Plant Physiology, November 1999, Vol. 121, pp. 977–986, www.plantphysiol.org © 1999 American Society of Plant Physiologists

Magnesium Sensitizes Slow Vacuolar Channels to Physiological Cytosolic Calcium and Inhibits Fast Vacuolar Channels in Fava Bean Guard Cell Vacuoles[1]

Zhen-Ming Pei*, John M. Ward[2], and Julian I. Schroeder

Department of Biology and Center for Molecular Genetics, University of California, San Diego, La Jolla, California 92093–0116

Vacuolar ion channels in guard cells play important roles during stomatal movement and are regulated by many factors including Ca^{2+}, calmodulin, protein kinases, and phosphatases. We report that physiological cytosolic and luminal Mg^{2+} levels strongly regulate vacuolar ion channels in fava bean (*Vicia faba*) guard cells. Luminal Mg^{2+} inhibited fast vacuolar (FV) currents with a K_i of approximately 0.23 mM in a voltage-dependent manner at positive potentials on the cytoplasmic side. Cytosolic Mg^{2+} at 1 mM also inhibited FV currents. Furthermore, in the absence of cytosolic Mg^{2+}, cytosolic Ca^{2+} at less than 10 μM did not activate slow vacuolar (SV) currents. However, when cytosolic Mg^{2+} was present, submicromolar concentrations of cytosolic Ca^{2+} activated SV currents with a K_d of approximately 227 nM, suggesting a synergistic Mg^{2+}-Ca^{2+} effect. The activation potential of SV currents was shifted toward physiological potentials in the presence of cytosolic Mg^{2+} 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 Ca^{2+} with an affinity in the submicromolar range and a cytosolic low-affinity Mg^{2+}-Ca^{2+} binding site. SV channels are predicted to contain a third binding site on the vacuolar luminal side, which binds Ca^{2+} and is inhibitory. In conclusion, cytosolic Mg^{2+} sensitizes SV channels to physiological cytosolic Ca^{2+} elevations. Furthermore, we propose that cytosolic and vacuolar Mg^{2+} concentrations ensure that FV channels do not function as a continuous vacuolar K^+ leak, which would prohibit stomatal opening.

Mg^{2+} is an abundant cytoplasmic cation in higher plants (Epstein, 1965), with concentrations of 2 to 10 mM in leaf cells (Leigh and Wyn Jones, 1986). Mg^{2+} 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 Mg^{2+}, 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 Mg^{2+} 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; Kerschbaum and Cahalan, 1999). Matsuda et al. (1987) and Vandenberg (1987) demonstrated the direct blockage of inward rectifier K^+ channels in animal cells by Mg^{2+}; however, little is known about how Mg^{2+} 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 Ca^{2+}-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 Ca^{2+}, whereas FV channels are inhibited by elevations in cytosolic Ca^{2+} (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 Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} (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-

[1] This work was supported by the National Science Foundation (grant no. MCB–9506191 to J.I.S.).

[2] Present address: Center for Plant Molecular Biology, University of Tübingen, D–72076 Tübingen, Germany.

* Corresponding author; e-mail zpei@biomail.ucsd.edu; fax 858–534–7108.

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:

$$I = I_{max} * [Mg^{2+}]^n/(K_i + [Mg^{2+}]^n)$$

where I is the degree of current inhibition, I_{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), pH 7.8, with 4 mM EGTA in all solutions. Free Ca^{2+} concentrations were calculated after accounting for 10 mM $MgCl_2$, ionic 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. Osmolalities of all solutions were adjusted to 600 mmol kg^{-1} by the addition of D-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

