoplasmic Side of a Guard Cell Vacuolar Membrane Patch Activates Single Ion Channels.

(A) Local perfusion with 5 μM free Ca^{2+} activated single ion channels in cytoplasmic-side-out patches of the vacuolar membrane. The vacuolar membrane potential was held at −50 mV on the cytoplasmic side. The arrow in this continuous trace indicates the start of local perfusion with 5 μM free Ca^{2+}. Local perfusion delay time was ⩾300 msec. Downward deflections represent cation currents directed from the vacuole to the cytoplasm (see arrow in **[B]**). The pH on the cytoplasmic membrane side was 7.2. C, O_1, and O_2 indicate the closed state and the opening of one and two channels, respectively. Solutions are as given in Methods.

(B) Single channel currents in another vacuolar membrane patch were activated by cytosolic Ca^{2+} under the same conditions given in **(A)** and are presented in a compressed time scale. At least three channels were present in the membrane patch.

In **(A)** and **(B)**, the recordings were filtered at 1 kHz.

channels in guard cell vacuoles was accurately determined at the single channel level. A cytoplasmic free Ca^{2+} concentration of 1 μM was utilized to activate the vacuolar membrane ion channels. As shown in Figure 2A, when the membrane potential was changed linearly from −100 to +100 mV over a period of 1.8 sec, single channel currents were activated over the entire voltage range. The single channel conductance and Ca^{2+} activation of these ion channels were identical to those presented in Figure 1. Single channel currents also remained activated at all potentials in the range of +100 to −100 mV during continuous recordings (n = 9 membrane patches). In detailed experiments, single channel current amplitudes were

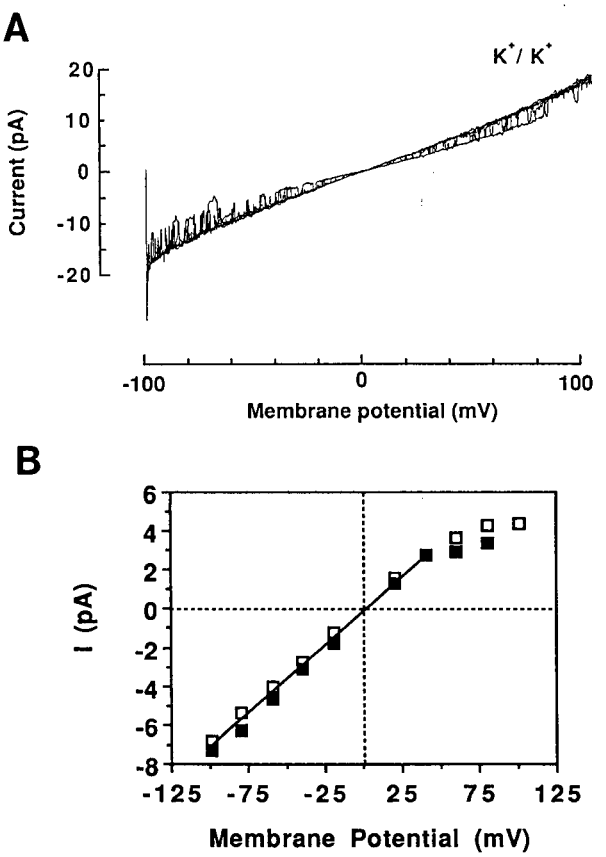

Figure 2. The Ca^{2+}-Activated Ion Channels in the Vacuolar Membrane Allow Large Influx and Efflux Currents.

Single channel currents were recorded using cytoplasmic-side-out membrane patches excised from guard cell vacuoles.

(A) The membrane potential was changed linearly from −100 to +100 mV over 1.8 sec. Five current records were superimposed; they were filtered at 200 Hz.

(B) The dependence of single channel current amplitude on applied membrane potential is shown. Single ion channel currents were recorded at the indicated membrane potentials, and current amplitudes were measured using Fetchan software. Data from two vacuole membrane patches are indicated by closed and open squares.

In **(A)** and **(B)**, free calcium was buffered at 1 μM with 4 mM EGTA and 3.48 mM $CaCl_2$ on the cytoplasmic side of the membrane at pH 7.2.

measured and plotted as a function of membrane potential (Figure 2B). Linear regression analysis was used to determine a single channel conductance of 70 pS (slope = 70 × 10^{-12} A/V), which indicated that these channels allowed large influx and efflux currents.

Single channel currents reversed at a membrane potential of +2.5 mV, close to the theoretical equilibrium potential for K^+ of 0 mV under these recording conditions. The equilibrium potential for Ca^{2+} was adjusted to +107 mV by imposing a physiological 4200-fold transmembrane gradient of free Ca^{2+}, after correction for ionic activities. Under these conditions, the recorded reversal potential of single channel currents of 2.5 mV revealed that these Ca^{2+}-activated ion channels were not significantly permeable to Ca^{2+} ions (Figures 2A and 2B).

To further characterize the ionic selectivity of these vacuolar membrane channels, K^+ ions on the cytoplasmic side of the membrane (bath solution) were replaced with various monovalent cations. The Cl^- concentration in the bath solution was maintained at 101 mM (see Methods). The selectivity of the Ca^{2+}-activated channels was determined from shifts in the reversal potential of single channel currents as well as the ability of the cations tested to permeate the vacuolar membrane.

When K^+ ions on the cytoplasmic side of the membrane were replaced by NH_4^+, K^+ currents from the vacuole into the cytosol were clearly resolved, as shown in Figures 3A and 3B. NH_4^+ permeated these ion channels to a lesser degree as indicated by less clearly resolved upward current deflections at positive vacuolar membrane potentials during voltage ramps (Figure 3A). Continuous recordings in the range from +60 to +100 mV allowed improved resolution of single channel currents at positive potentials (Figures 3B and 3C, top trace) and indicated a measurable NH_4^+ permeability of the Ca^{2+}-activated vacuolar channels (n = 5 membrane patches). The reversal potential of single channel currents was shifted to more positive potentials, indicating that these channels were selective for K^+ over NH_4^+. The reversal potential, determined by a linear regression fit to the data (from −60 to +100 mV), was +19.6 mV (Figure 3B). From this reversal potential, a permeability ratio for K^+ to NH_4^+ of 2.2 to 1 was calculated.

To determine the Rb^+ permeability of these ion channels, K^+ ions on the cytosolic membrane side were replaced with Rb^+ by bath perfusion (n = 5 membrane patches). With Rb^+ at the cytosolic side of the membrane, only downward current deflections that represented K^+ flux from the vacuole into the cytoplasm at negative potentials were clearly resolved, as shown in Figures 3D and 3E (n = 5 membrane patches). Rubidium ions were largely impermeant to these ion channels because measurable currents from the cytosol into the vacuole were also not observed during continuous recordings at positive holding potentials (n = 4 membrane patches).

The dependence of the amplitude of single channel currents on membrane potential for K^+ flux was linear with an extrapolated reversal potential of 13.8 mV and a single channel conductance of 68 pS (Figure 3E). These data indicated that these ion channels were selective for K^+ over Rb^+ (calculated K^+ to Rb^+ permeability ratio of ~1.7:1). Reversal potential measurements reflect the ability of ions to enter the channel pore and can be independent of ion permeation through the channel (Hille, 1992). The data presented in Figures 3D and 3E indicated that Rb^+ entered the ion channel pore but did not significantly pass through these channels. Consistent with these observations, the open probability of these Ca^{2+}-activated vacuolar K^+ channels at negative potentials was greatly reduced by Rb^+ (Figure 3D and data not shown), indicating that Rb^+ on the cytosolic side of the membrane may partially block these K^+ channels.

