Osmo-Sensitive and Stretch-Activated Calcium-Permeable Channels in *Vicia faba* Guard Cells Are Regulated by Actin Dynamics¹[OA]

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In responses to a number of environmental stimuli, changes of cytoplasmic \([\text{Ca}^{2+}]_{\text{cyt}}\) in stomatal guard cells play important roles in regulation of stomatal movements. In this study, the osmo-sensitive and stretch-activated (SA) \([\text{Ca}^{2+}]\) channels in the plasma membrane of *Vicia faba* guard cells are identified, and their regulation by osmotic changes and actin dynamics are characterized. The identified \([\text{Ca}^{2+}]\) channels were activated under hypotonic conditions at both whole-cell and single-channel levels. The channels were also activated by a stretch force directly applied to the membrane patches. The channel-mediated inward currents observed under hypotonic conditions or in the presence of a stretch force were blocked by the \([\text{Ca}^{2+}]\) channel inhibitor Gd³⁺. Disruption of actin filaments activated SA \([\text{Ca}^{2+}]\) channels, whereas stabilization of actin filaments blocked the channel activation induced by stretch or hypotonic treatment, indicating that actin dynamics may mediate the stretch activation of these channels. In addition, \([\text{Ca}^{2+}]\) imaging demonstrated that both the hypotonic treatment and disruption of actin filaments induced significant \([\text{Ca}^{2+}]\) elevation in guard cell protoplasts, which is consistent with our electrophysiological results. It is concluded that stomatal guard cells may utilize SA \([\text{Ca}^{2+}]\) channels as osmo sensors, by which swelling of guard cells causes elevation of \([\text{Ca}^{2+}]_{\text{cyt}}\) and consequently inhibits overswellling of guard cells. This SA \([\text{Ca}^{2+}]\) channel-mediated negative feedback mechanism may coordinate with previously hypothesized positive feedback mechanisms and regulate stomatal movement in response to environmental changes.

Stomata form pores on leaf surfaces that facilitate \(\text{CO}_2\) uptake for photosynthesis and regulate transpirational water vapor loss. A number of stimuli, such as light, \(\text{CO}_2\), drought, humidity, and the phytohormone abscisic acid (ABA), regulate the aperture of the stoma by controlling the turgor of the two guard cells that surround each stomatal pore (for review, see Mansfield et al., 1990; Assmann, 1993). Turgor changes are driven by fluxes of \(\text{K}^+\) and anions through ion channels in the plasma and vacuolar membranes, \(\text{Suc}\) accumulation/removal, and metabolism between starch and malate (for review, see Assmann, 1993; MacRobbie, 1998). An increase of \([\text{Ca}^{2+}]_{\text{cyt}}\) has been shown to be a common and key intermediate, both inactivating inward \(\text{K}^+\) channels and activating slow anion channels (Schroeder and Hagiwara, 1989), which leads to stomatal closure (for review, see Blatt, 2000; McAinsh et al., 2000). More recent studies demonstrated that \([\text{Ca}^{2+}]_{\text{cyt}}\) oscillations in guard cells are important for stomatal closure movements (Allen et al., 2000, 2001). The changes of \([\text{Ca}^{2+}]_{\text{cyt}}\) result from both \([\text{Ca}^{2+}]\) release from and sequestration to intracellular stores (such as tonoplasts) and \([\text{Ca}^{2+}]\) entry and efflux across the plasma membrane (PM; Köhler et al., 2003; for review, see MacRobbie, 1998; Hetherington and Brownlee, 2004). \([\text{Ca}^{2+}]\) efflux from tonoplasts through ion channels has been studied extensively. Two tonoplast \([\text{Ca}^{2+}]\)-permeable ion channels, the fast- and slow-vacuole channels, exist in stomatal guard cells and are regulated by inositol 1,4,5-triphosphate (Allen et al., 1995), calcineurin (Allen and Sanders, 1995), and cyclic ADP-ribose (Leckie et al., 1998). Compared to the studies on tonoplast \([\text{Ca}^{2+}]\)-permeable ion channels and the substantial body of evidence on regulation of \([\text{Ca}^{2+}]_{\text{cyt}}\) in guard cells (Ward et al., 1995; Ng et al., 2001; for review, see Assmann, 1993; MacRobbie, 1998; Schroeder et al., 2001), little is known about the regulatory mechanisms for \([\text{Ca}^{2+}]\) channels in the PM of guard cells (Hamilton et al., 2000; Pei et al., 2000; Köhler et al., 2003). Hydrogen peroxide (\(\text{H}_2\text{O}_2\)) has been demonstrated to mediate ABA signaling to increase \([\text{Ca}^{2+}]_{\text{cyt}}\) in guard cells by activating \([\text{Ca}^{2+}]\) channels in the PM (Pei et al., 2000; Klüsener et al., 2002), whereas there is also a line of evidence suggesting that the ABA and \(\text{H}_2\text{O}_2\) pathways diverge further

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downstream in their actions on the outward K<sup>+</sup> channels, questioning the role of H<sub>2</sub>O<sub>2</sub> as a critical second messenger regulating guard cell ion channels in response to ABA (Köhler et al., 2003).