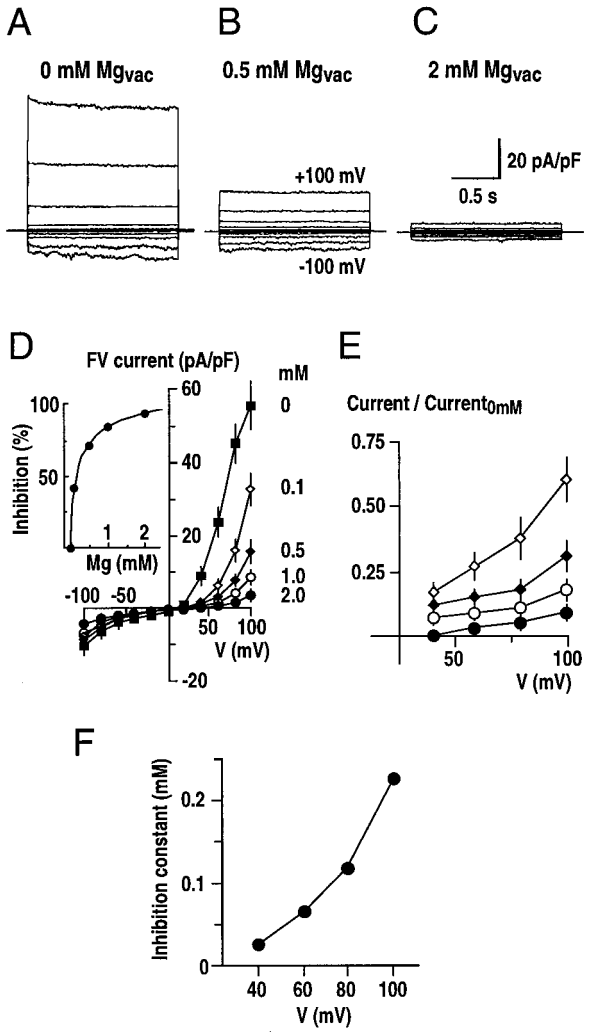

Figure 1. Fast-activating vacuolar currents inhibited by vacuolar Mg^{2+} in fava bean guard cells. A through C, Three representative whole-vacuole recordings are shown at Mg^{2+} concentrations of 0 mM (A), 0.5 mM (B), and 2 mM (C) in the pipette (luminal) solution. Membrane potential was stepped from −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-vacuolar 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 $MgCl_2$ of 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+} ($Current_{0\ mM}$). 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").

Schroeder, 1994) was avoided by buffering cytosolic Ca^{2+} to nominally zero. The steady-state current-voltage characteristics were similar to FV channel-mediated currents previously described in beet root vacuoles (Hedrich and Neher, 1987), barley mesophyll vacuoles (Tikhonova et al., 1997), and fava bean guard cell vacuoles (Allen and Sanders, 1996; Allen et al., 1998). The instantaneous FV currents were carried by monovalent cations including K^+ and Cs^+ (data not shown) as shown for FV currents (Allen and Sanders, 1996; Tikhonova et al., 1997).

Whole-vacuolar currents were analyzed at 0 to 2 mM vacuolar Mg^{2+} concentrations (Fig. 1). FV current amplitudes were reduced by increasing the vacuolar Mg^{2+} concentration from 0 (Fig. 1A) to 0.5 mM (Fig. 1B) or 2 mM (Fig. 1C). FV currents measured at five different vacuolar Mg^{2+} concentrations confirmed the strong down-regulation of FV currents by vacuolar Mg^{2+} (Fig. 1D). The average effect of vacuolar Mg^{2+} shows a 14.3- ± 2.1-fold decrease of FV currents at +100 mV by increasing vacuolar Mg^{2+} from 0 to 2 mM (Fig. 1D). FV currents at negative potentials were also reduced (2.4- ± 0.4-fold). A Hill curve could be fitted to the currents at +100 mV showing a K_i of approximately 0.23 mM and a Hill coefficient of 0.67 (Fig. 1D, inset), indicating that FV current amplitudes are inhibited by vacuolar Mg^{2+} within the physiological range (Yazaki et al., 1988). The Hill coefficient of 0.67 is consistent with one Mg^{2+} binding site per FV channel.

Whole-vacuole currents measured at different vacuolar Mg^{2+} concentrations were normalized to the control currents measured in the absence of Mg^{2+}, and plotted as a function of applied voltage (Fig. 1E). Voltage-dependent block was observed at positive membrane potentials, with a continuous decrease in current by decreasing the voltage from +100 to +40 mV. Furthermore, the apparent K_i at different membrane potentials also shows the voltage dependence of Mg^{2+} block (Fig. 1F).

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+}.

