Magnesium Sensitizes Slow Vacuolar Channels to Physiological Cytosolic Calcium and Inhibits Fast Vacuolar Channels in Fava Bean Guard Cell Vacuoles

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Vacuolar ion channels in guard cells play important roles during stomatal movement and are regulated by many factors including Ca2+, calmodulin, protein kinases, and phosphatases. We report that physiological cytosolic and luminal Mg2+ levels strongly regulate vacuolar ion channels in fava bean (Vicia faba) guard cells. Luminal Mg2+ inhibited fast vacuolar (FV) currents with a Kd of approximately 0.23 mM in a voltage-dependent manner at positive potentials on the cytoplasmic side. Cytosolic Mg2+ at 1 mM also inhibited FV currents. Furthermore, in the absence of cytosolic Mg2+, cytosolic Ca2+ at less than 10 μM did not activate slow vacuolar (SV) currents. However, when cytosolic Mg2+ was present, submicromolar concentrations of cytosolic Ca2+ activated SV currents with a Kd of approximately 227 nM, suggesting a synergistic Mg2+-Ca2+ effect. The activation potential of SV currents was shifted toward physiological potentials in the presence of cytosolic Mg2+ concentrations. The direction of SV currents could also be changed from outward to both outward and inward currents. Our data predict a model for SV channel regulation, including a cytosolic binding site for Ca2+ with an affinity in the submicromolar range and a cytosolic low-affinity Mg2+-Ca2+ binding site. SV channels are predicted to contain a third binding site on the vacuolar luminal side, which binds Ca2+ and is inhibitory. In conclusion, cytosolic Mg2+ sensitizes SV channels to physiological cytosolic Ca2+ elevations. Furthermore, we propose that cytosolic and vacuolar Mg2+ concentrations ensure that FV channels do not function as a continuous vacuolar K+ leak, which would prohibit stomatal opening.

Mg2+ is an abundant cytoplasmic cation in higher plants (Epstein, 1965), with concentrations of 2 to 10 mM in leaf cells (Leigh and W. Jones, 1986). Mg2+ ions exist as free cations and are also sequestered in internal organelles, bound by cytosolic proteins, or complexed with small organic molecules. Many enzymes require or are strongly activated by Mg2+, for example, plasma membrane ATPases, protein kinases, type-2C phosphatases, glutathione synthase, and RuBP carboxylase (Marschner, 1995; Leube et al., 1998). The important role of Mg2+ as a regulator of various ion channels is well established in animal cells (Agus and Morad, 1991; Flatman, 1991; Murphy et al., 1991; Hille, 1992; Chuang et al., 1997; Kirschbaum and Cahalan, 1999). Matsuda et al. (1987) and Vandenberg (1987) demonstrated the direct blockage of inward rectifier K+ channels in animal cells by Mg2+; however, little is known about how Mg2+ affects ion channel activities in plant cells.

Two types of ion channels have been characterized in most plant vacuolar membranes studied to date. These are the Ca2+-permeable, cation-selective slow vacuolar (SV) channels and the cation-selective fast vacuolar (FV) channels (Hedrich and Neher, 1987; Weiser et al., 1991; Bethke and Jones, 1994; Ward and Schroeder, 1994; Allen and Sanders, 1996). SV channels are activated by cytosolic Ca2+, whereas FV channels are inhibited by elevations in cytosolic Ca2+ (Allen and Sanders, 1996).

FV channels show instantaneous currents in response to voltage pulses (Hedrich and Neher, 1987; Allen and Sanders, 1996; Tikhonova et al., 1997). FV channels are cation-selective (Allen and Sanders, 1996; Tikhonova et al., 1997). The functions of FV channels remain unknown (Allen and Sanders, 1997), although proposals of functions have been made on the basis of their properties, including mediating K+ release from guard cell vacuoles during stomatal closing (Allen and Sanders, 1996). However, at physiological resting cytosolic Ca2+ concentrations of 0.1 to 0.2 μM, FV current activities can be very high (Hedrich and Neher, 1987; Allen and Sanders, 1996; Tikhonova et al., 1997). This raised the possibility that FV channels need to be further down-regulated by factors other than Ca2+ in order to maintain vacuolar membrane ion gradients. Recently, physiological polyamine levels have been shown to partially down-regulate FV channels (Brüggemann et al., 1998; Dobrovinskaya et al., 1999).