These vacuolar membrane ion channels were strongly selective for K^+ over Na^+, Cs^+, and Li^+ as shown in Figure 4. When K^+ in the cytosolic solution was replaced with these ions, single channel currents did not reverse at potentials up to +100 mV, and only K^+ currents from the vacuole into the cytosol were observed (Figures 4A to 4C). These data indicated that Na^+, Cs^+, or Li^+ did not measurably permeate these ion channels and that K^+ ions were ⩾50 times more permeant than Na^+, Cs^+, or Li^+ ions. The permeability sequence determined from reversal potential measurements for the Ca^{2+}-activated K^+ channels was $K^+ > Rb^+ > NH_4^+ >> Cs^+ \approx Na^+ \approx Li^+$. The highly selective vacuolar K^+ channels were therefore named VK channels.

Calcium-Activated K^+ Currents in Whole Guard Cell Vacuoles

To determine whether the highly selective VK channels could provide a physiologically significant pathway for large K^+ fluxes from the vacuole into the cytoplasm during Ca^{2+}-dependent signaling in guard cells, the sum of all K^+ channel currents carried across the vacuolar membrane of single guard cell vacuoles was recorded using the whole-vacuole recording technique (Hedrich and Neher, 1987). Voltage clamp recordings were performed to analyze the regulation of K^+ channel currents by cytoplasmic Ca^{2+} and pH. As shown in Figure 5 (inset), voltage pulses from −100 to +100 mV were applied to single guard cell vacuoles. Figure 5A shows the typical low but measurable level of background ionic currents crossing the vacuolar membrane of whole guard cell vacuoles when the cytoplasmic Ca^{2+} concentration was buffered to <10 nM (indicated as 0 Ca^{2+}).

When the Ca^{2+} concentration on the cytoplasmic side of the membrane (bath solution) was increased to 1 μM (Figure 5B) or 5 μM (Figure 5C), large currents were recorded (n = 15). These Ca^{2+}-induced whole-vacuole currents activated instantaneously upon application of voltage steps to positive and negative potentials (Figures 5B and 5C), consistent with the activation of Ca^{2+}-dependent single K^+ channels. Calcium-activated K^+ currents showed a weak voltage dependence, which is indicated by saturation of ionic currents at positive potentials (Figure 5D) similar to that observed for single channel currents (Figure 2).

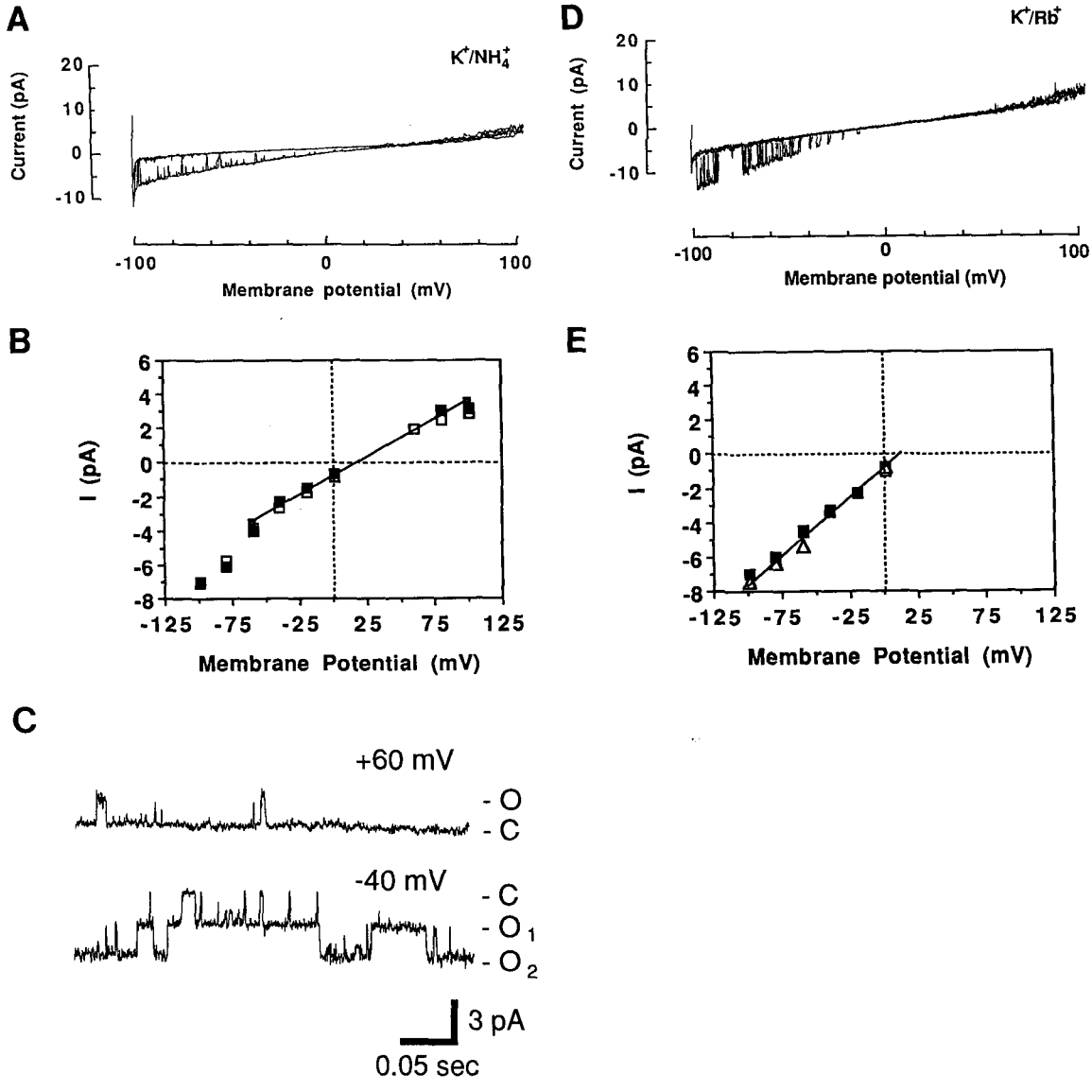

Figure 3. The Ca^{2+}-Activated Vacuolar Membrane Ion Channels Are Selective for K^+ over NH_4^+ and Rb^+.

Potassium ions in the bath solution (cytoplasmic side) were replaced entirely by Rb^+ or NH_4^+ to determine the cationic selectivity. The cytoplasmic free Ca^{2+} concentration was buffered to 1 μM (see legend to Figure 2), and other components of solutions are as given in the Methods.

(A) The cytoplasmic solution contained 100 mM NH_4Cl. The vacuolar membrane potential was changed linearly from −100 to +100 mV over 1.8 sec. Five current records were superimposed; they were filtered at 200 Hz.

(B) The cytoplasmic solution contained 100 mM NH_4Cl. Single ion channel current amplitude was measured during continuous recordings at the indicated membrane potentials using Fetchan software and was plotted as a function of vacuolar membrane potential. Data from two vacuolar membrane patches are indicated by the open and closed squares.

(C) Representative current records for data presented in **(B)**. The membrane potential is indicated. C, O_1, and O_2 indicate the closed state and the opening of one and two channels, respectively. Solutions are as given in **(B)**. Current records were filtered at 1 kHz.

(D) The cytoplasmic solution contained 100 mM RbCl. As given in **(A)**, the vacuolar membrane potential was changed linearly from −100 to +100 mV over 1.8 sec. Five current records were superimposed; they were filtered at 200 Hz.

(E) The cytoplasmic solution contained 100 mM RbCl. Single ion channel current amplitudes were determined during continuous recordings and plotted as a function of vacuolar membrane potential. Data from three vacuolar membrane patches are indicated by closed squares, open squares, and open triangles.

In **(A)** to **(E)**, single channel currents were recorded using cytoplasmic-side-out patches of vacuolar membrane.