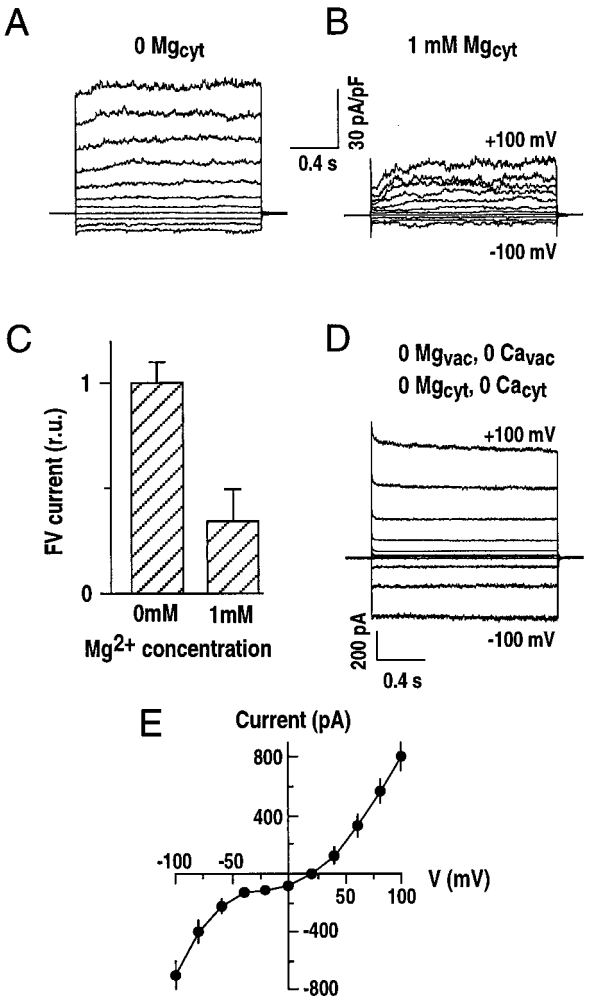

Figure 2. Cytosolic Mg^{2+} inhibits FV current. A and B, Whole-vacuole currents recorded in the absence (0 Mg_{cyt}; A) or presence of Mg^{2+} (1 mM Mg_{cyt}; B) in bath (cytosolic) solutions of the same vacuole. The holding potential was −40 mV. The pipette solution contained 20 mM KCl, 2 mM EGTA, 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 μM $CaCl_2$, 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.

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; McAinsh 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 μM 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 μM 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 (○) 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 from 10 nM to 50 μM with a constant cytosolic Mg^{2+} concentration of 10 mM (Fig. 5). At 10 nM Ca^{2+}, SV currents were not activated (Fig. 5A). Strikingly, when the Ca^{2+} concentration was subsequently increased to 50 nM, 150 nM, and up to 1 μM, SV currents measured in the same vacuole were gradually activated (Fig. 5). At 10 and 50 μM Ca^{2+}, SV currents were close to saturation (Fig. 5). In contrast, in the absence of Mg^{2+} in the bath solution, physiological concentrations of Ca^{2+} could not activate SV currents (Fig. 5B, ●). A Hill curve could be fitted to the

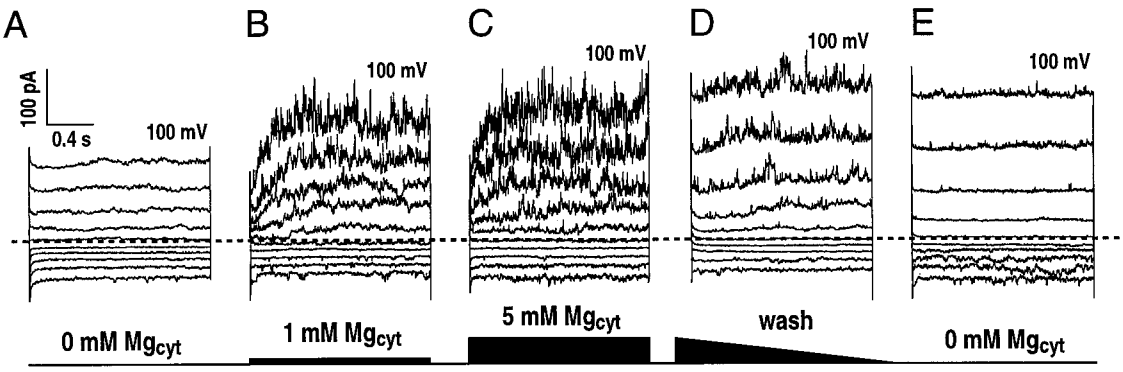

Figure 3. Possible up-regulation of slowly activating vacuolar currents by cytosolic Mg^{2+} in fava bean guard cells. Whole-vacuole currents measured from one vacuole at different Mg^{2+} concentrations in the bath solution. The holding potential was −40 mV with an interval time between pulses of 1 s. Standard bath and pipette solutions (see "Materials and Methods") were used with varying Mg^{2+} concentrations (0, 1, and 5 mM) in the bath. Note that 10 μM CaCl was added to the bath solution. A, Current recordings started in a bath solution containing no added Mg^{2+}. B and C, Bath solutions containing 1 mM (B) and 5 mM Mg^{2+} (C) were subsequently added by local perfusion (see "Materials and Methods"). D and E, The vacuole was then perfused with a bath solution containing no added Mg^{2+}. Similar experiments were repeated on eight vacuoles.

data for cytosolic Ca^{2+} concentrations from 10 nM to 1 μM, 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 μM 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 μM 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+}.