Voltage- and time-dependent SV channels, as well as vacuolar K+ selective (VK) channels, are activated by cytosolic Ca2+ (Hedrich and Neher, 1987; Bethke and Jones, 1994; Ward and Schroeder, 1994; Allen and Sanders, 1996; Pottosin et al., 1997). In addition, SV channels are regulated by ATP, calmodulin, protein kinases, and phosphatases (Weiser et al., 1991; Bethke and Jones, 1994, 1997; Allen and Sanders, 1995). Although a significant anion permeability of SV channels had been proposed (Coyaud et al., 1987; Hedrich and Kurkdjian, 1988; Schulz-Lessdorf and Hedrich, 1995), detailed studies unequivocally demonstrated the cation selectivity of SV channels with negligible anion permeability (Colom-
bo et al., 1988; Ward and Schroeder, 1994; Ward et al., 1995; Allen and Sanders, 1996; Pottosin et al., 1997). Studies showed substantial Ca$^{2+}$ and Mg$^{2+}$ permeabilities of SV currents (Ward and Schroeder, 1994; Allen and Sanders, 1996). Therefore, SV channels are cation selective with poor selectivity among monovalent cations (K$^+$, Na$^+$, and Cs$^+$) and divalent cations (Ca$^{2+}$, Mg$^{2+}$, and Ba$^{2+}$). The finding that Ca$^{2+}$-activated SV channels are Ca$^{2+}$ permeable has led to the suggestion that these channels may provide an important mechanism not only for K$^+$ transport but also for Ca$^{2+}$-induced Ca$^{2+}$ release (Ward and Schroeder, 1994). A recent study showed that conditions favoring Ca$^{2+}$ release from vacuoles decrease the SV channel open probability, leading to a counter-hypothesis in which SV channels cannot mediate Ca$^{2+}$-induced Ca$^{2+}$ release from vacuoles (Pottosin et al., 1997).

At physiological cytosolic Ca$^{2+}$ concentrations, SV channel activities are generally negligible in many plants (Hedrich and Neher, 1987; Ward and Schroeder, 1994; Barkla and Pantoja, 1996; Allen and Sanders, 1997; Allen et al., 1998). Moreover, the activation potentials of SV channels lie positive of physiological vacuolar membrane potentials of 0 to −40 mV (Hedrich and Neher, 1987; Sze et al., 1992; Bethke and Jones, 1994; Ward and Schroeder, 1994; Allen and Sanders, 1996; Pottosin et al., 1997; Allen et al., 1998). A study on fava bean (Vicia faba) guard cell vacuoles led to the suggestion that cytosolic Mg$^{2+}$ activates SV channels in the absence of cytosolic Ca$^{2+}$ (Allen and Sanders, 1996). However, a more recent study on barley mesophyll vacuoles suggested that Mg$^{2+}$ does not activate SV channels (Pottosin et al., 1997).

The findings that physiological cytosolic Ca$^{2+}$ concentrations do not activate SV channels and over-stimulate FV channels have led to difficulties in predicting their functions in vivo. In the present study, we demonstrate that at physiological concentrations, Mg$^{2+}$ down-regulates vacuolar membrane FV channels in fava bean guard cells, which may provide an efficient down-regulation mechanism of FV channels in vivo. Interestingly, cytosolic Mg$^{2+}$ sensitized SV channels to physiological concentrations of cytosolic Ca$^{2+}$, and data presented here clarify the controversy of Mg$^{2+}$ activation of SV channels raised previously.

MATERIALS AND METHODS

Isolation of Fava Bean Guard Cell Vacuoles

Fava bean (Vicia faba) plants were grown in a controlled environment growth chamber (model E15, Conviron, Asheville, NC) with 16-h light/8-h dark cycle. Guard cell protoplasts were isolated from 3- to 4-week-old plants by enzymatic digestion of leaf epidermal strips, as previously described (Kruse et al., 1989; Ward and Schroeder, 1994). Vacuoles were released from guard cell protoplasts by osmotic shock and purified using a Ficoll density gradient (Ward and Schroeder, 1994).

Patch Clamp and Data Acquisition

Patch-clamp pipettes were prepared from soft glass capillaries (Kimax 51, Kimble, Toledo, OH), and pulled on a multi-stage programmable puller. Giga-Ω seals between electrode and the vacuolar membrane (>15 GΩ) were obtained by gentle suction. The patch-clamp technique was applied to isolated guard cell vacuoles as previously described (Pei et al., 1996). The whole-vacuole configuration, analogous to the whole-cell configuration (Hamill et al., 1981), was attained by applying high-voltage pulses (usually ±500 mV, 25 ms for each) and slight suction to the interior of the pipette (Pei et al., 1996).

Vacuoles were voltage clamped using an amplifier (Axopatch 200, Axon Instruments, Foster City, CA). All membrane potentials are specified as the potential on the cytosolic side relative to the vacuolar side (Bertl et al., 1992). Data were analyzed using AXOGRAPH software (3.5, Axon Instruments). Statistical analyses were performed using EXCEL (5.0, Microsoft, Redmond, WA). Data are the means ± se. In Figure 1D, the average percentage of inhibition of SV currents at +100 mV by vacuolar Mg$^{2+}$ is fitted to a Hill equation:

\[ I = I_{\text{max}} \cdot \frac{[\text{Mg}^{2+}]^{n}}{(K_{i} + [\text{Mg}^{2+}]^{n})} \]

where \( I \) is the degree of current inhibition, \( I_{\text{max}} \) is the maximum current inhibition, [Mg$^{2+}$] is the Mg$^{2+}$ concentration on the vacuolar side, \( n \) is the Hill coefficient, and \( K_{i} \) is the inhibition constant.