Stomatal response to osmotic stress is regulated via a feedback mechanism (Liu and Luan, 1998). It has been hypothesized that some components, such as stretch-activated (SA) ion channels in the PM of guard cells could sense the osmotic change-induced cell turgor changes or membrane stretch and further transduce osmotic signals (MacRobbie, 1995). It is also suggested that the ABA-dependent and ABA-independent pathways might work together to regulate guard cell turgor under osmotic stress conditions (Liu and Luan, 1998). Voltage-dependent K<sup>+</sup> channels and slow anion channels have been shown to be regulated by water stress-induced ABA signal, which contributes to regulation of guard cell turgor and stomatal aperture (Assmann and Wu, 1994; Schwartz et al., 1994; Pei et al., 1997, 1998). On the other hand, osmo-sensitive voltage-dependent K<sup>+</sup> channels, regulated by the osmo gradient across the PM of guard cells, have been considered to be a positive feedback loop in an ABA-independent osmo-sensing pathway that accelerates stomatal movements (Liu and Luan, 1998). Both osmotic stress and physiological cell swelling during stomatal opening may cause stretch forces imposed on the PM of guard cells. SA Ca<sup>2+</sup>-permeable channels (SA Ca<sup>2+</sup> channels) have been suggested to contribute to regulation of turgor pressure in Vicia guard cells (Cosgrove and Hedrich, 1991). However, regulatory mechanisms of SA Ca<sup>2+</sup> channels in the PM of guard cells and their roles in signal remain to be elucidated.

Actin microfilaments are dynamic cellular components and they can be assembled or disassembled into actin monomers at spatially defined sites of a living cell during physiological processes. It is established that in animal cells, as a signal transducer, dynamic structural changes in actin microfilaments contribute to several signaling processes involving regulation of ion channel activities (Schwiebert et al., 1994; Cantiello, 1997; for review, see Janmey, 1998). In plants, it has become evident that actin microfilaments also function in signal transduction networks (for review, see Volkmann and Baluška, 1999). Actin dynamics has been proposed to be involved in regulation of Ca<sup>2+</sup> oscillations and the establishment of [Ca<sup>2+</sup>]<sub>i</sub> gradient in pollen tubes (Fu et al., 2001; Wang et al., 2004). In stomatal guard cells, actin microfilaments respond to physiological stimuli, including light and ABA (Eun and Lee, 1997; Lemichez et al., 2001), and an increase in [Ca<sup>2+</sup>]<sub>i</sub> mediates ABA-induced disruption of actin filaments (Hwang and Lee, 2001). Furthermore, disruption of actin filaments by cytochalasin D (CD), an inhibitor of actin cytoskeleton polymerization, has been shown to up-regulate the osmo-sensitive inward K<sup>+</sup> channels and enhance stomatal opening (Kim et al., 1995; Eun and Lee, 1997; Liu and Luan, 1998). The actin cytoskeleton is suggested to serve as an osmo sensor, targeting K<sup>+</sup> channels for turgor regulation in a positive feedback loop in guard cells (Liu and Luan, 1998). Similar to the observation in animal cells (Glogauer et al., 1995), actin dynamics in stomatal guard cells may play an important role in regulating SA Ca<sup>2+</sup> channels and thus modulating stomatal movements. In this study, we report SA Ca<sup>2+</sup>-permeable channels in the PM of Vicia faba guard cell, which are sensitive to osmotic changes or stretch forces and are regulated by actin dynamics. These SA Ca<sup>2+</sup>-permeable channels may, at least in part, account for the osmo-sensitive macroscopic Ca<sup>2+</sup> currents across the PM of guard cells and serve as an osmotic-sensing target in a negative feedback loop. This negative feedback loop, together with the positive feedback loop proposed previously (Liu and Luan, 1998), may explain the mechanisms for osmo regulation of stomatal movements and oscillation (Cowan et al., 1997).

RESULTS

Identification of the Osmo-Sensitive and Voltage-Dependent Ca<sup>2+</sup>-Permeable Channels in Vicia Guard Cells

Considering that Ba<sup>2+</sup> has been commonly used for Ca<sup>2+</sup> channel identification (Hamilton et al., 2000; Pei et al., 2000), Ba<sup>2+</sup> was used in our patch-clamping experiments as the major charge-carrying ion to identify Ca<sup>2+</sup> channels, unless otherwise indicated. Under the control conditions with an osmolality at 500 mosmol for both bath and pipette solutions, inward currents with small amplitude were observed (Fig. 1, trace 5, n = 12). When the bath isotonic solutions (osmolality at 500 mosmol) were changed to hypertonic solutions with an osmolality at 600 mosmol, the currents were inhibited by approximately 30% (Fig. 1, trace 2, n = 12). In contrast, the inward currents were dramatically increased when the bath isotonic solutions (osmolality at 500 mosmol) were replaced with hypotonic bath solutions (osmolality at 400 mosmol; Fig. 1, trace 5, n = 12). Both the inhibition and activation of the inward currents were reversible when the bath solutions were changed between the hypertonic and the isotonic (control) solutions or between the hypotonic and the isotonic solutions. Furthermore, when Ba<sup>2+</sup> was substituted by Ca<sup>2+</sup> in the hypotonic bath solution, the observed currents appeared similar to the currents when Ba<sup>2+</sup> were used under the same conditions (Fig. 1, trace 4, n = 4; compared to trace 5). The results indicate that these channels are Ca<sup>2+</sup> permeable and activated or inhibited under the hypotonic or the hypertonic conditions, respectively.