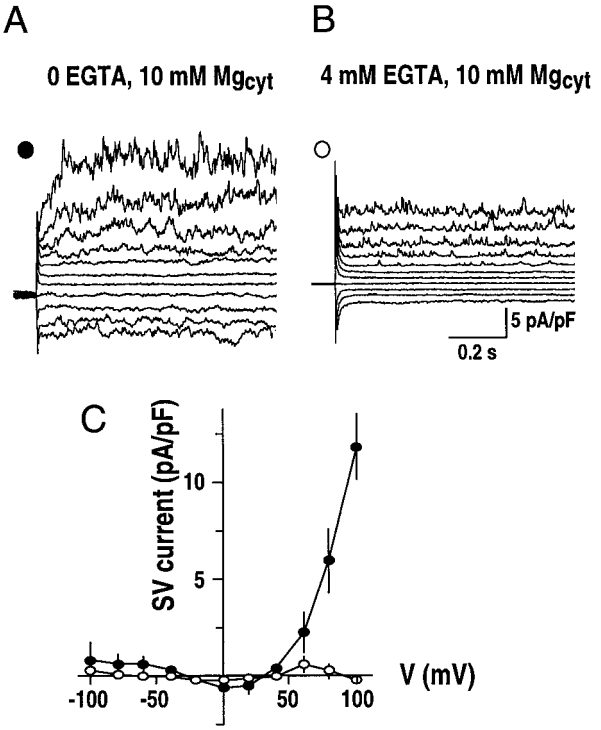

Figure 4. Cytosolic EGTA inhibits Mg^{2+} activation of SV currents. A and B, Whole-vacuole currents recorded in the absence (A) or presence (B) of 4 mM EGTA in the bath solutions in one vacuole. Standard pipette and bath solutions were used without or with the addition of 4 mM EGTA. C, Current-voltage 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 Pantoja, 1996; Bethke and Jones, 1997; Pottosin et al., 1997), whereas at physiological vacuolar potentials (from 0 to −40 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 −40 mV (Fig. 7, A and B). In some vacuoles (two out of nine), the activation potential was shifted dramatically to −90 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.