Experimental Solutions

The standard solutions used in patch-clamp experiments were composed of 200 mM KCl and 20 mM HEPES-Tris, pH 8.0, in the bathing medium (cytosolic side), and 20 mM KCl, 2 mM EGTA, and 5 mM HEPES-Tris, pH 7.0, in the pipette (vacuolar side) unless otherwise noted. Free cytosolic Ca$^{2+}$ concentrations ranging from 10 nM to 1 μM were buffered with EGTA. Total CaCl$_2$ concentrations in bath solutions (Fig. 5) were changed to give the indicated cytosolic free Ca$^{2+}$ of 10 nM (0.8 mM total CaCl$_2$ concentration), 50 nM (2 mM), 150 nM (3 mM), and 1 μM (3.8 mM), at pH 7.8, with 4 mM EGTA in all solutions. Free Ca$^{2+}$ concentrations were calculated after accounting for 10 mM MgCl$_2$ stoichiometric strength, and temperature (24°C) with CALCV22 software (Foehr et al., 1993). For 10 and 50 μM Ca$^{2+}$ in Figure 5, 10 and 50 μM CaCl$_2$ were added to the bath solution without the addition of the Ca$^{2+}$ buffer EGTA, as these concentrations lie outside the range of effective EGTA-buffering capacity. The bath solution was exchanged either by bath perfusion using a peristaltic pump (Rainin, Woburn, MA) or by a local perfusion pipette. Osmolarities of all solutions were adjusted to 600 mmol kg$^{-1}$ by the addition of α-sorbitol.

RESULTS

Inhibition of Vacuolar FV Channels by Luminal Mg$^{2+}$

At zero cytosolic Ca$^{2+}$, vacuolar currents were almost entirely instantaneous and were larger at positive potentials (on the cytoplasmic side of the membrane) compared with negative potentials (Fig. 1A). The activation of previously described Ca$^{2+}$-activated VK channels (Ward and
Inhibition of Vacuolar FV Channels by Cytosolic Mg$^{2+}$

Experiments were designed to analyze whether, in addition to vacuolar Mg$^{2+}$ (Fig. 1), cytosolic Mg$^{2+}$ affects FV currents. A local perfusion system was used that allowed multiple changes of cytosolic solutions bathing single vacuoles. In the whole-vacuole configuration with zero Mg$^{2+}$ on the cytosolic side, large instantaneous currents were recorded (Fig. 2A). When Mg$^{2+}$ (1 mM) was applied to the cytosolic side, FV currents were decreased dramatically at both positive and negative vacuolar potentials (Fig. 2B). Activation of time-dependent SV currents in Figure 2B will be described later. Quantitative analysis showed a 3-fold inhibition of instantaneous currents by varying cytosolic Mg$^{2+}$ from 0 to 1 mM at +100 mV (Fig. 2C). These results indicate that both luminal (Fig. 1) and cytosolic Mg$^{2+}$ (Fig. 2) down-regulate FV currents at both positive and negative vacuolar potentials. As predicted, elimination of Mg$^{2+}$ and Ca$^{2+}$ from both the luminal and cytosolic sides gave rise to large FV currents (Fig. 2D), further illustrating the inhibitory effects of Mg$^{2+}$ and Ca$^{2+}$.

Figure 1. Fast-activating vacuolar currents inhibited by vacuolar Mg$^{2+}$ in fava bean guard cells. A through C, Three representative whole-vacuole recordings are shown at Mg$^{2+}$ concentrations of 0 mM (A), 0.5 mM (B), and 2 mM (C) in the pipette (luminal) solution. Membrane potential was stepped from −100 to +100 mV in 20-mV increments from a holding potential of 0 mV. In all traces, the vacuolar ion currents have been normalized to the whole-vacuole capacitance (pA/pF). The solutions for FV current measurement contained 100 mM KCL, 4 mM EGTA, 10 mM HEPES-Tris, pH 7.5, in the bathing medium (cytosolic side), and 100 mM KCL, 5 mM CaCl$_2$, 5 mM MES-Tris, pH 5.5, with varied MgCl$_2$, from 0 to 2 mM in the pipette (vacuolar side). D, Average current-voltage relationships from experiments performed as in A through C at Mg$^{2+}$ concentrations of 0, 0.1, 0.5, 1.0, and 2.0 mM in the pipette solution. FV currents were measured as the instantaneous component of whole-vacuole currents (n = 3–5 vacuoles per Mg$^{2+}$ concentration; whole-vacuole capacitance = 9.7 ± 3.4 pF). Inset, Average percentage of inhibition of SV currents at +100 mV is plotted as a function of the concentrations of vacuolar Mg$^{2+}$ and fitted to a Hill equation. E, Voltage dependence of vacuolar Mg$^{2+}$ block. Average whole-vacuole currents in the presence of vacuolar Mg$^{2+}$ as in D were normalized to currents in the absence of Mg$^{2+}$ (Current$_{0mM}$). Symbols are as in D. F, Mg$^{2+}$ inhibition constant ($K_i$) plotted as a function of the applied membrane potentials. Inhibition constants at +40 to +100 mV were obtained using the Hill equation (see “Materials and Methods”).
Does Cytosolic Mg$^{2+}$ Activate SV Currents?