To further confirm the nature of the recorded inward currents, Gd<sup>3+</sup>, an inorganic blocker of Ca<sup>2+</sup>-permeable channels, was applied. Lemtiri-Chlieh et al. (2003) reported that the 100 μM Gd<sup>3+</sup> dramatically blocks PM
Ca\textsuperscript{2+}-permeable channels in \textit{V. faba} guard cell protoplasts but with no effect on the inward K\textsuperscript{+} channel currents. Ding and Pickard (1993) showed that Gd\textsuperscript{3+} blocks mechanosensory (SA) Ca\textsuperscript{2+}-selective channels in epidermal cells. In this study, addition of 100 \textmu M Gd\textsuperscript{3+} to the bath solutions dramatically inhibited the activation of the currents by the hypotonic treatment (Fig. 1, trace 1, \(n = 5\); compared to trace 5). The reversal potential of the inward currents activated by hypotonic bath solution was approximately 12 mV, which is close to the theoretical equilibrium potential for Ba\textsuperscript{2+} (\(E_{Ba} = 14.9\) mV) and far from that for Cl\textsuperscript{−} under the given conditions (\(E_{Cl} = -34.6\) mV).

These results demonstrate that the recorded osmo-regulated inward currents are predominantly carried by an influx of Ba\textsuperscript{2+} or Ca\textsuperscript{2+} through Ca\textsuperscript{2+}-permeable channels in the PM of \textit{Vicia} guard cell protoplasts. These osmo-sensitive Ca\textsuperscript{2+} channels in guard cells are inhibited under hypertonic conditions and activated under hypotonic conditions.

**Actin Dynamics Modulates the Osmo Regulation of the Whole-Cell Ba\textsuperscript{2+} Channel Activity**

Actin dynamics has been demonstrated to regulate various types of ion channels in animal and plant cells as discussed in the introduction. To investigate if actin dynamics are involved in the osmo regulation of Ca\textsuperscript{2+}-permeable channels in guard cells, the actin polymerization inhibitor CD and the actin filament stabilizer phalloidin were applied to test their effects on activity of the osmo-regulated Ca\textsuperscript{2+}-permeable channels. Compared to the control (the isotonic bath solutions; Fig. 2A, trace 2, \(n = 8\)), the hypertonic treatment exerted a slight inhibition of the whole-cell inward Ba\textsuperscript{2+} currents (Fig. 2A, trace 1, \(n = 8\)). Addition of 20 \textmu M CD to the hypertonic bath solutions dramatically increased the inward Ba\textsuperscript{2+} currents (Fig. 2A, trace 3, \(n = 8\)). Conversely, application of 100 \textmu M phalloidin to...
the pipette solutions had no effect on the inward currents under the isotonic conditions (the osmolality of both bath and pipette solutions at 500 mosmol; Fig. 2B, trace 2 versus trace 1, \( n = 6 \)). Furthermore, in the presence of phalloidin, activation of the inward currents by the hypotonic treatment was abolished (Fig. 2B, trace 3 versus trace 1, \( n = 6 \)). These results demonstrate that the effect of osmo gradients on the inward currents was mediated by actin dynamics. The blockage in depolymerization of actin filaments abolishes activation of \( Ca^{2+} \)-permeable channels by hypotonic treatment, which suggests that the hypotonic treatment may first induce depolymerization of actin filaments and consequently cause activation of the \( Ca^{2+} \)-permeable channels in guard cells.

**Characterization of the SA \( Ca^{2+} \) Channels in the PM of Guard Cells**

Under the control conditions, the inward channel activity in the isolated outside-out membrane patches was not observed at any voltage tested (from 60 mV to \(-120 \) mV) without application of a pressure-mediated stretch to the membrane (the top trace in Fig. 3A shows the recording at \(-60 \) mV without application of a stretch). However, the inward currents were elicited at \(-60 \) mV after application of a positive pressure to the outside-out membrane patch (blowing into the interior of the glass pipette) and the channel activity increased along with the increase of the applied pressure from 3 to 15 kPa (Fig. 3A). For example, the open probability (\( N_{Po} \), as defined in "Materials and Methods") was increased by nearly 200% (from 0.31–0.91) when the applied pressure was increased from 9 to 15 kPa (Fig. 3, B and C). This pressure- or stretch-induced activation of the channels was reversible. The channel activity disappeared once the pressure was released (back to 0 kPa; see bottom trace of Fig. 3A).

As shown in Figure 4, the current amplitude (Fig. 4, A and B) and the \( N_{Po} \) (Fig. 4, A and C) of the SA channels are both voltage dependent when a 9-kPa positive pressure was applied to an outside-out membrane patch via the pipette. The derived single-channel conductance was 19.7 pS and the reversal potential was near 15 mV (Fig. 4B, \( n = 8 \)). The value of reversal potential is close to the theoretical \( E_{Ba} \) under the given conditions (\( E_{Ba} = 14.9 \) mV, \( E_{Cl} = -34.6 \) mV), indicating that the single-channel currents can be ascribed to an influx of Ba\(^{2+} \) through the \( Ca^{2+} \)-permeable channels. This notion was further supported by the result that the single-channel conductance increased (Fig. 5A) and the reversal potential shifted to more positive values (Fig. 5B) along with the increase of Ba\(^{2+} \) concentration in the bath solutions. The relationship for single-channel conductance versus Ba\(^{2+} \) concentration was well fitted by the Michaelis-Menten kinetic equation with a \( K_m \) at approximately 1.98 mM (Fig. 5A), which is similar to that reported previously (Hamilton et al., 2000). The results presented in Figure 5B also show that the measured reversal potentials merged well with the calculated Nernst potentials.