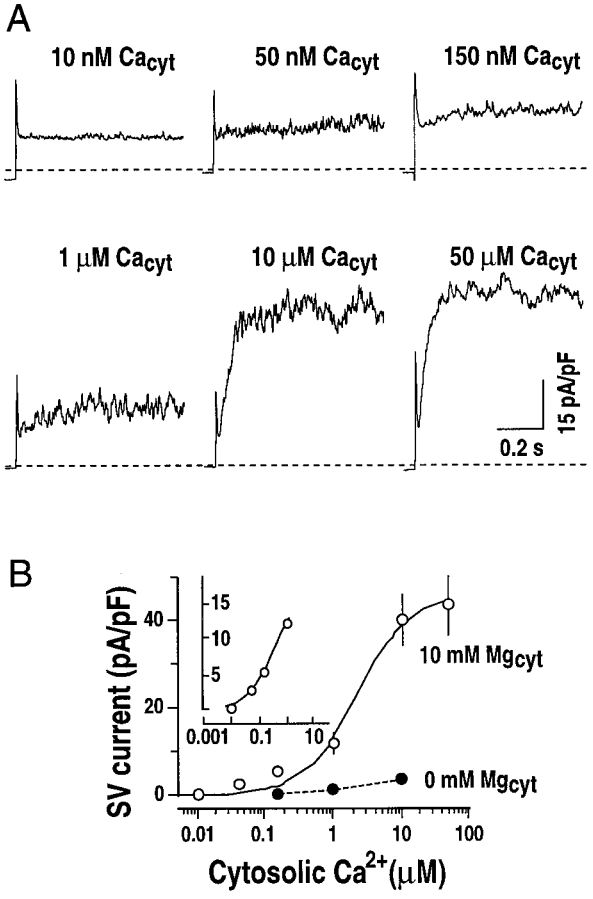

Figure 5. Cytosolic Mg^{2+} sensitizes SV channels to cytosolic Ca^{2+}. A, Representative whole-vacuole currents recorded at different cytosolic Ca^{2+} concentrations in two separate vacuoles. In one vacuole, cytosolic Ca^{2+} concentrations were changed from 10 nM to 1 μM by either local or bath perfusion (vacuolar capacitance = 3.5 pF). In another vacuole, 10 μM Ca^{2+} in the bath solution was replaced by 50 μM Ca^{2+} (vacuolar capacitance = 4.2 pF). Only current traces at +100 mV are shown. Dashed lines show zero current levels. Pipette solution contained 20 mM KCl, 2 mM EGTA, and 5 mM HEPES-Tris, pH 7. Bath solution contained 200 mM KCl, 10 mM $MgCl_2$, and 20 mM HEPES-Tris, pH 8.0, with varying free Ca^{2+} concentrations of 0, 10 nM, 50 nM, 150 nM, 1 μM, 10 μM, and 50 μM (see "Materials and Methods" for details). B, Effect of cytosolic Mg^{2+} on cytosolic Ca^{2+} activation of SV currents at +100 mV as performed in A. In control experiments, SV currents were recorded at 0 mM Mg^{2+} in bath solutions (●). Values are from three to eight vacuoles (capacitance = 4.7 ± 1.2 pF). A Hill curve is fitted to the data for the SV currents activated by Ca^{2+} at 10 mM cytosolic Mg^{2+}. Data obtained at 10 nM to 1 μM cytosolic Ca^{2+} are shown in the inset (K_d approximately 227 nM).

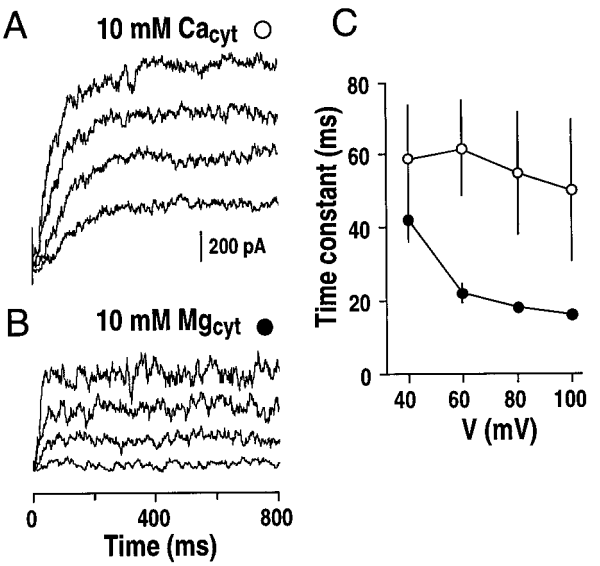

Figure 6. Effect of cytosolic Mg^{2+} on the activation time course of SV currents. A and B, SV currents recorded at saturated cytosolic 10 mM Ca^{2+} (A) are compared with SV currents at saturated cytosolic 10 mM Mg^{2+} (B). For Mg^{2+} activation of SV currents, 10 μM $CaCl_2$ was added to the bath solution to saturate the proposed high-affinity Ca^{2+} binding site (see "Discussion"). Standard pipette and bath solutions (see "Materials and Methods") were used with the addition of 10 mM $CaCl_2$ in A and with the addition of 10 mM $MgCl_2$ in B. C, Fitted time constants of the activation of SV currents plotted against the applied vacuolar membrane potentials (n = 3 vacuoles for each condition). Symbols are as in A and B.

DISCUSSION

In animal cells, Mg^{2+} blocks many cation channels, which includes Ca^{2+} channels, various inward-rectifier K^+