In fava bean guard cells, at high cytosolic Ca$^{2+}$ concentrations SV currents are the major vacuolar conductance. However, the cytosolic Ca$^{2+}$ concentration required for SV current activation is larger than known resting cytosolic Ca$^{2+}$ levels and the upper limit of free cytosolic Ca$^{2+}$ concentrations measured during Ca$^{2+}$-dependent signal transduction (Ward and Schroeder, 1994; Bush, 1995; Allen and Sanders, 1996; McAlinsh et al., 1997). This has contributed to difficulties in predicting the physiological roles of SV channels. We therefore designed experiments to determine whether the Ca$^{2+}$ sensitivity of SV activation could be modified. At 10 $\mu$m cytosolic Ca$^{2+}$, instantaneous currents were recorded in the absence of cytosolic Mg$^{2+}$ (Fig. 3A).

Cytosolic Mg$^{2+}$ of 1 mM was applied by local perfusion in the continued presence of 10 $\mu$m Ca$^{2+}$. Interestingly, time-dependent SV currents were increased dramatically at positive vacuolar potentials (Fig. 3B). The Mg$^{2+}$ concentration was then further increased to 5 mM. SV currents were similar in magnitude to those at 1 mM cytosolic Mg$^{2+}$ (Fig. 3C). Finally cytosolic Mg$^{2+}$ was removed by slow bath perfusion, during which the time-dependent SV currents vanished (Fig. 3, D and E), while instantaneous currents increased (Fig. 3, D and E), also confirming the inhibitory effect of cytosolic Mg$^{2+}$ on FV currents described in Figure 2. These data suggest that Mg$^{2+}$ might up-regulate SV current as previously proposed (Allen and Sanders, 1996). However, in a recent study, no Mg$^{2+}$ activation of SV currents was found in barley mesophyll vacuoles, and Mg$^{2+}$ activation of SV channels described previously (Allen and Sanders, 1996) were concluded to be an artifact (Pottosin et al., 1997). To further examine these differences among previous reports, we investigated whether Mg$^{2+}$ activation of SV currents depends on the presence of physiological levels of cytosolic Ca$^{2+}$.

**Mg$^{2+}$ Sensitizes SV Currents to Cytosolic Ca$^{2+}$**

To test whether cytosolic Ca$^{2+}$ is necessary for activation of SV currents by Mg$^{2+}$ in fava bean guard cells, whole-vacuolar currents were measured at 10 mM cytosolic Mg$^{2+}$ in the absence or presence of the Ca$^{2+}$ buffer EGTA (4 mM) in the bath solution (Fig. 4). Small time-dependent SV currents were observed in the absence of EGTA (Fig. 4A). However, in the presence of EGTA, SV currents were reduced (Fig. 4B). Figure 4C (C) shows the dramatic reduction in time-dependent SV currents at positive potentials, when EGTA was added to the cytosolic side. These data (Figs. 3 and 4) indicate the possibility that cytosolic Mg$^{2+}$ might modify the sensitivity of SV channels to cytosolic Ca$^{2+}$.

To analyze quantitatively whether Mg$^{2+}$ could shift the sensitivity of SV activation to physiological cytosolic Ca$^{2+}$ concentrations and to determine cytosolic Ca$^{2+}$ concentrations required for Mg$^{2+}$ activation of SV currents, SV currents were measured over a range of cytosolic Ca$^{2+}$ concentrations and to determine cytosolic Ca$^{2+}$ concentrations required for Mg$^{2+}$ activation of SV currents. SV currents by Mg$^{2+}$ were normalized as 1 (64.2 ± 4.5 pA/pF; n = 11 vacuoles for each condition; whole-vacuole capacitance = 8.3 ± 1.2 pF). r.u., Relative unit. D, Representative whole-vacuole FV currents recorded in the absence of Mg$^{2+}$ and Ca$^{2+}$ on both cytosolic and vacuolar membrane sides (n > 15 vacuoles). The solution contained 200 mM KCl, 5 mM HEPES-Tris, pH 7.0, in the pipette and 50 mM KCl, 2 mM EGTA, and 20 mM HEPES-Tris, pH 8.0, in the bath. No Mg$^{2+}$ or Ca$^{2+}$ was added to solutions. E, Current-voltage relationships as recorded in D. Currents from five representative recordings are averaged and plotted as a function of the applied membrane potentials.