When Ba\(^{2+} \) in the bath solution was substituted with \( Ca^{2+} \), similar voltage-dependent SA currents were observed with application of a 9-kPa positive pressure to an outside-out membrane patch (Fig. 4D). Compared to the results presented in Figure 4, A to C, the \( Ca^{2+} \) current amplitude and the \( N_{Po} \) of the channels were similarly voltage dependent (Fig. 4, D–F). The single-channel conductance and the reversal potential of the inward \( Ca^{2+} \) currents were 11.8 pS and 23 mV, respectively (Fig. 4E, \( n = 4 \)). The relative permeabilities of Ba\(^{2+} \) and \( Ca^{2+} \) were calculated by the simplified Goldman-Hodgkin-Katz equation (Hille, 1993) and the derived ratio of \( PCa^{2+}/PBa^{2+} \) was 1.91.

**Figure 3.** Analysis of strength-dependent SA channel activity in an isolated outside-out membrane patch. A. Current traces were recorded from the same outside-out membrane patch at \(-60 \) mV under different stretches between 0 and 15 kPa, as indicated. Stretch was applied to the membrane patch by exerting a positive pressure to the interior of the glass pipette. The bath and pipette solutions for the control conditions were used. Dotted lines indicate the state of the channel, and the letters c or o stand for the closed or open state, respectively. B and C. \( N_{Po} \), chart derived from the data recorded at 9 and 15 kPa, respectively. The \( N_{Po} \) values were the averaged results of the recordings from six membrane patches.
Figure 6 shows reversible inhibitory effects of Gd$^{3+}$, a Ca$^{2+}$ channel blocker, on the activity of the identified SA channels. The NPo of the channels was significantly reduced in an inhibitor concentration-dependent manner. Under the application of a 9-kPa positive pressure to the membrane patch at $-60$ mV, the NPo of the SA channels was decreased by nearly $60\%$ or $95\%$ after the addition of $10\mu M$ (Fig. 6, B and F) or $100\mu M$ Gd$^{3+}$ (Fig. 6, C and G) compared to the control (Fig. 6, A and E), respectively. The removal of Gd$^{3+}$ from the bath solution resulted in restoration of the channel activity (Fig. 6, D and H).

Figure 7 presents results showing that the SA Ca$^{2+}$ channels are similarly regulated by actin dynamics compared to the whole-cell recording results as shown in Figure 2. Under the application of a 9-kPa positive pressure to the outside-out membrane patch at $-60$ mV, addition of $20\mu M$ CD dramatically increased the activity of the SA Ca$^{2+}$ channels (Fig. 7, B and G versus A and F; $n = 7$), suggesting that CD-induced depolymerization of actin filaments enhanced the sensitivity of the channels to the stretch. Not surprisingly, the presence of $100\mu M$ phalloidin in the pipette solutions significantly impaired the CD’s stimulatory effects on channel activation (Fig. 7, C–E, H, and J).

**SA of the Ca$^{2+}$-Permeable Channels Are Regulated by Actin Dynamics**

As described above, the SA Ca$^{2+}$ channels recorded at the single-channel levels shared similar electrophysiological properties with the osmo-sensitive whole-cell inward currents. Figure 7 presents results showing that the SA Ca$^{2+}$ channels are similarly regulated by actin dynamics compared to the whole-cell recording results as shown in Figure 2. Under the application of a 9-kPa positive pressure to the outside-out membrane patch at $-60$ mV, addition of $20\mu M$ CD dramatically increased the activity of the SA Ca$^{2+}$ channels (Fig. 7, B and G versus A and F; $n = 7$), suggesting that CD-induced depolymerization of actin filaments enhanced the sensitivity of the channels to the stretch. Not surprisingly, the presence of $100\mu M$ phalloidin in the pipette solutions significantly impaired the CD’s stimulatory effects on channel activation (Fig. 7, C–E, H, and J).

**Osmo Regulation of the SA Ca$^{2+}$ Channels under the Cell-Attached Configuration**

Osmoregulation of the SA Ca$^{2+}$ channels was also investigated under the cell-attached configuration. As shown in Figure 8, channel activity was activated by...
would regulate the $[\text{Ca}^{2+}]_{\text{cyt}}$ in guard cells. Most of the tested protoplasts (approximately 80%) were loaded successfully with Fluo 3-AM and the ones with relative stronger fluorescence were chosen for further experiments. Under control conditions (incubation solutions containing 0.1% dimethyl sulfoxide as solvent for CD), the fluorescence of the protoplasts remained at a stable level during a 60-min observation (Fig. 9A). Addition of 20 μM CD in the incubation solution significantly increased the fluorescence intensity after 20 min (Fig. 9B), while addition of 100 μM Gd3⁺ impaired the CD-induced increase of fluorescence (Fig. 9C). The results indicate that CD-induced depolymerization of actin filaments may elevate $[\text{Ca}^{2+}]_{\text{cyt}}$ by stimulating Ca²⁺ influx through Ca²⁺ channels in the PM. Similarly, hypertonic treatment (400 mosmol compared to 500 mosmol as the control) increased the fluorescence intensity in guard cell cytoplasm (Fig. 9D), while this hypertonicity-induced increase of fluorescence intensity was blocked by 100 μM Gd3⁺ (Fig. 9E). In addition, a hypotonic treatment (600 mosmol compared to 500 mosmol as the control) did not significantly affect the fluorescence intensity (Fig. 9F), while subsequent hypotonic treatment significantly increased the fluorescence intensity (Fig. 9F). The addition of 20 μM CD significantly increased the fluorescence intensity even under the hypertonic condition (Fig. 9G). Figure 9H shows the time kinetics of the changes in the relative fluorescence intensity in the guard cell protoplasts under the various treatments, as indicated in the legends of Figure 9. The results of $[\text{Ca}^{2+}]_{\text{cyt}}$ imaging presented in Figure 9 correlate well with those from the patch-clamping experiments, and both results lead to the same conclusion that actin dynamics mediates osmotic regulation of Ca²⁺ channel-facilitated Ca²⁺ influx across the PM of guard cells.