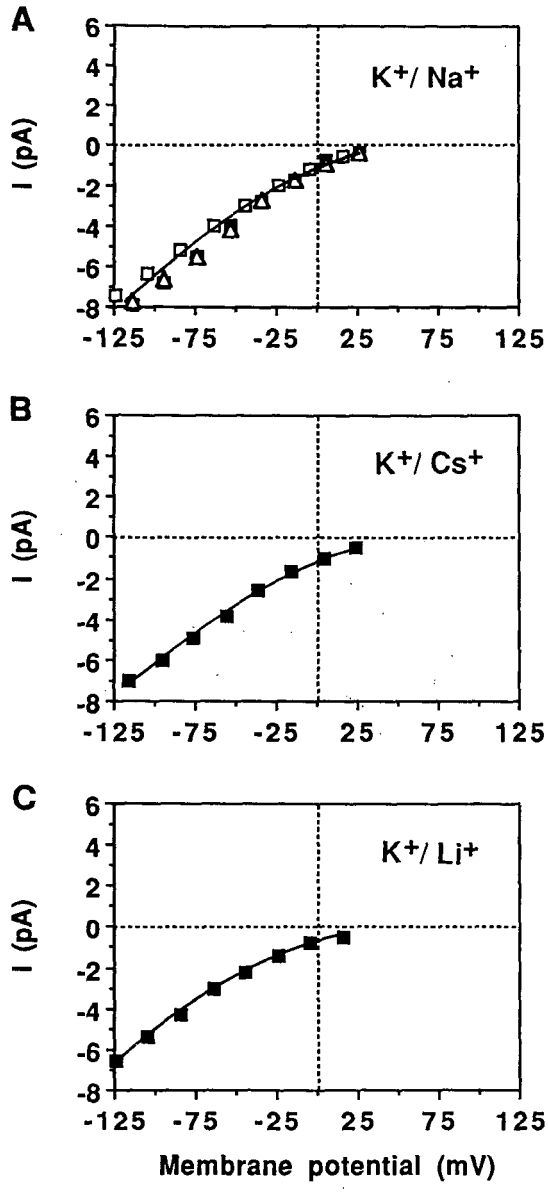

Figure 4. The Ca^{2+}-Activated Channels Are Highly Selective for K^+.

Single channel currents were recorded using cytoplasmic-side-out membrane patches from guard cell vacuoles. Downward current deflections represent K^+ flux from the vacuolar side to the cytoplasmic side of the vacuolar membrane. The cytoplasmic free Ca^{2+} concentration was buffered to 1 μM (see legend to Figure 2), and other components of solutions are as given in the Methods, except that KCl on the cytoplasmic side was replaced with chloride salts by bath perfusion as follows.

(A) The bath solution (cytosolic side) contained 100 mM NaCl. Data from three vacuolar membrane patches are indicated by open squares, closed squares, and open triangles.

(B) The bath solution contained 100 mM CsCl. Data from one vacuolar membrane patch are indicated by closed squares.

(C) The bath solution contained 100 mM LiCl. Data from one vacuolar membrane patch are indicated by closed squares.

The K^+ permeability of Ca^{2+}-activated currents was analyzed at the whole-vacuole level. The lack of time-dependent deactivation (tail currents) of VK channels required measurements of zero-current potentials, which result from the sum of all vacuolar conductances. Under 100 mM symmetrical KCl concentrations, reversal potentials of whole-vacuole Ca^{2+}-activated currents were +9 mV, which is close to the K^+ equilibrium potential (E_{K+} = 0 mV) and diverged from imposed Ca^{2+} equilibrium potentials (E_{Ca2+} = +107 mV in Figure 5B; E_{Ca2+} = +86 mV in Figure 5C, after correction for ionic activities). To further analyze for K^+ over Cl^- selectivity of whole-vacuole Ca^{2+}-activated currents, a transmembrane gradient of KCl was applied. As shown in Figure 5E, the whole-vacuole zero-current potential shifted to ~+42 mV. Under these recording conditions, the equilibrium potential for K^+ was +70 mV and that for Cl^- was −57 mV, after correction for ionic activities. The deviation of the zero-current potential from E_{K+} can be attributed to a background conductance of uncharacterized selectivity in whole vacuoles (Figure 5A). When K^+ in the bath solution was replaced by 100 mM Rb^+, whole-vacuole Ca^{2+}-activated instantaneous currents were blocked (n = 3 vacuoles, data not shown); this correlates to Rb^+ block of single channel VK channels (Figure 3D). The Ca^{2+} activation, K^+ permeability, and time and voltage dependence of whole-vacuole currents (Figure 5) correlated to the properties of single Ca^{2+}-activated K^+ channels. These data indicated that the whole-vacuole Ca^{2+}-activated currents were carried to a large extent by the above described Ca^{2+}-activated and largely voltage-independent VK channels.

The large amplitude of these whole-vacuole K^+ currents (Figure 5) and the occurrence of multiple single VK channels in most membrane patches (Figures 1 through 4) indicated that VK channels were present in high density in the guard cell vacuolar membrane. Elevation of the cytosolic free Ca^{2+} concentration to 1 μM on the cytoplasmic side of the membrane was sufficient for >50% activation of these vacuolar K^+ channels (Figures 5B and 5D). The high selectivity of these VK channels for K^+ together with the vacuolar membrane potential generated by the vacuolar proton pumps suggested that increases in the cytoplasmic Ca^{2+} concentration would result in K^+ release from the vacuole mediated by these channels. Based on these findings, VK channels could provide a physiologically significant pathway for K^+ flux from the vacuole into the cytoplasm during stomatal closure (see Discussion).

Regulation of Vacuolar Ca^{2+}-Activated K^+ Channels by Cytoplasmic pH

Changes in cytosolic pH have been measured in response to ABA and may play a major regulatory function in guard cell responses to hormones (Irving et al., 1992; Blatt and Armstrong, 1993). To determine the effects of cytoplasmic pH on the vacuolar Ca^{2+}-activated K^+ channels, whole-vacuole recordings were performed at high Ca^{2+} concentrations on the cytoplasmic side (1 mM unbuffered Ca^{2+} in the bath solution). This