**Figure 2.** Cytosolic Mg$^{2+}$ inhibits FV current. A and B, Whole-vacuole currents recorded in the absence (0 Mg$_{\text{cyt}}$; A) or presence of Mg$^{2+}$ (1 mM Mg$_{\text{cyt}}$; B) in bath (cytosolic) solutions of the same vacuole. The holding potential was −40 mV. The pipette solution contained 20 mM KCl, 2 mM EGTA, and 5 mM HEPES-Tris, and the bath solution contained 100 mM KCl, 20 mM HEPES-Tris, pH 8.0, with the addition of 10 $\mu$m CaCl$_2$, in the absence or presence of 1 mM MgCl$_2$. Whole-vacuole capacitance = 8.4 pF. C, Average FV currents at +100 mV as recorded in A and B. Currents recorded in the absence of cytosolic Mg$^{2+}$ were normalized as 1 (64.2 ± 4.5 pA/pF; n = 11 vacuoles for each condition; whole-vacuole capacitance = 8.3 ± 1.2 pF). r.u., Relative unit. D, Representative whole-vacuole FV currents recorded in the absence of Mg$^{2+}$ and Ca$^{2+}$ on both cytosolic and vacuolar membrane sides (n > 15 vacuoles). The solution contained 200 mM KCl, 5 mM HEPES-Tris, pH 7.0, in the pipette and 50 mM KCl, 2 mM EGTA, and 20 mM HEPES-Tris, pH 8.0, in the bath. No Mg$^{2+}$ or Ca$^{2+}$ was added to solutions. E, Current-voltage relationships as recorded in D. Currents from five representative recordings are averaged and plotted as a function of the applied membrane potentials.
data for cytosolic Ca\(^{2+}\) concentrations from 10 nM to 1 \(\mu\)M, showing a \(K_d\) of approximately 227 nM for a Hill coefficient of 0.95 (Fig. 5B, inset). The Hill coefficient of approximately 1 indicates binding of one Ca\(^{2+}\) ion per SV channel. These data demonstrate that physiological concentrations of Ca\(^{2+}\) can activate SV currents, if Mg\(^{2+}\) is also present on the cytosolic side, showing a sensitization of the SV channel to Ca\(^{2+}\) by cytosolic Mg\(^{2+}\).

**Differential Activation Time Course of SV Currents by Saturating Cytosolic Ca\(^{2+}\) or Mg\(^{2+}\)**

The data presented above suggested two ways to activate SV currents: first by high concentrations of cytosolic Ca\(^{2+}\) alone and second by combining cytosolic Mg\(^{2+}\) with low physiological concentrations of Ca\(^{2+}\). To test whether these two putative mechanisms of SV channel activation were kinetically distinguishable, experiments were designed using saturating Ca\(^{2+}\) (10 mM) in the absence of Mg\(^{2+}\); or using saturating Mg\(^{2+}\) (10 mM) in the presence of 10 \(\mu\)M cytosolic Ca\(^{2+}\). Under these two conditions, activation time courses for SV currents were different (Fig. 6, A and B). The time constants for SV current activation by Mg\(^{2+}\) in the presence of 10 \(\mu\)M Ca\(^{2+}\) were approximately three times more rapid than by Ca\(^{2+}\) alone (Fig. 6C), further supporting the hypothesis that there are two distinct mechanisms for the activation of SV channels (see “Discussion”).

Varying the luminal Mg\(^{2+}\) concentration had no effect on SV currents in fava bean guard cells (\(n = 6\); data not shown), which was also demonstrated in barley mesophyll vacuoles (Pottosin et al., 1997), suggesting that SV channels are not regulated by luminal Mg\(^{2+}\).

**Figure 4.** Cytosolic EGTA inhibits Mg\(^{2+}\) activation of SV currents. A and B, Whole-vacuole currents recorded in the absence (A) or presence (B) of 4 mM EGTA in the bath solutions in one vacuole. Standard pipette and bath solutions were used without or with the addition of 4 mM EGTA. C, Current-voltage relationships from experiments performed in the absence or presence of 4 mM EGTA as in A and B. SV currents were measured as time-dependent components of whole-vacuole currents. Symbols are as given in A and B (\(n = 8\); whole-vacuole capacitance = 7.1 ± 2.6 pF).
Shifting SV Activation to Physiological Potentials and Modification of Outward-Rectifying Properties

In previous studies, steady-state SV currents have only been activated at positive vacuolar potentials (Ward and Schroeder, 1994; Allen and Sanders, 1996; Barkla and Panttoja, 1996; Bethke and Jones, 1997; Pottosin et al., 1997), whereas at physiological vacuolar potentials (from 0 to $-40 \text{ mV}$; Sze et al., 1992) SV currents are vanishingly small. Experiments were designed to test whether the activation potential of SV currents could be significantly shifted to negative vacuolar potentials within the physiological range. To maximize SV channel activation, we designed a pipette solution containing 20 mM KCl and 4 mM EGTA (to eliminate the inhibitory effect of Ca$^{2+}$ from the luminal side on SV channels (Allen and Sanders, 1996; Pottosin et al., 1997), and a bath solution containing 200 mM KCl, 10 mM CaCl$_2$, and 2 mM MgCl$_2$. Under these conditions the activation potential was shifted to potentials of about $-60$ to $-240 \text{ mV}$ (Fig. 7, A and B). In some vacuoles (two out of nine), the activation potential was shifted dramatically to $-90 \text{ mV}$. Both inward and outward currents were recorded, and the time-dependent activation of SV channels was not altered (Fig. 7, C and D). A similar modification of the rectification property of the SV current has also been found in barley mesophyll cells (Pottosin et al., 1997).