**DISCUSSION**

Stomatal movements are controlled by turgor-driven guard cell volume changes. Voltage-dependent K⁺ channels have been implicated critically in these processes by serving as osmotic-sensing targets in guard cells for a positive feedback loop (Liu and Luan, 1998). In this study, we report SA Ca²⁺ channels in the PM of *V. faba* guard cells, which are activated by mechanical stretch applied to the PM either by changing osmotic concentration of the bath solutions or by directly exerting a pressure to the PM via micropipettes. More importantly, the observed osmo regulation of the channels is mediated by actin dynamics. Depolymerization of actin filaments results in activation of the channels, whereas the inhibition of actin depolymerization blocked the activation of Ca²⁺-permeable channels.

Compared to the Ca²⁺-permeable channels in *V. faba* guard cells reported by Hamilton et al. (2000), the channels shown in this study may share similar characteristics, although it can be only clarified by further

**Actin Dynamics Regulates $[\text{Ca}^{2+}]_{\text{cyt}}$ in Guard Cell Protoplasts**

Fluo 3-AM, a fluorescent calcium indicator, was employed to test if actin dynamics or osmotic change was involved in $[\text{Ca}^{2+}]_{\text{cyt}}$ regulation. The results of the protoplasts treated with Fluo 3-AM imaging showed that actin depolymerization blocked the activation of Ca²⁺ channels. The actin filaments were depolymerized by the addition of the actin depolymerization agent cytochalasin D (CD), which increased the fluorescence intensity in guard cell cytoplasm (Fig. 9A). Addition of 20 μM CD significantly increased the fluorescence intensity after 20 min (Fig. 9B), while addition of 100 μM Gd3⁺ impaired the CD-induced increase of fluorescence (Fig. 9C). Therefore, the results indicate that CD-induced depolymerization of actin filaments may elevate $[\text{Ca}^{2+}]_{\text{cyt}}$ by stimulating Ca²⁺ influx through Ca²⁺ channels in the PM. Similarly, hypertonic treatment (400 mosmol compared to 500 mosmol as the control) increased the fluorescence intensity in guard cell cytoplasm (Fig. 9D), while this hypertonicity-induced increase of fluorescence intensity was blocked by 100 μM Gd3⁺ (Fig. 9E). In addition, a hypotonic treatment (600 mosmol compared to 500 mosmol as the control) did not significantly affect the fluorescence intensity (Fig. 9F), while subsequent hypotonic treatment significantly increased the fluorescence intensity (Fig. 9F). The addition of 20 μM CD significantly increased the fluorescence intensity even under the hypertonic condition (Fig. 9G). Figure 9H shows the time kinetics of the changes in the relative fluorescence intensity in the guard cell protoplasts under the various treatments, as indicated in the legends of Figure 9. The results of $[\text{Ca}^{2+}]_{\text{cyt}}$ imaging presented in Figure 9 correlate well with those from the patch-clamping experiments, and both results lead to the same conclusion that actin dynamics mediates osmotic regulation of Ca²⁺ channel-facilitated Ca²⁺ influx across the PM of guard cells.

**Figure 5.** Single-channel conductance and reversal potential of the SA channels are dependent on extracellular Ba²⁺ concentration. Control solutions were used except that various concentrations of Ba²⁺ were added to the bath solutions. Data in both A and B are presented as mean ± se (n = 8). A, The relationship of single-channel conductance versus extracellular Ba²⁺ concentration derived from the single-channel recordings at -60 mV and 9 kPa positive pressure applied to the outside-out membrane patches. The curve is well fitted by the Michaelis-Menten equation. B, Comparison of the measured reversal potentials (black circles) with the theoretical equilibrium potentials (white circles) calculated by Nernst equation.
channel molecular identification, given that the experimental conditions were similar in two independent studies in spite of some minor differences. First, the channels in both studies are hyperpolarization activated. Second, although Hamilton et al. (2000) did not mention osmo regulation of the channels, there was an osmolality difference (about 40 mosmol) across the membrane (200 mosmol and 240 mosmol for the bath and pipette solutions, respectively) and both bath and pipette solutions had rather low osmolality in their experiments. Thus, it may be hypothesized that the channel activity shown in their study might be also osmo regulated. Third, the current amplitudes of the channels in the study by Hamilton et al. (2000) are much greater than that we show in this study, possibly due to different osmolality of solutions and other recording conditions.