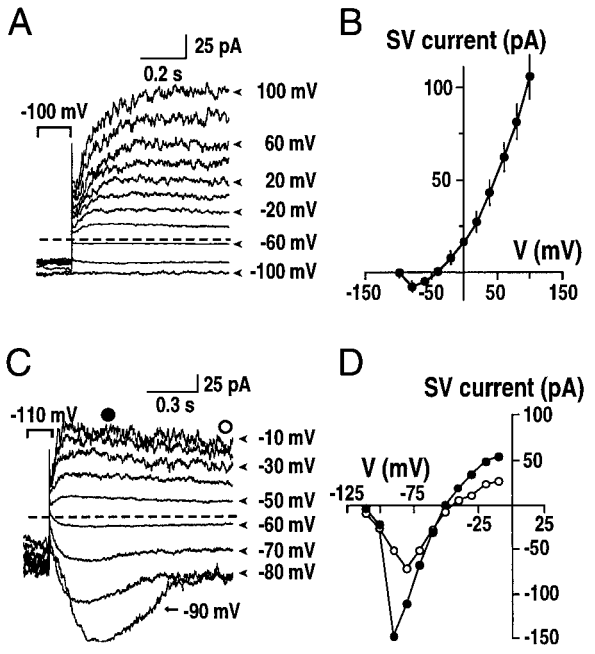

Figure 7. Shifting SV activation to physiological vacuolar potentials and changing rectification property. A and B, Whole-vacuolar SV currents (A) and current-voltage relationship (B). The pipette solution contained 20 mM KCl, 2 mM EGTA, and 5 mM HEPES-Tris, pH 7.0. The bath contained 200 mM KCl, 10 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 inward SV currents were recorded occasionally ($n = 2$ of 9 vacuoles) at negative membrane potentials (C). D, Current-voltage relationship of vacuoles showing bi-directional 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.

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 μM 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 divalent 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 μM), 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

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_d of approximately 227 nM) 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 μM Ca^{2+} to bind to the high-affinity site (A_1) (Fig. 6), indicating additional ion-specific effects.

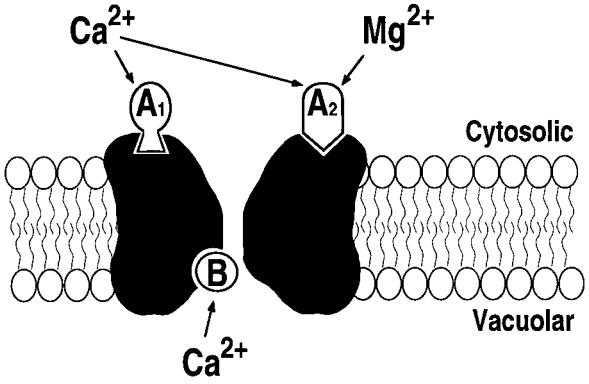

Figure 8. Simplified model for the regulation of SV channels by cytosolic and luminal Ca^{2+} and Mg^{2+} in fava bean guard cells. A_1, High-affinity Ca^{2+}-binding site on the cytosolic side, which is not activated by Mg^{2+}. A_2, Low-affinity binding site on the cytosolic side, which can be occupied 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.

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

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. Further research on mechanisms that could shift the voltage dependence of SV channels, such as cytosolic Mg^{2+} (Fig. 7), calmodulin, redox agents (Bethke and Jones, 1994; Carpaneto et al., 1999), and other regulators, may lead to the identification of additional mechanisms that allow Ca^{2+}-induced Ca^{2+} release. Taken together, our data show that Mg^{2+} can play an important role in the regulation of vacuolar ion channels. These findings raise an additional question of whether cytosolic Mg^{2+} activities change during stomatal movements.

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.

ACKNOWLEDGMENTS

We thank Gethyn J. Allen for comments, Sébastien Thomine and David A. Lee for reading the manuscript, and Walter Gassmann, Martin Schwarz, and Walter B. Kelly for technical support during experiments and helpful discussions.

Received February 24, 1999; accepted July 12, 1999.

LITERATURE CITED

Agus ZS, Morad M (1991) Modulation of cardiac ion channels by magnesium. Annu Rev Physiol **53:** 299–307

Alberts B (1998) The cell as a collection of protein machines: preparing the next generation of molecular biologists. Cell **92:** 291–294

Allen GJ, Amtmann A, Sanders D (1998) Calcium-dependent and calcium-independent K^+ mobilization channels in *Vicia faba* guard cell vacuoles. J Exp Bot **49:** 305–318

Allen GJ, Muir SR, Sanders D (1995) Release of Ca^{2+} from individual plant vacuoles by both $InsP_3$ and cyclic ADP-ribose. Science **268:** 735–737

Allen GJ, Sanders D (1995) Calcineurin, a type 2B protein phosphatase, modulates the Ca^{2+}-permeable slow vacuolar ion channel of stomatal guard cells. Plant Cell **7:** 1473–1483

Allen GJ, Sanders D (1996) Control of ionic currents in guard cell vacuoles by cytosolic and luminal calcium. Plant J **10:** 1055–1069

Allen GJ, Sanders D (1997) Vacuolar ion channels of higher plants. Adv Bot Res **25:** 217–252

Assmann SM (1993) Signal transduction in guard cells. Annu Rev Cell Biol **9:** 345–375

Barkla BJ, Pantoja O (1996) Physiology of ion transport across the tonoplast of higher plants. Annu Rev Plant Physiol Plant Mol Biol **47:** 159–184

Bertl A, Blumwald E, Coronado R, Eisenberg R, Findlay G, Gradman D, Hille B, Kohler K, Kolb HA, MacRobbie E (1992) Electrical measurements on endomembranes. Science **258:** 873–874