These results suggest that SV currents can activate at physiological vacuolar potentials under specific ionic conditions, and that SV channels may carry both inward and outward currents in vivo depending on conditions. We used extreme experimental conditions to show that the activation potential of SV channels could be strongly shifted (Fig. 7). The variation in activation potential (Fig. 7) suggests that additional unknown factors exist that can greatly shift the activation potential of SV channels.

**DISCUSSION**

In animal cells, Mg$^{2+}$ blocks many cation channels, which includes Ca$^{2+}$ channels, various inward-rectifier K$^+$
channels, N-methyl-D-Asp receptor channels, ryanodine receptor-Ca\(^{2+}\) release channels, and Ca\(^{2+}\) release-activated Ca\(^{2+}\) channels (Nowak et al., 1984; Matsuda et al., 1987; Vandenberg, 1987; Agus and Morad, 1991; Hille, 1992; Laver et al., 1997; Kerschbaum and Cahalan, 1999). However, in plant cells, regulation of ion channels by Mg\(^{2+}\) has not yet been studied in detail. In guard cells, inward-rectifying K\(^{+}\) currents are not blocked by cytosolic Mg\(^{2+}\) (Schroeder, 1995), and the only ionic current shown to be activated by Mg\(^{2+}\) is a cation current in beet vacuoles, which has been proposed to be a shunt conductance for the vacuolar H\(^+\)-ATPase (Davies and Sanders, 1995). In this study, we show that in fava bean guard cells, Mg\(^{2+}\) strongly regulates two major vacuolar currents: down-regulating the vacuolar FV currents from both the cytosolic and luminal sides (Figs. 1 and 2) and up-regulating vacuolar SV currents from the cytosolic side (Figs. 4 and 6). The regulation of vacuolar ion channels by Mg\(^{2+}\) may play an important role in guard cells, as ion transport processes across the vacuolar membrane are essential for stomatal movements (MacRobbie, 1981, 1998; Assmann, 1993; Ward et al., 1995; Allen and Sanders, 1997).

**Inhibition of Guard Cell Vacuolar FV Currents by Both Cytosolic and Luminal Mg\(^{2+}\)**

Systematic studies of the effect of Ca\(^{2+}\) on FV channels in fava bean guard cell and barley mesophyll vacuoles have shown that cytosolic and vacuolar Ca\(^{2+}\) inhibits FV channels (Allen and Sanders, 1996; Tikhonova et al., 1997). Higher concentrations of Mg\(^{2+}\) are required to inhibit FV currents (Figs. 1 and 2). For half inhibition from the luminal side, Mg\(^{2+}\) concentrations of approximately 230 \(\mu\)M were required (Fig. 1). In a previous study of VK currents, 2 mM Mg\(^{2+}\) was used to exclude FV currents (Ward and Schroeder, 1994), while Mg\(^{2+}\)-free conditions result in large FV currents (Allen and Sanders, 1996). These results suggest a divergent ion-binding/block site on the luminal side of FV channels.

In animal cells, both Mg\(^{2+}\) and spermine block inward-rectifier K\(^{+}\) channels and cause voltage-dependent inward rectification (Matsuda et al., 1987; Vandenberg, 1987; Hille, 1992; Fakler et al., 1995). In the case of NMDA receptors in neurons, Mg\(^{2+}\) and spermine share a regulatory site (Paoletti et al., 1995). Similarly, FV channels are inhibited by both Mg\(^{2+}\), as shown here, and spermine in barley vacuoles (Figs. 1 and 2; Brüggemann et al., 1998; Dobrovinskaya et al., 1999). The inhibition of FV channels by vacuolar Mg\(^{2+}\) is voltage dependent (Fig. 1), whereas spermine inhibition is voltage independent (Brüggemann et al., 1998; Dobrovinskaya et al., 1999), suggesting that Mg\(^{2+}\) and spermine may not share the same binding site or that the inhibitory mechanisms are different. Whether the inhibitory effects of Mg\(^{2+}\) and spermine are additive in FV channel regulation or if Mg\(^{2+}\) and spermine share an inhibitory site will require further investigation.

**Mg\(^{2+}\) Sensitizes SV Channels to Physiological Cytosolic Ca\(^{2+}\) Levels and a Model for SV Activation with Two Binding Sites**

SV currents in many plant cell types are activated at cytosolic Ca\(^{2+}\) concentrations (for example >100 \(\mu\)M), which are >100-fold higher than known resting levels (Ward and Schroeder, 1994; Barkla and Pantoja, 1996; Allen and Sanders, 1996, 1997). The high cytosolic Ca\(^{2+}\) levels required for SV channel activation have contributed to the difficulty in assigning a physiological function to the channels. Information on mechanisms that modify the Ca\(^{2+}\) sensitivity of SV channel activation could further our understanding of SV function in guard cells. Mg\(^{2+}\) activation of SV channels has been proposed in fava bean guard cells (Allen and Sanders, 1996). However, Pottosin et al. (1997) reported that the SV channel activation in barley mesophyll vacuoles is due to Ca\(^{2+}\) contamination of the cytosolic bath solution, and that Mg\(^{2+}\) does not activate SV channels.