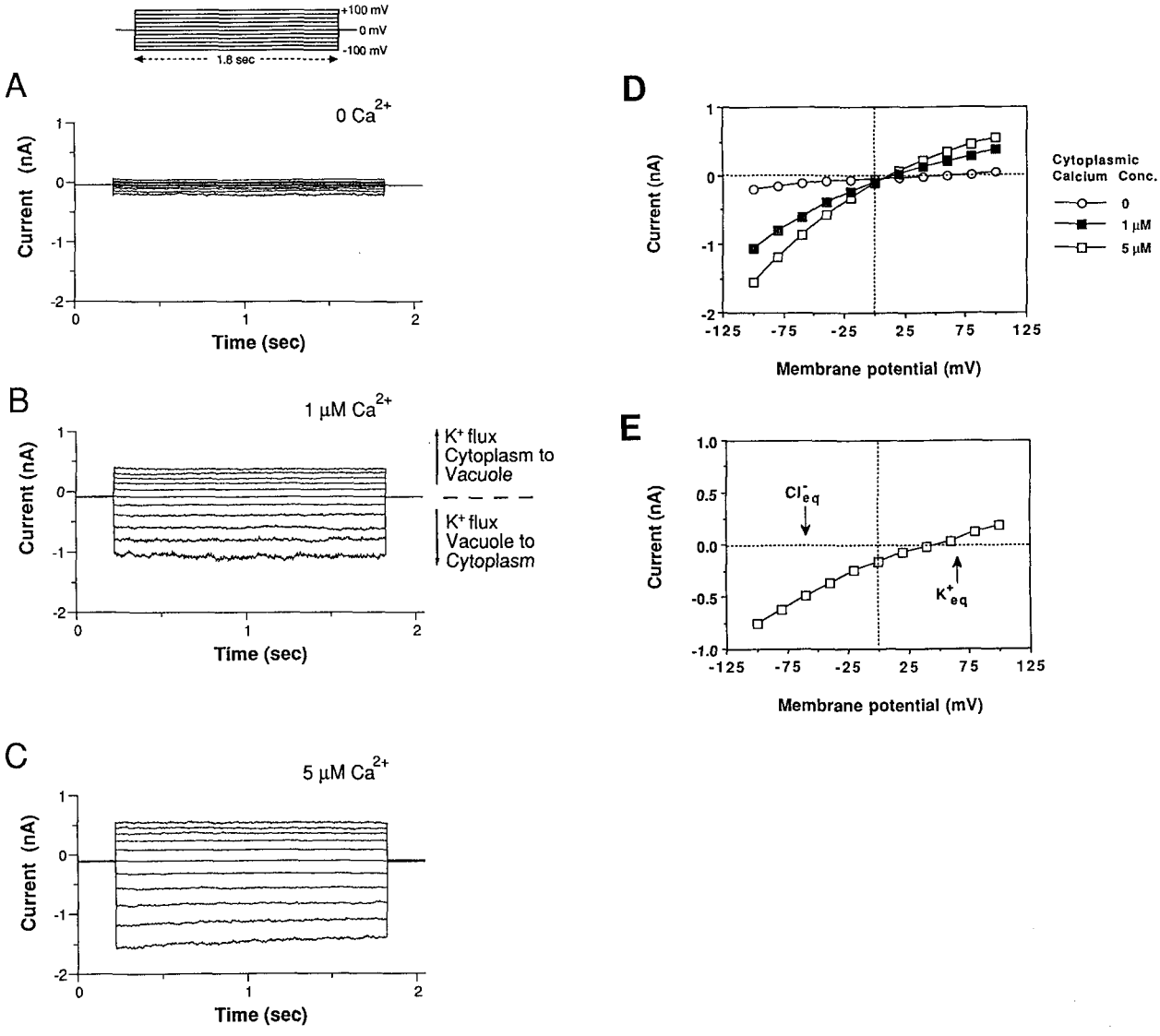

Figure 5. Increasing Cytoplasmic Ca^{2+} Concentration within the Physiological Range Activates Ion Channel Currents in the Vacuolar Membrane of Guard Cells.

The inset shows that from a holding potential of 0 mV, 1.8-sec voltage pulses were applied between −100 and +100 mV on the cytosolic side of the membrane in increments of 20 mV. Whole-vacuole currents were recorded. Downward deflections in current represent K^+ flux directed from the vacuolar side to the cytoplasmic side of the membrane as indicated in **(B)**.

(A) Low background currents at submicromolar cytoplasmic Ca^{2+} concentration are shown. The bath solution (cytoplasmic side) contained 4 mM EGTA at pH 7 without additional Ca^{2+}.

(B) Large inward- and outward-directed currents were activated by increasing the free Ca^{2+} concentration at the cytoplasmic side of the vacuolar membrane to 1 μM at pH 7.0.

(C) The free Ca^{2+} concentration in the bath solution was buffered to 5 μM.

(D) Current-voltage relationships from **(A)**, **(B)**, and **(C)** are shown. Average current amplitudes across the vacuolar membrane measured are plotted as a function of applied membrane potentials. In **(A)** to **(C)**, current records were filtered at 2 kHz. Conc., concentration.

(E) Whole-vacuole Ca^{2+}-activated currents were recorded in the presence of an imposed KCl gradient. The bath solution (cytoplasmic side) contained 20 mM Tris, 10 mM KCl, 4 mM EGTA, 5 μM free Ca^{2+}, and the pH was adjusted to 7.0 with Mes. The pipette solution contained 5 mM Mes, 200 mM KCl, 2 mM $MgCl_2$, 5 mM $CaCl_2$, and the pH was adjusted to 5.5 with Tris. The equilibrium (eq) potentials for K^+ and Cl^- (+70 mV and −57 mV, respectively, after correction for ionic activities) are indicated.

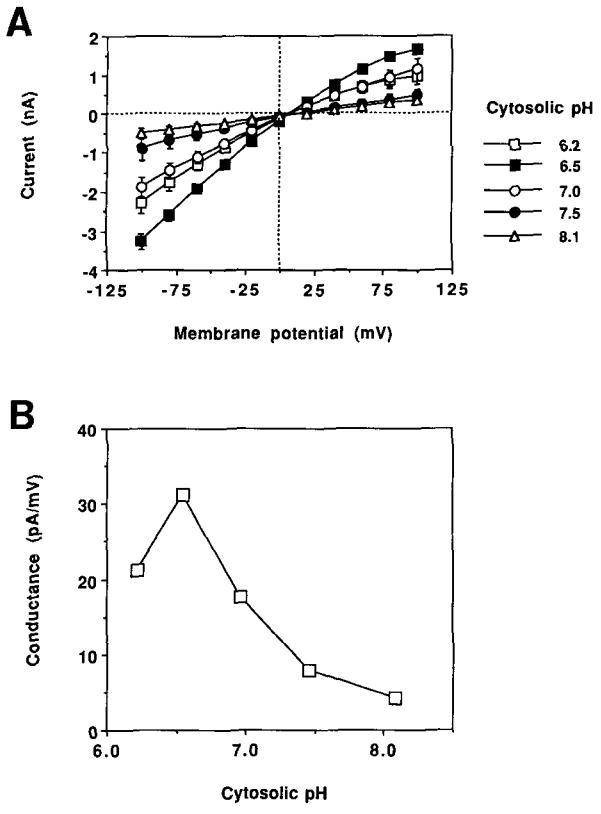

Figure 6. Ca^{2+}-Activated K^+ Currents in the Guard Cell Vacuole Membrane are pH Dependent.

(A) Whole-vacuole instantaneous currents are plotted as a function of membrane potential for each bath pH (± SE, n = 3 to 5 vacuoles at each pH value). The bath solution (cytoplasmic side) contained 1 mM Ca^{2+} without the addition of EGTA. The bath solution pH was adjusted using Mes. Other solution components are as described in the Methods. Whole-vacuole currents were recorded following the voltage protocol of Figure 5.

(B) Conductance values, indicated by open squares, were derived by linear regression analysis of currents (between −100 and +40 mV) presented in **(A)**.

saturates the Ca^{2+} binding sites functional in Ca^{2+}-dependent activation of these ion channels and excludes the possible effect of pH on Ca^{2+} binding. Ca^{2+}-activated instantaneous vacuolar K^+ channel currents showed a strong dependence on cytosolic pH (n = 18 vacuoles). Figure 6A shows the dependence of whole-vacuole K^+ currents on the imposed vacuolar membrane potential at five cytosolic pH values. A plot of the K^+ conductance (Figure 6B) shows a peak of channel activity at pH 6.5 and a marked pH dependence through the physiological range. Thus, the physiologically occurring cytoplasmic alkalinization during stomatal closing (Irving et al., 1992) may down-regulate K^+ flux from the vacuole into the cytoplasm mediated by the vacuolar K^+ channels (see Discussion).

Guard Cell SV-Type Channels Are Ca^{2+} Permeable

The ionic selectivity, voltage independence, and the time and Ca^{2+} dependences of the above described VK ion channels are highly distinct from less selective voltage-dependent SV ion channels (Hedrich and Neher, 1987). In addition, the Ca^{2+} activation mechanism of VK channels differs significantly from that of SV-type channels (Weiser et al., 1991; Bertl et al., 1992b; Bethke and Jones, 1994; J.M. Ward and J.I. Schroeder, unpublished data). The high K^+ selectivity of VK channels suggests that their activation results in K^+ flux from the vacuole into the cytosol, which in turn could cause a positive shift in the vacuolar membrane potential. Under conditions of elevated cytoplasmic Ca^{2+}, such a positive shift in vacuolar membrane potential could activate SV channels in the vacuolar membrane (Hedrich and Neher, 1987). Therefore, to understand the role of VK channels, it was important to determine which physiological ions permeate SV channels in guard cells.