**SA Ca\(^{2+}\) Channels May Function as an Osmotic Signal Transducer in Stomatal Guard Cells in Vivo**

SA channels or mechano-sensitive channels have been reported in a wide variety of species from bacteria and yeasts to animals and plants (Morris, 1990; Garrill et al., 1996; Ramahaleo et al., 1996; Sachs, 1997; Hamill and Martinac, 2001). SA Ca\(^{2+}\) channels, as signal transducers, activated by mechanical stimuli, including stretch or osmotic swelling imposed on the PM, have been implicated in a number of physiological processes, such as cell movement, cell volume and turgor regulation (Pickard and Ding, 1993), and gravitropism (White and Broadley, 2003). The voltage-dependent Ca\(^{2+}\)-permeable channels in the PM of *V. faba* guard cells identified in this study are activated by stretch forces applied to the PM of guard cells. The pressures applied to the isolated membrane patches in our experiments may be much lower than the turgor pressure present in stomatal guard cells in vivo. A turgor pressure between 3 and 4 MPa has been suggested to be common for guard cells surrounding an open stoma (Franks, 2003). As demonstrated in yeast (*Saccharomyces cerevisiae*) and animal cells, SA channels are membrane tension dependent rather than pressure dependent (Gustin et al., 1988). According to Laplace’s law, \(T = Pd/4\) (where \(T\) is tension on the membrane, \(P\) is applied pressure, and \(d\) is cell diameter), the larger the cell, the greater the sensitivity to osmotic pressure changes (Gustin et al., 1988). In most of our patch-clamping experiments (Figs. 4–6 and 7), a 9-kPa positive pressure was applied to the membrane patches to activate SA Ca\(^{2+}\) channels. Such a pressure gives a tension force at 7.5 relative units on the membrane patches, assuming that the membrane bleb formed at the tip of a micropipette averages approximately 2 µm

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**Figure 6.** Reversible inhibition of the SA channel activity by Ca\(^{2+}\) channel blocker Gd\(^{3+}\). A to D, Current traces recorded from the same outside-out membrane patch in the presence of a 9-kPa positive pressure at −60 mV with addition of different concentrations of Gd\(^{3+}\) in the bath solutions as indicated. Different concentrations of Gd\(^{3+}\) were added to (B and C) or washed out (D) from the control bath solutions. Dotted lines indicate the state of the channels and the letters c or o stand for the closed or open state, respectively. E to H, NPo charts derived from the corresponding recordings of the SA Ca\(^{2+}\) currents as shown in A to D, respectively.
in diameter. Given that an average diameter of Vicia guard cell protoplasts is approximately 15 μm, 4 MPa turgor pressure in a guard cell is equivalent to a tension of 10,000 units on the PM of the guard cell according to Laplace’s law. Although most of the turgor pressure-generated tension force is borne by the cell wall (Cosgrove and Hedrich, 1991) and the actual membrane tension in guard cells is much lower than this, it is reasonable to believe that such turgor pressure-induced membrane tension is sufficient to activate SA Ca\(^{2+}\) channels in the PM of guard cells in vivo.

**Potential Roles of the Osmo-Regulated Ca\(^{2+}\) Channels in Regulation of Stomatal Movements**

A K\(^{+}\) channel-based positive feedback model has been proposed to explain the osmo-regulation mechanisms for stomatal movements (Liu and Luan, 1998). In this model, during the initial opening of illuminated stomata, the H\(^{+}\) pump in the PM of guard cells is activated, resulting in a more negative membrane potential that activates the inward K\(^{+}\) currents (\(I_{\text{Kin}}\)), and K\(^{+}\) influx takes place accompanied by water influx, making guard cell swell. Cell swelling further activates \(I_{\text{Kin}}\) and therefore accelerates K\(^{+}\) and water influxes and consequently stimulates stomatal opening (Liu and Luan, 1998). One may question for this osmo-regulation model what would stop continuous swelling of guard cells. The results presented in this study suggest that the osmo-sensitive inward Ca\(^{2+}\) channels may mediate Ca\(^{2+}\) influx and subsequent [Ca\(^{2+}\)]\(_{\text{cyt}}\) elevation in response to turgor pressure-induced membrane tension and consequently inhibit further swelling of guard cells by inhibiting K\(^{+}\) influx (Assmann, 1993; Ward et al., 1995) and activating Cl\(^{-}\) efflux (Schroeder et al., 2001). Thus, this model provides further explanation for how the stomatal movements are finely controlled by the internal mechanisms.

It is known that cytoplasmic Ca\(^{2+}\) elevation may inhibit \(I_{\text{Kin}}\) and stimulate slow anion channels (such as...
Cl\textsuperscript{−} channels), thus contributing to regulation of stomatal movement (for review, see Schroeder et al., 2001). Therefore, the transporters for controlling \([\text{Ca}^{2+}]_{\text{cyt}}\) may play important roles during stomatal movement. SA \text{Ca}^{2+} channels have been reported previously in the PM of *V. faba* guard cells (Cosgrove and Hedrich, 1991), although their role had not been well defined at that time. As discussed earlier, these SA \text{Ca}^{2+} channels may operate in vivo and thus play roles in regulation of stomatal movement. In this study, we present an osmosensing negative feedback mechanism mediated by \([\text{Ca}^{2+}]_{\text{cyt}}\). During the initial stage of stomatal opening, the SA \text{Ca}^{2+} channels are activated by cell swelling that is caused by the influxes of K\textsuperscript{+} and water. As a result, \([\text{Ca}^{2+}]_{\text{cyt}}\) tends to be elevated, thereby inhibiting the H\textsuperscript{+} pump (Kinoshita et al., 1995) and \(I_{\text{Kin}}\), which in turn decreases cell turgor. On the other hand, elevated \([\text{Ca}^{2+}]_{\text{cyt}}\) disrupts the actin filaments (Hwang and Lee, 2001), thus in turn enhancing the sensitivity of the SA \text{Ca}^{2+} channels to swelling (Glogauer et al., 1995; this study) and accelerating \([\text{Ca}^{2+}]_{\text{cyt}}\) elevation. The interaction between disruption of actin filaments and elevation of \([\text{Ca}^{2+}]_{\text{cyt}}\) may form a negative loop to limit the sustained activation of \(I_{\text{Kin}}\) and cell swelling. During the initiation of stomatal closure induced by darkness, SA \text{Ca}^{2+} channels may be activated by the existing cell turgor, contributing to regulation of increase or oscillation of \([\text{Ca}^{2+}]_{\text{cyt}}\), which initiates the signaling events for stomatal closure (McAinsh et al., 1995; Allen et al., 2000, 2001). The activation of slow anion channels and inactivation of \(I_{\text{Kin}}\) by the increase of \([\text{Ca}^{2+}]_{\text{cyt}}\), together with depolarization-driven K\textsuperscript{+} efflux, cause cell shrinking, which in turn inactivates SA \text{Ca}^{2+} channels. Therefore, SA \text{Ca}^{2+} channels may be also actively functioning during stomatal closure. The possible complex interaction between the positive and negative feedback mechanisms by which a number of ion transporters are regulated may explain stomatal oscillation under variable environmental conditions (Cowan et al., 1997).