Bethke PC, Jones RL (1994) Ca^{2+}-calmodulin modulates ion channel activity in storage protein vacuoles of barley aleurone cells. Plant Cell **6:** 277–285

Bethke PC, Jones RL (1997) Reversible protein phosphorylation regulates the activity of the slow-vacuolar ion channel. Plant J **11:** 1227–1235

Boller T, Wiemken A (1986) Dynamics of vacuolar compartmentation. Annu Rev Plant Physiol **37:** 137–164

Brüggemann LI, Pottosin II, Schönknecht G (1998) Cytoplasmic polyamines block the fast-activating vacuolar cation channel. Plant J **16:** 101–106

Bush DS (1995) Calcium regulation in plant cells and its role in signaling. Annu Rev Plant Physiol Plant Mol Biol **46:** 95–122

Cancela JM, Churchill GC, Galione A (1999) Coordination of agonist-induced Ca^{2+}-signalling patterns by NAADP in pancreatic acinar cells. Nature **398:** 74–76

Carpaneto A, Cantù AM, Gambale F (1999) Redox agents regulate ion channel activity in vacuoles from higher plant cells. FEBS Lett **442:** 129–132

Chuang H-h, Jan YN, Jan LY (1997) Regulation of IRK3 inward rectifier K^+ channel by m1 acetylcholine receptor and intracellular magnesium. Cell **89:** 1121–1132

Colombo R, Cerana R, Lado P, Peres A (1988) Voltage-dependent channels permeable to K^+ and Na^+ in the membrane of *Acer pseudoplatanus* vacuoles. J Membr Biol **103:** 227–236

Coyaud L, Kurkdjian A, Kado R, Hedrich R (1987) Ion channels and ATP-driven pumps involved in ion transport across the tonoplast of sugar beet vacuoles. Biochim Biophys Acta **902:** 263–268

Davies JM, Sanders D (1995) ATP, pH and Mg^{2+} modulate a cation current in *Beta vulgaris* vacuoles: a possible shunt conductance for the vacuolar H^+-ATPase. J Membr Biol **145:** 75–86

Dobrovinskaya OR, Muniz J, Pottosin II (1999) Inhibition of vacuolar ion channels by polyamines. J Membr Biol **167:** 127–140

Epstein E (1965) Mineral metabolism. *In* J Bonner, JE Varner, eds, Plant Biochemistry. Academic Press, London, pp 438–466

Fakler B, Brändle U, Glowatzki E, Weidemann S, Zenner H-P, Ruppersberg JP (1995) Strong voltage-dependent inward rectification of inward rectifier K^+ channels is caused by intracellular spermine. Cell **80:** 149–154

Flatman PW (1991) Mechanisms of magnesium transport. Annu Rev Physiol **53:** 259–271

Foehr KJ, Warchol W, Gratzl M (1993) Calculation and control of free divalent cations in solutions used for membrane fusion studies. Methods Enzymol **221:** 149–157

Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording form cells and cell-free membrane patches. Pflügers Arch **391:** 85–100

Hedrich R, Flügge UI, Fernandez JM (1986) Patch-clamp studies of ion transport in isolated plant vacuoles. FEBS Lett **204:** 228–232

Hedrich R, Kurkdjian A (1988) Characterization of an anion-permeable channel from sugar beet vacuoles: effect of inhibitors. EMBO J **7:** 3661–3666

Hedrich R, Neher E (1987) Cytoplasmic calcium regulates voltage-dependent ion channels in plant vacuoles. Nature **329:** 833–836

Hille B (1992) Ionic Channels of Excitable Membranes, Ed 2. Sinauer Associates, Sunderland, MA

Kerschbaum HH, Cahalan MD (1999) Single-channel recording of a store-operated Ca^{2+} channel in Jurkat T lymphocytes. Science **283:** 836–839

Kruse T, Tallman G, Zeiger E (1989) Isolation of guard cell protoplasts from mechanically prepared epidermis of *Vicia faba* leaves. Plant Physiol **90:** 1382–1386

Laver DR, Owen VJ, Junankar PR, Taske NL, Dulhunty AF, Lamb GD (1997) Reduced inhibitory effect of Mg^{2+} on ryanodine receptor Ca^{2+} release channels in malignant hyperthermia. Biophys J **73:** 1913–1924

Leckie CP, McAinsh MR, Allen GJ, Sanders D, Hetherington AM (1998) Abscisic acid-induced stomatal closure mediated by cyclic ADP-ribose. Proc Natl Acad Sci USA **95:** 15837–15842