To clarify these controversial conclusions, our results show that in the presence of EGTA, Mg\(^{2+}\) does not activate SV currents in fava bean guard cells (Fig. 3, B and C), indicating that Mg\(^{2+}\) activation in the previous study can be explained by residual free Ca\(^{2+}\), because no Ca\(^{2+}\) chelators were added to the cytosolic membrane side (Allen and Sanders, 1996). The conclusion that cytosolic Mg\(^{2+}\) does

![Figure 7. Shifting SV activation to physiological vacuum potentials and changing rectification property. A and B. Whole-vacuolar SV currents (A) and current-voltage relationship (B). The pipette solution contained 20 mM KCl, 2 mM EGTA, and 5 mM HEPES-Tris, pH 7.0. The bath contained 200 mM KCl, 10 mM CaCl\(_2\), 2 mM MgCl\(_2\), and 20 HEPES-Tris, pH 8.0. Similar currents were recorded in seven of nine vacuoles. C. Under the same conditions as in A, time-dependent peak and steady-state amplitudes of SV currents. This behavior was observed in two of nine vacuoles recorded under these conditions. As symbolized in C, ○ and ● show the time-dependent peak and steady-state amplitudes of SV currents, respectively.](image-url)
not modulate SV channels was based on experiments with 1 to 2 mM EGTA and no Ca\(^{2+}\) added to the cytosolic solutions (Pottosin et al., 1997). Interestingly, however, in our experiments within the range of cytosolic Ca\(^{2+}\) concentrations at which SV currents were not normally activated, the addition of Mg\(^{2+}\) led to SV current activation (Fig. 4), indicating a synergistic effect between Mg\(^{2+}\) and Ca\(^{2+}\). Our data show that Mg\(^{2+}\) sensitizes SV channels to physiological levels of cytosolic Ca\(^{2+}\). Ba\(^{2+}\) did not activate SV channels in fava bean guard cell vacuoles (Schulz-Lessdorf and Hedrich, 1995), but did activate SV channels in beet vacuoles (Pantoja et al., 1992).

Based on our results, a simplified model for SV channel regulation in fava bean guard cells is proposed (Fig. 8), which includes two activating cytosolic sites and one inhibitory luminal site. First, low concentrations of cytosolic Ca\(^{2+}\) cannot activate SV channels in the absence of cytosolic Mg\(^{2+}\), whereas in the presence of cytosolic Mg\(^{2+}\), these low concentrations of Ca\(^{2+}\) (A\(_1\); Fig. 8) are necessary and sufficient to activate SV channels, implying a synergistic Mg\(^{2+}\)-binding site (A\(_2\); Fig. 8). Second, Mg\(^{2+}\) alone cannot activate SV channels, indicating that a high-affinity (K\(_A\) of approximately 227 mM) Ca\(^{2+}\)-binding site (A\(_1\)) is required and is different from the Mg\(^{2+}\)-binding site (A\(_2\)). Mg\(^{2+}\) cannot compete with Ca\(^{2+}\) for A\(_1\) binding. Both A\(_1\) and A\(_2\) need to be occupied for SV channel activation. Third, a high concentration of cytosolic Ca\(^{2+}\) alone can activate SV channels (Ward and Schroeder, 1994; Allen and Sanders, 1996), suggesting (for a simple model) that cytosolic Ca\(^{2+}\) can bind to both sites A\(_1\) and A\(_2\). In addition, our results showed that the time course of SV current activation differed when using saturating Ca\(^{2+}\) (10 mM) to bind both sites (A\(_1\) and A\(_2\)) in the absence of Mg\(^{2+}\) as opposed to using saturating Mg\(^{2+}\) (10 mM) binding to the low-affinity site (A\(_2\)) in the presence of 10 mM Ca\(^{2+}\) to bind to the high-affinity site (A\(_1\)) (Fig. 6), indicating additional ion-specific effects.

Finally, luminal Ca\(^{2+}\) inhibits SV currents by shifting the activation potential (Figs. 7B versus 4C; also see Allen and Sanders, 1996; Pottosin et al., 1997), indicating that Ca\(^{2+}\) ion binding on the luminal side (B; Fig. 8) is inhibitory and thus limits large Ca\(^{2+}\) release currents that could be toxic. Vacuolar Mg\(^{2+}\) did not affect SV (data not shown), indicating that Mg\(^{2+}\) does not compete for this inhibitory Ca\(^{2+}\)-binding site (B).