Liu and Luan (1998) reported that hypotonic or hypertonic condition caused gradual swelling or
shrinking of Vicia guard cell protoplasts, respectively, but both swelling and shrinking of the cells saturated during the given time period. According to the K\textsuperscript{+}-channel-based positive feedback model (Liu and Luan, 1998), the protoplasts will continuously swell at hypotonic condition or shrink at hypertonic condition. The swelling or shrinking of stomatal guard cells may be compromised by increased or decreased [Ca\textsuperscript{2+}]\textsubscript{cyt} as explained by the osmo-sensing negative feedback mechanism mediated by [Ca\textsuperscript{2+}]\textsubscript{cyt} presented in this study.

Actin Cytoskeleton May Serve as an Osmo Sensor for Regulation of Ca\textsuperscript{2+} Channels in Guard Cells

As discussed by Liu and Luan (1998), the actin cytoskeleton may serve as an osmo sensor and target osmo-sensitive K\textsuperscript{+} channels in guard cells. This study shows that the actin cytoskeleton may also transduce osmotic signals to SA Ca\textsuperscript{2+} channels under hypotonic conditions (induced, e.g., by high humidity or high water potential in vivo). On the other hand, we also show that the actin cytoskeleton may act as a stress sensor for regulation of SA Ca\textsuperscript{2+} channels in the PM under hypertonic stress that may occur under drought or low-humidity conditions (for review, see Raschke, 1975; Zeiger, 1983; Grantz, 1990). Water stress-induced ABA triggers an elevation or oscillation in [Ca\textsuperscript{2+}]\textsubscript{cyt} in guard cells that is critical for maintenance of stomatal closure (for review, see Schroeder et al., 2001). Ca\textsuperscript{2+} influx through Ca\textsuperscript{2+} channels across the PM has been proposed to be involved in water stress- and ABA-induced signaling of stomatal closure (Hamilton et al., 2000; Pei et al., 2000). H\textsubscript{2}O\textsubscript{2} and protein phosphorylation were implicated to mediate ABA signaling in regulating Ca\textsuperscript{2+} channels in the PM (Hamilton et al., 2000; Pei et al., 2000; Köhler and Blatt, 2002). External ABA or Ca\textsuperscript{2+} induces a disintegration of actin filaments in guard cells (Eun and Lee, 1997; Eun et al., 2001; Hwang and Lee, 2001; Lemichez et al., 2001). ABA-induced
actin disruption in guard cells was demonstrated to be mediated by \([\text{Ca}^{2+}]_{\text{cyt}}\) and by protein phosphorylation and dephosphorylation (Hwang and Lee, 2001). In pollen tubes, tip F-actin and tip-focused calcium gradients oscillate in the opposite phase (Fu et al., 2001), indicating that high \([\text{Ca}^{2+}]_{\text{cyt}}\) might play a vital role in regulating actin dynamics and vice versa. Moreover, a study has shown that actin disruption stimulates \(\text{Ca}^{2+}\) channels in fibroblasts (Glogauer et al., 1995). In this study, we demonstrate that actin dynamics regulates \([\text{Ca}^{2+}]_{\text{cyt}}\) by modulating SA \(\text{Ca}^{2+}\) channels in the PM of guard cells. Therefore, the interplay between the dynamics of these two vital cellular components may be an important regulatory mechanism by which guard cells sense environmental changes and regulate stomatal movement.

MATERIALS AND METHODS

Guard Cell Protoplast Preparation

\(\text{Vicia faba}\) plants were grown in soil mixture in a growth chamber under 12-h light (100 \(\mu\)mol \(m^{-2}\) \(s^{-1}\)) and 12-h dark cycle and temperatures of 22°C and 15°C for daylight and night, respectively. Plants were watered twice a week with tap water and relative humidity was kept at approximately 50%. Guard cell protoplasts were prepared by an enzymatic method as described previously (Wang et al., 1998), with slight modification. Briefly, abaxial epidermis were peeled off from fully expanded leaves of 3- to 4-week-old plants. Mesophyll cells were brushed off from the epidermal strips in distilled water, then the epidermis was transferred to enzyme solution I containing 0.7% (w/v) cellulysin (Calbiochem), 0.1% (w/v) polyvinylpyrrolidone-40, 0.3% (w/v) bovine serum albumin, and 0.5 mM ascorbic acid (freshly added from stock solution) and incubated in a rotary water bath at 21°C with rotation speed at 160 rpm for 30 min. The partially digested epidermal strips were thoroughly washed with the basic solution on 220-µm nylon mesh and transferred to the enzyme solution II (basic solution plus 1.5% [w/v] cellulase RS [Yakult], 0.3% [w/v] bovine serum albumin, 0.2% [w/v] Pectolyase Y-23 [Seishin Pharmaceutical], and 0.5 mM ascorbic acid, pH 5.5) and incubated in a rotary water bath at 21°C with rotation speed at 60 rpm for 40 min. Released protoplasts were collected and washed twice by filtration and centrifugation at 800 rpm for 5 min. The isolated protoplasts were resuspended in the basic solution and kept on ice in the dark for at least 1 h before use for patch-clamping and \([\text{Ca}^{2+}]_{\text{cyt}}\) measurements.