Leigh RA, Wyn Jones RG (1986) Cellular compartmentation in plant nutrition: the selective cytoplasm and the promiscuous vacuole. *In* B Tinker, A Lauchli, eds, Advances in Plant Nutrition 2. Praeger Scientific, New York, pp 249–279

Leube MP, Grill E, Amrhein N (1998) ABI1 of *Arabidopsis* is a protein serine/threonine phosphatase highly regulated by the proton and magnesium ion concentration. FEBS Lett **424:** 100–104

MacRobbie EAC (1981) Effects of ABA on "isolated" guard cells of *Commelina communis* L. J Exp Bot **32:** 563–572

MacRobbie EAC (1998) Signal transduction and ion channels in guard cells. Phil Trans R Soc Lond B **353:** 1475–1488

Marschner H (1995) Mineral Nutrition of Higher Plants, Ed 2. Academic Press, London

Matsuda H, Saigusa A, Irisawa H (1987) Ohmic conductance through the inward rectifying K^+ channel and blocking by Mg^{2+}. Nature **325:** 156–159

McAinsh MR, Brownlee C, Hetherington AM (1997) Calcium ions as second messengers in guard cell signal transduction. Physiol Plant **100:** 16–29

McAinsh MR, Hetherington AM (1998) Encoding specificity in Ca^{2+} signaling systems. Trends Plant Sci **3:** 32–36

Murphy E, Freudenrich CC, Lieberman M (1991) Cellular magnesium and Na^+/Mg^{2+} exchange in heart cells. Annu Rev Physiol **53:** 273–287

Nowak L, Bregestovski P, Ascher P, Herbet A, Prochiantz A (1984) Magnesium gates glutamate-activated channels in mouse central neurons. Nature **307:** 462–465

Pantoja O, Gelli A, Blumwald E (1992) Voltage-dependent calcium channels in plant vacuoles. Science **255:** 1567–1570

Paoletti P, Neyton J, Ascher P (1995) Glycine-independent and subunit-specific potentiation of NMDA responses by extracellular Mg^{2+}. Neuron **15:** 1109–1120

Pei Z-M, Ward JM, Harper JF, Schroeder JI (1996) A novel chloride channel in *Vicia faba* guard cell vacuoles activated by the serine/threonine kinase, CDPK. EMBO J **15:** 6564–6574

Pottosin II, Tikhonova LI, Hedrich R, Schönknecht G (1997)Slowly activating vacuolar channels can not mediate Ca^{2+}-induced Ca^{2+} release. Plant J **12:** 1387–1398

Schroeder JI (1995) Magnesium-independent activation of inward-rectifying K^+ channels in *Vicia faba* guard cells. FEBS Lett **363:** 157–160

Schulz-Lessdorf B, Hedrich R (1995) Protons and calcium modulate SV-type channels in the vacuolar-lysosomal compartment: channel interaction with calmodulin inhibitors. Planta **197:** 655–671

Sze H, Ward JM, Lai S (1992) Vacuolar H^+-translocating ATPases from plants: structure, function, and isoforms. J Bioenerg Biomembr **24:** 371–381

Tikhonova LI, Pottosin II, Dietz K-J, Schönknecht G (1997) Fast-activating cation channel in barley mesophyll vacuoles: inhibition by calcium. Plant J **11:** 1059–1070

Vandenberg CA (1987) Inward rectification of a potassium channel in cardiac ventricular cells depends on internal magnesium ions. Proc Natl Acad Sci USA **84:** 2560–2564

Ward JM, Pei Z-M, Schroeder JI (1995) Roles of ion channels in initiation of signal transduction in higher plants. Plant Cell **7:** 833–844

Ward JM, Schroeder JI (1994) Calcium-activated K^+ channel and calcium-induced calcium release by slow vacuolar ion channels in guard cell vacuoles implicated in the control of stomatal closure. Plant Cell **6:** 669–683

Weiser T, Blum W, Bentrup FW (1991) Calmodulin regulates the Ca^{2+}-dependent slow-vacuolar ion channel in the tonoplast of *Chenopodium rubrum* suspension cells. Planta **185:** 440–442

Yazaki Y, Asukagawa N, Ishikawa Y, Ohta E, Sakata M (1988) Estimation of cytoplasmic free Mg^{2+} levels and phosphorylation potentials in mung bean root tips by in vivo ^{31}P NMR spectroscopy. Plant Cell Physiol **29:** 919–924