**Proposed Physiological Roles of Mg\(^{2+}\) Regulation of Vacuolar Currents**

The vacuole constitutes ≥90% of the guard cell volume and functions as a storage organelle for solutes that are important for osmoregulation during stomatal movements (Boller and Wiemken, 1986; Assmann, 1993). More than 90% of the K\(^{+}\) and anions released from guard cells during stomatal closing must first be released from vacuoles into the cytosol (MacRobbie, 1981). Studies show that FV channels can mediate both inward and outward currents with large amplitudes (Fig. 2D), which would lead to vacuolar K\(^{+}\) release when V-type ATPases are active. Cytosolic Ca\(^{2+}\) and polyamines down-regulate FV channels (Hedrich and Neher, 1987; Allen and Sanders, 1996; Tikhonova et al., 1997). The half-inhibitory concentration of cytosolic Ca\(^{2+}\) for FV currents is about 6 μM (Tikhonova et al., 1997), which is higher than known physiological levels of free Ca\(^{2+}\) (Bush, 1995; McAinsh and Hetherington, 1998). In contrast to Ca\(^{2+}\), the half-inhibitory concentration of cytosolic Mg\(^{2+}\) was about 230 μM (Fig. 1), which lies within the physiological range of free Mg\(^{2+}\) concentrations (400 μM; Yazaki et al., 1988), suggesting that Mg\(^{2+}\) might play a major role in the down-regulation of FV channels in vivo during stomatal opening or cell expansion.

A recent study concluded that due to vacuolar Ca\(^{2+}\) block (Fig. 8B), SV channels cannot mediate Ca\(^{2+}\)-induced Ca\(^{2+}\) release (Pottosin et al., 1997). However, this model represents a negative hypothesis based on a lack of observation, which, given the complexity of biological systems, may be oversimplified (for review, see Alberts, 1998). This hypothesis (Pottosin et al., 1997) did not consider shifts in the Ca\(^{2+}\) sensitivity of SV channels to cytosolic Mg\(^{2+}\) (Fig. 7), the effects of the K\(^{+}\) gradient across the vacuolar membrane (Fig. 7), nor effects of malate gradients proposed to shift SV activation (Hedrich et al., 1986). Cellular regulation mechanisms of the SV channel, such as calmodulin (Bethke and Jones, 1994), redox agents (Carpaneto et al., 1999), and protein phosphorylation (Allen and Sanders, 1995; Bethke and Jones, 1997) might also shift the voltage dependence of SV channels, and were not considered (Pottosin et al., 1997).

In addition, deactivating time-dependent (tail) SV currents have been shown to unequivocally mediate Ca\(^{2+}\) efflux from vacuoles (Ward and Schroeder, 1994; Ward et al., 1995). Therefore, the conclusion that SV channels cannot mediate Ca\(^{2+}\) release (Pottosin et al., 1997) is not consistent with these direct recordings. Transient stimulation of second-messenger (cADPR and InP\(_3\))-activated Ca\(^{2+}\) selective channels in the vacuolar membrane (Allen et al., 1995; Leckie et al., 1998; Cancela et al., 1999) will polarize the

**Figure 8.** Simplified model for the regulation of SV channels by cytosolic and luminal Ca\(^{2+}\) and Mg\(^{2+}\) in fava bean guard cells. A\(_1\), High-affinity Ca\(^{2+}\)-binding site on the cytosolic side, which is not activated by Mg\(^{2+}\). A\(_2\), Low-affinity binding site on the cytosolic side, which can be occupied by either Mg\(^{2+}\) or Ca\(^{2+}\). B, Vacuolar Ca\(^{2+}\)-binding site, which is not affected by vacuolar Mg\(^{2+}\). For the activation of SV channels, both activation sites A\(_1\) and A\(_2\) need to be occupied (see “Discussion”). The cytosolic and vacuolar membrane sides are labeled.
vacuolar potential to positive voltages, which in turn activates SV channels. Subsequently, deactivation of second-messenger-activated Ca\(^{2+}\)-selective channels (Allen et al., 1995; Leckie et al., 1998) could produce Ca\(^{2+}\)-induced Ca\(^{2+}\) release via tail currents. Rapid vacuolar membrane repolarization could also be mediated by the combination of activated VK channels and proton pumps and/or anion efflux from vacuoles (Ward et al., 1995). In addition, data in Figure 7 show yet to be identified conditions that shift the voltage dependence of SV channels. Furthermore, both vacuolar and cytosolic Mg\(^{2+}\) activities change during stomatal movements. These findings raise an additional question of whether cytosolic Mg\(^{2+}\) activities change during stomatal opening.

**CONCLUSION**

The effects of both cytosolic and vacuolar Mg\(^{2+}\) on SV and FV channels have been systematically investigated. The present study shows that even if cytosolic Mg\(^{2+}\) concentrations do not change, physiological levels of Mg\(^{2+}\) ions provide a major mechanism for sensitizing SV channels to stimulus-induced elevations in cytosolic Ca\(^{2+}\) during signal transduction. Furthermore, both vacuolar and cytosolic Mg\(^{2+}\) ensure that FV channels do not function as a continuous leak for K\(^{+}\) ions, which would prevent stomatal opening.

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