Under the recording conditions imposed here, higher cytosolic Ca^{2+} concentrations (10 to 100 μM Ca^{2+}) were necessary to activate voltage-dependent SV channels as shown in Figure 7 (n = 19 vacuoles) than for Ca^{2+} activation of the largely voltage-independent VK channel (compare to Figure 5). In 100 mM symmetrical KCl, SV channels had a single channel conductance of ~155 pS, and channel openings were observed only at positive holding potentials, which confirmed previous findings (Hedrich et al., 1988; Amodeo et al., 1994). This single channel conductance was ~2.2-fold higher than the conductance of VK channels at negative potentials (Figure 2). As shown in Figure 7A, at positive potentials both VK and SV channel openings were recorded in the same patch of vacuolar membrane. At a holding potential of +80 mV (Figure 7A, top trace), openings of two VK ion channels (smaller amplitude) and one SV channel (larger amplitude) are shown. Under these recording conditions, with 1 mM Ca^{2+} in the bath (cytoplasmic side), the outward VK channel amplitudes (at +80 mV, top trace) were slightly smaller than with 1 μM Ca^{2+} in the bath (see Figure 2). However, at negative potentials (Figure 7A, bottom trace) single VK ion channel current amplitude was the same as with 1 μM cytosolic Ca^{2+} (Figure 2).

To determine the ionic selectivity of the SV ion channel currents, the reversal of SV channel deactivation (tail current) was analyzed at the whole vacuole level (n = 11 vacuoles), as shown in Figures 7B and 7C. With 100 mM KCl in the bath solution (cytoplasmic side), when positive vacuolar membrane potentials were applied, typical SV channel currents were recorded (Figure 7B; Hedrich and Neher, 1987). The vacuolar membrane was then stepped to less positive potentials and tail currents were measured. Under these recording conditions, the K^+ equilibrium potential was extremely negative because K^+ was present only at the cytosolic side of the membrane. However, time-dependent whole-vacuole currents were observed to reverse at +14 mV (Figure 7B), and single SV channel currents reversed at +5.8 mV (Figure 7E). These data indicated that in addition to K^+ (Kolb et al., 1987; Colombo et al., 1988;

Amodeo et al., 1994), SV channels were also permeable to either Ca^{2+} or Cl^-.

From detailed studies on other tissues as well as in guard cells, SV channels are known to be nonselective with respect to monovalent cations (Kolb et al., 1987; Colombo et al., 1988; Amodeo et al., 1994). In this study, we therefore focused on the physiologically important issue of whether guard cell SV channels were also permeable to Ca^{2+} (Bertl et al., 1992b; Pantoja et al., 1992), Cl^- ions (Hedrich et al., 1986; Hedrich and Kurkdjian, 1988), or both. To clearly distinguish whether Ca^{2+}, Cl^-, or both ions permeated guard cell SV channels, experiments were performed without K^+ in the bath or pipette solutions and with a $CaCl_2$ gradient across the vacuolar membrane (see Figure 7C inset). As shown in Figure 7C, application of positive vacuolar membrane potentials activated SV currents. Under these recording conditions, the equilibrium potential for Cl^- was ~ -59 mV and that for Ca^{2+} was +29 mV, respectively, after correction for ionic activities. The reversal potential of peak tail currents (Hille, 1992) was interpolated at +32 mV (Figure 7C). Using the same solutions, single SV channel currents reversed at +26.6 mV (Figure 7F). This indicated that guard cell SV channels were selective for Ca^{2+} and not significantly permeable to Cl^-. The calculated permeability ratio using the Goldman-Hodgkin-Katz equation (Hille, 1992) of Ca^{2+} to K^+ derived for the data presented in Figure 7B, after correction for ionic activities, was 5.3 to 1. Based on the decreased amplitude and lower noise of whole-vacuole SV channel recordings performed in the absence of K^+ in the bath solution (compare Figures 7B and 7C), the single channel conductance of SV channels was expected to be lower than 155 pS (Figure 7A) with Ca^{2+} as the only permeant ion. This was confirmed in single channel recordings (Figures 7D to 7F).

DISCUSSION

The aperture of stomatal pores in the leaf epidermis is regulated by turgor changes in pairs of guard cells. The large central vacuole plays a central role in guard cell turgor regulation by accumulating and releasing K^+ (Humble and Raschke, 1971; MacRobbie, 1981, 1990) and anions (MacRobbie, 1981). However, information concerning the molecular mechanisms responsible for controlling and mediating fluxes of osmotically important solutes across the vacuolar membrane of guard cells has been limited. Stomatal closure depends on large fluxes of K^+ and anions from the vacuole into the cytoplasm and subsequently to the extracellular space. Based on the physiological membrane potential generated by vacuolar membrane proton pumps of -20 to -50 mV, channel-mediated K^+ flux from the vacuole into the cytoplasm is thermodynamically favored. In contrast, vacuolar K^+ accumulation, which is important for stomatal opening, would require the inactivation of vacuolar K^+ channels and the operation of active K^+ transport mechanisms (Sze et al., 1992; Rea and Poole, 1993).

VK Channel Activation Can Initiate Vacuolar Ion Release

Treatments that increase the guard cell cytoplasmic Ca^{2+} concentration lead to stomatal closure, indicating that Ca^{2+} concentration change is a central regulator of guard cell turgor (Gilroy et al., 1990). Increases in the cytoplasmic Ca^{2+} concentration to >1 μM occur in response to ABA (McAinsh et al., 1990; Schroeder and Hagiwara, 1990) and are sufficient to activate VK channels (Figures 1 and 5). The vacuolar Ca^{2+}-activated channels are highly selective for K^+ (Figures 2, 3, and 4). The activation of VK channels would result in a flux of K^+ into the cytosol, causing a shift in the vacuolar membrane potential to more positive potentials on the cytosolic side of the vacuolar membrane, as indicated in Figure 8. Elevated Ca^{2+} together with this initial positive shift in membrane potential, due to VK channel activation, would activate voltage-dependent SV channels (Hedrich et al., 1986) that allow Ca^{2+} efflux from the vacuole.

SV ion channels are activated only at vacuolar membrane potentials of $\sim$0 mV or more positive on the cytoplasmic membrane side (Hedrich and Neher, 1987). Due to the large physiological gradient of Ca^{2+} across the vacuolar membrane, activation of vacuolar Ca^{2+}-permeable ion channels at membrane potentials in the physiological range would result in Ca^{2+} flux from the vacuole into the cytoplasm. Therefore, VK channel activation may be a critical step in controlling stomatal closure by (1) mediating vacuolar K^+ release and (2) depolarizing the vacuolar membrane to potentials at which SV channels are activated, thereby allowing Ca^{2+} and additional K^+ efflux from the vacuole into the cytosol (Figure 8). This increase in cytoplasmic Ca^{2+} would sustain VK and SV channel activation and provides a mechanism for Ca^{2+}-induced Ca^{2+} release, which may have additional regulatory roles during stomatal closing (e.g., Schroeder and Hagiwara, 1989; Hedrich et al., 1990). Further research is necessary to elucidate the regulatory mechanisms in guard cells responsible for shifts in the activation potential of SV ion channels to vacuolar membrane potentials within the physiological range of 0 to -20 mV (Hedrich and Neher, 1987; Bertl et al., 1992b; Bethke and Jones, 1994).