Patch-Clamping Recordings and Data Analysis

Standard whole-cell and single-channel recording techniques were applied in this study. All experiments were conducted at room temperature (approximately 22°C) under dim light. Glass micropipettes were made by using a two-step puller (model PP-83, Narishige) and fire polished by a microforge (model MF-83, Narishige). For the control conditions of both whole-cell and outside-out single-channel recordings, the bath solutions contained 50 mM BaCl\(_2\), 0.1 mM diithiothreitol, 10 mM MES/Tris, pH 5.6, and the osmolality was adjusted to 500 mosmol with sorbitol. The pipette solutions were composed of 10 mM BaCl\(_2\), 0.1 mM diithiothreitol, 2 mM 1,2-bis(2-aminoophenoxy)ethane-N,N,N',N'-tetraacetic acid, and 10 mM HEPES/Tris, pH 7.1, and the osmolality was adjusted to 500 mosmol with sorbitol. For some specific treatments, BaCl\(_2\) was replaced with CaCl\(_2\), or some reagents (such as GdCl\(_3\), Cd, phalloidin, etc.) were added into the solutions, or the osmolality of the solutions was adjusted. All changes made with solution composition or osmolality are indicated in the text or in figure legends.

For the whole-cell recordings, the seal resistance between the membrane and the micropipette was greater than 2 GΩ in all experiments. Membrane potential was clamped to 0 mV, and current data were acquired when the voltages were clamped from −100 mV to 40 mV using a voltage-ramp method at a speed of 0.014 mV/ms or 1 mV/ms, as indicated in figure legends, at 5 min after obtaining whole-cell configuration. Data were filtered at 1 kHz (1 ms/sample) before storage onto the disc of the computer. For the single-channel recordings from the outside-out membrane patches, the bath and pipette solutions were the same as those for the whole-cell recording experiments. For the cell-attached recordings, the pipettes were filled with the bath solution used for the whole-cell recording experiments. The seal resistance was no less than 10 GΩ for all excised-patch and cell-attached recordings. In the recordings from the outside-out membrane patches, the SA \(\text{Ca}^{2+}\) channels were activated by a positive pressure applied to the interior of a glass pipette. For the cell-attached recordings, SA \(\text{Ca}^{2+}\) channels were activated by a negative pressure (suction) applied to the interior of the glass pipettes. The strength of blow or suction was monitored by a barometer connected to the micropipette buffered via a water column. The data of single-channel recordings were analyzed with Fetchan software, and then, Simplex-LSQ fitting method and Gaussian fitting included in Pstal software were applied. After being fitted with graphic seeds, the area of the column was calculated for further calculation of channel \(N_P\). Because most of the tested membrane patches showed multiple channels, \(N_P\) were expressed as \(N_P\), where \(N\) represents the number of channels existing in the membrane patches and \(P\) represents the open probability of a single channel. The \(N_P\) values were calculated using the equation \(N_P = [A_0 + A_1 + A_1 + A_1 + \ldots + A_n]/[A_0 + A_1 + A_1 + A_1 + \ldots + A_n]\), as described previously (López-López et al., 1998). Patch-clamp recordings were performed using an Axopatch-200B amplifier (Axon Instruments) connected to a microcomputer via an interface (TL-1 DMA Interface, Axon Instruments). pClAMP software (Version 6.0.4, Axon Instruments) was used to acquire and analyze the whole-cell and single-channel currents. SigmaPlot software was used to draw I-V plots and data analysis.

Cytosolic Calcium Measurements

A \(\text{Ca}^{2+}\) fluorescent dye, Fluo 3-AM, was used to monitor changes in relative \([\text{Ca}^{2+}]_{\text{cyt}}\) in guard cell protoplasts. Protoplasts were isolated as described above and then incubated in a solution containing 10 µM Fluo 3-AM, 0.2% [w/v] pluronic F-127 (freshly added from 1 mM stock solution), 5 mM MES, pH 5.0 (adjusted with Tris), with osmolality adjusted to 500 mosmol with sorbitol, and incubated in the dark for 1.5 h at 4°C. This incubation resulted in 80% of the protoplasts being successfully loaded with Fluo3. The preincubated protoplasts were washed with and kept in a solution containing 10 mM MES, pH 6.0, 50 mM KCl, 1 mM CaCl\(_2\) with osmolality adjusted to 500 mosmol with sorbitol. Fluo3 fluorescence was imaged under a laser scanning confocal microscope (Bio-Rad, MRC-1024, equipped with Krypton/Argon laser light). The wavelengths of excitation and emission light were 488 nm and 515 nm, respectively. Three-dimensional scanning was applied with 1-µm Z-series project steps in 2-min cycles, and the three-dimensional reconstruction was used to display the variations of cytosolic calcium, as shown in Figure 9.

Chemicals

All chemicals were obtained from Sigma unless otherwise indicated in the text.

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LITERATURE CITED


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