Long-Term Vacuolar K^+ Release Mediated by VK Channels

ABA-induced elevations in cytosolic Ca^{2+} in guard cells persist for 15 min or longer (McAinsh et al., 1990; Schroeder and Hagiwara, 1990) and would promote long-term activation of VK channels. As described above, the activation of VK channels results in a shift in vacuolar membrane potential to more positive potentials and reduces the driving force for further K^+ release. Therefore, long-term K^+ flux into the cytosol mediated by selective VK channels would require a mechanism to generate a negative vacuolar membrane potential on the cytosolic membrane side. Electrogenic H^+ pumps in the vacuolar

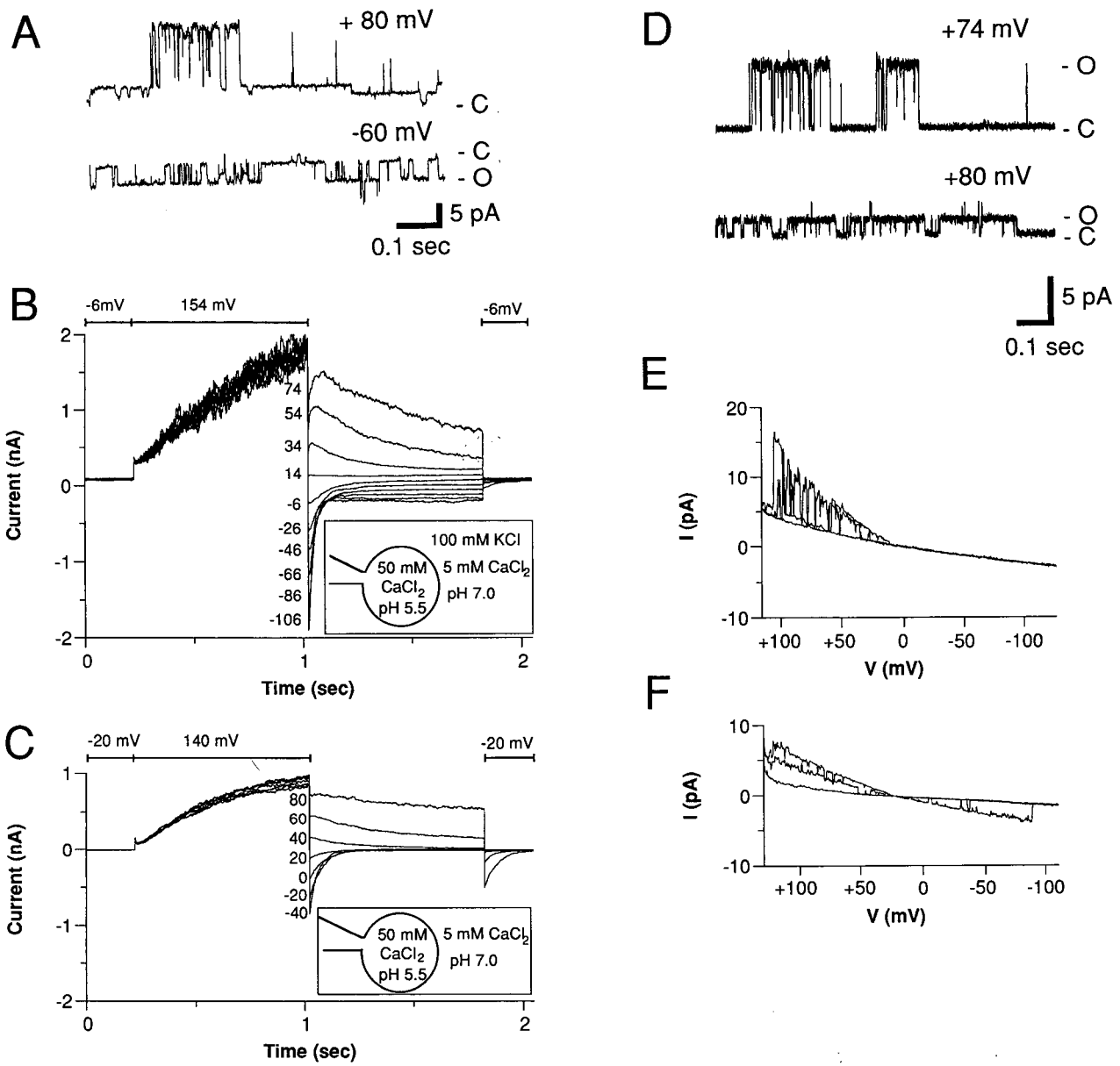

Figure 7. Guard Cell SV-Type Channels Are Ca^{2+} Selective.

(A) Single channel VK and SV currents were recorded in the same cytoplasmic-side-out vacuolar membrane patch. Representative recordings at +80 mV and −60 mV, indicated at the right, are presented. The bath solution (cytoplasmic side) contained 100 mM KCl, 1 mM $CaCl_2$, 20 mM Tris, and the pH was adjusted to 7.2 with Mes. The pipette solution is as described in the Methods. C and O indicate the closed state and one channel opening, respectively. The recordings were filtered at 1 kHz.

(B) From a holding potential of −6 mV, indicated at the top, SV currents were activated by a voltage step to 154 mV. Tail currents were recorded at the indicated membrane potentials. The pipette solution (vacuole lumen side) contained 50 mM $CaCl_2$ (as shown in the inset), 5 mM Mes, and the pH was adjusted to 5.5 with Tris. The bath solution (cytoplasmic side) contained 100 mM KCl, 5 mM $CaCl_2$ (as shown in the inset), 10 mM Tris, and the pH was adjusted to 7.0 with Mes. The measured liquid junction potential was +6.2 mV (see Methods).

(C) The Ca^{2+} selectivity of SV channels was determined using tail current analysis of whole-vacuole currents. The pipette solution is the same as given in **(B)**.

(D) Single SV ion channel current amplitude is decreased when Ca^{2+} is the only permeant ion. Using the solutions given in **(B)**, single channel conductance was 117 pS in cytoplasmic-side-out vacuolar membrane patches (top trace and **[E]**). When single SV channel currents were recorded using solutions without K^+, as given in **(C)**, single channel conductance was reduced to 16 pS (bottom trace and **[F]**). Holding potentials are indicated at the right. In **(B)** to **(D)**, current records were filtered at 2 kHz.

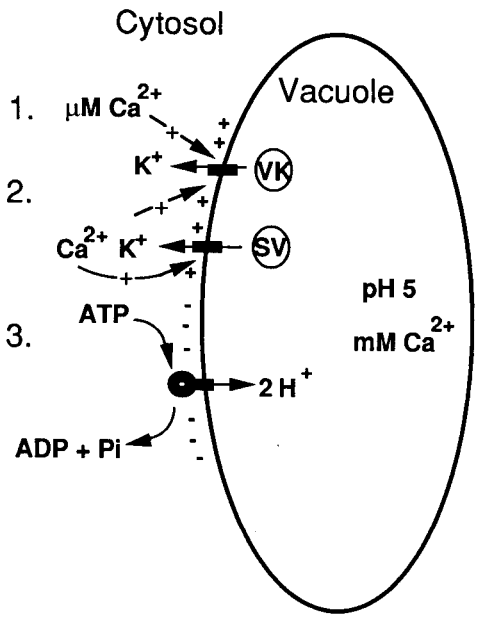

Figure 8. Schematic Representation of a Model for Molecular Mechanisms Controlling and Producing K^+ Efflux and Ca^{2+} Release from the Guard Cell Vacuole during Stomatal Closing.

Steps 1, 2, and 3 represent Ca^{2+}-activated VK channels, Ca^{2+}-activated SV channels, and H^+-translocating ATPase activities, respectively, as discussed in the text.

membrane (Sze et al., 1992; Rea and Poole, 1993) provide a mechanism for driving long-term K^+ release from guard cell vacuoles (Figure 8). In addition, enhanced vacuolar H^+ pumping due to VK channel activation can contribute to the slow alkalization of the guard cell cytosol that occurs in response to ABA (Irving et al., 1992).

At a cytoplasmic Ca^{2+} concentration of 1 μM and at physiological vacuolar membrane potentials of −10 mV, whole-vacuole K^+ currents in the range of −150 pA were measured (Figure 5). Based on a spherical guard cell vacuole diameter of 15 μm, a decrease in vacuolar K^+ concentration by 200 mM could occur within 7 min, which lies well within the physiologically observed time frame of stomatal closure (Outlaw, 1983). This estimate illustrates that the VK channels identified in this report can account for rates of vacuolar K^+ release required for stomatal closure.

ABA causes a slow alkalization of the cytosolic pH of guard cells (Irving et al., 1992; Blatt and Armstrong, 1993). The VK ion channels displayed a pH dependence within the physiological range (Figure 6). At an alkaline cytoplasmic pH of 8.0, K^+ current amplitudes were ~25% of those recorded at pH 7.0. Although the VK currents were greatly reduced, a decrease in vacuolar K^+ concentration by 200 mM, which is mediated by VK ion channels, could occur within 30 min at pH 8.0. The negative regulation of VK channels, although not complete, indicated that K^+ permeation through SV channels (Figures 7B, 7C, and 8) should contribute to long-term K^+ release during stomatal closing. Data suggesting that $^{86}Rb^+$ efflux from guard cell vacuoles occurs during ABA-induced stomatal closing (MacRobbie, 1981, 1990) further support a combined role of VK and SV channels in long-term K^+ release (Figures 3D and 3E).

Ca^{2+} Permeability of SV Channels

SV channels have been characterized as nonselective Ca^{2+}-activated voltage-dependent channels in the vacuolar membrane of various plant tissues (Coyaud et al., 1987; Hedrich and Neher, 1987; Kolb et al., 1987; Colombo et al., 1988; Hedrich and Kurkdjian, 1988; Maathuis and Prins, 1990; Weiser et al., 1991). However, the Ca^{2+} permeability of plant SV channels has not been directly addressed in previous studies. The Ba^{2+} permeability of voltage- and time-dependent SV channels from sugar beet storage tissue (Pantoja et al., 1992) provided a first indication that channels of this class may function as a pathway for voltage-dependent Ca^{2+} release from vacuoles, although this explanation was not considered by the authors of that report. Ca^{2+}-permeable ion channels have been reported in the vacuolar membrane of sugar beets that differ strongly from SV channels in voltage dependence, showing enhanced activation at negative potentials, and may represent an additional class of Ca^{2+}-release channels (Johannes et al., 1992). The monovalent cation selectivity of SV channels has been demonstrated in various tissues in detailed studies (Kolb et al., 1987; Colombo et al., 1988; Weiser et al., 1991; Amodeo et al., 1994). The data presented here support these findings and further show that SV channels in guard cells have a

Figure 7. (continued).

(E) Single SV channel currents recorded from cytoplasmic-side-out patches showed selectivity for Ca^{2+} over K^+. Solutions are as given in **(B)**. From a holding potential of +74 mV, the vacuolar membrane potential was changed linearly from +114 to −126 mV over 750 msec. Single channel currents reversed at 5.8 ± 1.1 mV (n = 4; Ca^{2+} to K^+ permeability ratio = 2.8:1). Three current records were superimposed that were filtered at 200 Hz.

(F) Single SV channels in cytoplasmic-side-out patches were Ca^{2+} permeable. Solutions are as given in **(C)**. From a holding potential of +90 mV, the vacuolar membrane potential was changed linearly from +130 to −110 mV over 750 msec. Single channel currents (three channels in patch) reversed at +26.6 ± 2.6 mV (n = 5). Three current records were superimposed that were filtered at 200 Hz. Note that in **(E)** and **(F)** positive membrane potentials are plotted on the left.

permeability ratio for Ca^{2+} to K^+ of ~3 to 1. It should be noted that the ionic permeability ratios of multi-ion pores can vary slightly depending on the ionic composition of the experimental solutions (Hille, 1992).

In this study, the voltage and time dependences, Ca^{2+} activation, ionic selectivity, and large single channel conductance of the currents (presented in Figure 7) are characteristic properties of SV channels. Experiments suggesting anion permeability of SV channels were performed under recording conditions that do not appear to completely separate anion and Ca^{2+} permeability contributions (see Hedrich et al., 1986; Coyaud et al., 1987). Other unknown factors such as the presence of other ion channels may also contribute to this discrepancy with all other SV channel ionic selectivity studies discussed above. When solutions were designed to distinguish Cl^- and Ca^{2+} permeability (Figures 7C and 7F), guard cell SV channels were determined to be Ca^{2+} permeable and not Cl^- permeable. Strictly speaking, a small Cl^- permeability (e.g., 5% of Ca^{2+} permeability) cannot be excluded by the experiments shown in Figures 7C and 7F, assuming a standard experimental error of ±3 mV when measuring reversal potentials. Similar results were obtained using mechanically isolated vacuoles from red beet storage tissue showing Ca^{2+} permeability, after correction for liquid junction potentials (n = 8 vacuoles; J.M. Ward and J.I. Schroeder, data not presented). In red beet vacuoles, the reversal potential of Ca^{2+}-activated SV currents was ~+30 mV after correction for liquid junction potentials using the conditions given for Figure 7C. This indicates that Ca^{2+} selectivity may be a common characteristic of SV channels from diverse tissues. Possible physiological roles for SV channels have remained a matter of debate. The data and the model presented here (Figures 7 and 8) lead us to propose that Ca^{2+}-induced Ca^{2+} release is a central physiological function of these ubiquitous plant channels.

Conclusions

The regulatory and biophysical properties of the VK channels identified here strongly suggest that this newly identified type of K^+ channel can provide a critical molecular control mechanism for initiating and maintaining K^+ transport from the vacuole into the guard cell cytosol during stomatal closure. This report provides evidence for a direct link in the signaling pathway between increases in cytoplasmic Ca^{2+}, which are known to result from physiological stimuli, such as ABA, and the initiation of K^+ release at the vacuolar membrane, which is required for stomatal closure. VK channel activation may provide positive shifts in vacuolar membrane potential required for SV channel activation. As determined here, guard cell SV channels are permeable to Ca^{2+} as well as K^+, and their activation may contribute to increases in cytosolic Ca^{2+} during stomatal closure. The presented model (Figure 8) provides a molecular basis for a close interaction between VK and SV ion channels and H^+ pumps required for stomatal closing. Further analysis of VK and SV channels should extend our understanding of the molecular mechanisms controlling K^+ and Ca^{2+} release from guard cell vacuoles during stomatal closing. Further research is required to determine whether the mechanisms described here can contribute to turgor regulation and vacuolar Ca^{2+}-induced Ca^{2+} release in higher plant cells in general.

METHODS

Isolation of Guard Cell Vacuoles

Guard cell protoplasts were isolated from leaves of *Vicia faba* (broad bean) as previously described (Schroeder and Fang, 1991). Plants were grown in a controlled environment growth chamber (model E15; Conviron, Asheville, NC) with a 12-hr light/12-hr dark cycle at a photon fluence rate of 100 μmol m^{-2} sec^{-1}. Epidermal tissue was isolated from four fully expanded leaves of 3- to 4-week-old plants using a modification of the blending procedure (Kruse et al., 1989). The leaves, after removal of the midvein, were blended (Waring, New Hartford, CT; 20,000 rpm) three times, 15 sec each, in deionized water at 4°C and collected on a 200-μm mesh. The epidermal tissue was then incubated in 5 mL of a medium containing 1% cellulase R10 and 0.5% macerozyme R10 (Yakult Honsha, Tokyo), 0.5% BSA, 0.4 M mannitol, 0.1 mM $CaCl_2$, 10 mM ascorbic acid, and 0.1% kanamycin sulfate at pH 5.7 for 16 hr at 23°C on a reciprocal shaker (0.5 Hz). Isolated guard cell protoplasts were then washed and purified in wash solution (0.5 M mannitol, 0.1 mM $CaCl_2$, and 0.1 mM KCl), as described previously (Schroeder and Fang, 1991).

The vacuoles were released from the guard cell protoplasts by osmotic shock (Wagner and Siegelman, 1975; Saunders and Conn, 1978) and purified using a Ficoll density gradient (Boller and Kende, 1979). To release vacuoles, guard cell protoplasts (in 1 mL of wash solution) were added to 5 mL of lysis buffer (10 mM Hepes-KOH, pH 8.0, 15% [w/v] Ficoll 400, 5 mM EDTA, 0.5% BSA, 2 mM DTT, the osmolality adjusted to 250 mmol kg^{-1} with ~150 mM sorbitol) at 23°C. This was overlayed immediately with 5 mL of a 1:1 mixture of lysis buffer (without BSA) and vacuole buffer (10 mM Hepes-KOH, pH 7.2, 2 mM DTT, the osmolality was adjusted to 540 mmol kg^{-1} with ~500 mM sorbitol). Finally, 2 mL of vacuole buffer was overlayed. After centrifugation at 250g for 20 min at 4°C, vacuoles (1 mL) were collected from the 0 to 7.5% Ficoll interface, stored at 4°C, and used for patch clamp experiments within 4 hr.

Patch Clamp Solutions and Data Acquisition

Glass pipettes coated with Sylgard (Dow Corning, Midland, MI) were filled with a solution containing 100 mM KCl, 2 mM $MgCl_2$, 5 mM $CaCl_2$, 5 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes), adjusted to pH 5.5 with Tris. Bath solutions contained 100 mM KCl and 20 mM Tris. The pH of bath solutions was adjusted using Mes to values indicated in the figure legends. The free Ca^{2+} concentration of the bath solution was buffered using 4 mM EGTA in the range of nominally 0 to 5 μM Ca^{2+}. Free Ca^{2+} in solutions was calculated using dissociation constants of EGTA for Ca^{2+} of 0.38 μM and 0.15 μM at pH 7.0 and 7.2, respectively (Tsien and Pozzan, 1989). For ionic selectivity experiments, 100 mM KCl was replaced by 100 mM concentrations of Cl^- salts by bath perfusion, as indicated in the legends to Figures 3 and

4. The osmolality of all solutions was adjusted to 600 mmol kg^{-1} with sorbitol. The whole-vacuole configuration (Hedrich and Neher, 1987), analogous to the whole-cell configuration, of the patch clamp technique (Hamill et al., 1981) was applied to isolated guard cell vacuoles.

Single channel recordings were made using cytoplasmic-side-out patches obtained by withdrawing the pipette from vacuoles in the whole-vacuole recording mode. Bath solutions were exchanged by perfusion (0.5 mL min^{-1}) using a peristaltic pump (Rainin, Woburn, MA). Potassium at the cytoplasmic side (bath solution) was replaced with other cations, whereas the Cl^- concentration of the bath solution was maintained at 101 mM. The Ca^{2+} concentration on the cytoplasmic side of membrane patch was rapidly changed using a local perfusion pipette as previously described (Pallotta et al., 1992).

Patch clamp recordings were performed using an Axopatch 200 amplifier (Axon Instruments, Foster City, CA). Data from whole-vacuole recordings were stored on-line via an Axolab interface (Axon Instruments) and a 25-MHz, 386-based microcomputer. Single channel currents were recorded on video tape following pulse-code modulation (model 200 PCM recorder; A.R. Vetter, Rebersburg, PA) and subsequently low-pass filtered with eight-pole Bessel characteristics, digitized, and stored as given above. All membrane potentials are specified as the potential on the cytoplasmic side relative to that on the vacuolar side (Bertl et al., 1992a). Current-voltage curves for whole-vacuole recordings were obtained by averaging current amplitude data obtained from 50 to 100 msec following initiation of voltage pulses. Single channel current amplitudes were measured using Fetchan software (Axon Instruments).

Liquid Junction Potential Correction

Proper correction for liquid junction potentials (LJP) was crucial for demonstrating the ionic selectivity of slow vacuolar (SV) channels in this study, as it has been previously for demonstrating that inward K^+ channels in guard cells reverse very close to E_{k+} when using K^+-glutamate solutions (Schroeder et al., 1987). LJPs were measured as described by Neher (1992) (see also Schroeder at al., 1987). In brief, as indicated in Figure 9, an agar bridge containing 3 M KCl was used as the bath ground. With the patch pipette (filled with the experimental pipette solution) and bath electrodes positioned in a chamber that also contained the pipette solution (Figure 9A) and the amplifier in zero-current clamp mode, the voltage reading was adjusted to zero. The electrodes were then moved to a second chamber containing the bath solution to be tested (Figure 9B), and the LJP was recorded as the zero-current potential. LJPs were less than ±5 mV for solutions used in Figures 1 through 6. The LJP for solutions used in experiments presented in Figure 7B was +6.2 mV and that for Figure 7C was +19.8 mV. Due to the sign conventions for vacuolar recordings (Bertl et al., 1992a) and the membrane orientation of cytoplasmic-side-out patches and whole vacuoles used here, the equation for LJP correction was $V_m = V_{experiment} - LJP$, where V_m is the corrected vacuolar membrane potential and $V_{experiment}$ is the noncorrected potential recorded in experiments. Note that this equation converts to $V_m = V_{experiment} + LJP$ for plasma membrane whole-cell and outside-out patch recordings or vacuolar inside-out patch recordings (for details, see Neher, 1992). Note that use of a bath electrode bridge containing the pipette solution does not correct for pipette LJPs (Pantoja et al., 1992) and that the pipette LJP does not shift when bath solutions are exchanged after seal formation (for details, see Neher, 1992).

The use of a 3 M KCl agar bridge minimizes bath electrode potential shifts to <1 mV (Neher, 1992), which is particularly important when Cl^- concentrations in the bath are changed during patch clamp experiments. However, the rapid diffusion of salts from agar to the bath solution requires that continuous bath perfusion be applied if a high salt agar bridge is used during patch recordings to avoid errors in ionic selectivity determinations resulting from increases in K^+ and Cl^- concentrations in the bath (see Schroeder and Fang, 1991; Neher, 1992). If low salt agar bridges are used, additional bath (not pipette) electrode LJP shifts can occur during bath exchange.

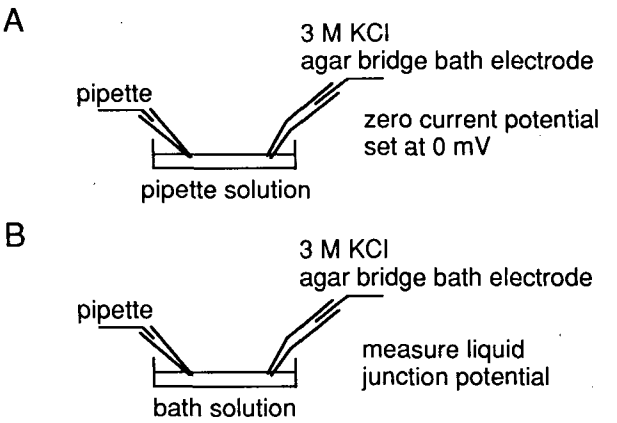

Figure 9. Measurement of Liquid Junction Potential.

(A) With the pipette and bath electrodes in a chamber containing the pipette solution, the zero-current potential is set to 0 mV using the patch clamp amplifier in current clamp mode.
(B) The pipette and bath electrodes are then moved to a chamber containing the bath (test) solution. In current clamp mode, the measured zero-current potential shift is equal to the liquid junction potential (see Methods).

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