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Voltage-Dependent K^+ Channels as Targets of Osmosensing in Guard Cells

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Guard cell turgor responds to the osmogradient across the plasma membrane and controls the stomatal aperture. Here, we report that guard cells utilize voltage-dependent K^+ channels as targets of the osmosensing pathway, providing a positive feedback mechanism for stomatal regulation. When exposed to a hypotonic condition, the inward K^+ current (I_{Kin}) was highly activated, whereas the outward K^+ current (I_{Kout}) was inactivated. In contrast, hypertonic conditions inactivated the I_{Kin} while activating I_{Kout}. Single-channel recording analyses indicated that an alteration in channel opening frequency was responsible for regulating I_{Kin} and I_{Kout} under different osmotic conditions. Further studies correlate osmoregulation of I_{Kin} with the pattern of organization of actin filaments, which may be a critical component in the osmosensing pathway in plant cells.

INTRODUCTION

A stoma consists of a pair of guard cells that integrate environmental and internal signals into an appropriate stomatal aperture that allows CO_2 influx for photosynthesis and limits water loss (Raschke, 1975; Zeiger, 1983; Zeiger et al., 1987). Regulation of stomatal aperture is a dynamic process that involves myriad signals and signal transmission pathways. The signals, including light, CO_2, humidity, and some plant hormones, target guard cell turgor, which is controlled by a number of ion pumps and channels (Assmann, 1993; Ward et al., 1995; Maathuis et al., 1997). Because guard cells have a unique cell wall structure that allows rapid swelling and shrinking, changes in cell turgor are often rapidly translated into changes in cell volume and stomatal aperture. Inward and outward rectifying K^+ channels carrying K^+ ions across the membrane play a critical role in turgor regulation of guard cells. Signals such as light and abscisic acid (ABA) are known to regulate stomatal movements through signaling pathways that directly or indirectly regulate K^+ channels (Assmann, 1993; Maathuis et al., 1997; MacRobbie, 1997).

Of all the signals to which the stomatal aperture responds, osmotic stress is unique in that both the initial stimulus and the final response are mediated by water; thus, it is considered to be a feedback regulation (reviewed in Grantz, 1990; Mansfield, 1990; Assmann, 1993). Studies have shown that stomatal closure in response to osmotic stress (such as low humidity or drought) consists of hydroactive (metabolic) and hydropassive (hydraulic) components (Grantz and Schwartz, 1988). The hydroactive component involves fluxes of osmolytes, especially K^+, across the cell membranes. The hydropassive process is mediated only by changes in external water potential, with the guard cells acting as osmometers (reviewed in Raschke, 1975). Although production of ABA plays an important role in the hydroactive process, evidence suggests the presence of an ABA-independent hydroactive pathway that involves K^+ fluxes (Raschke, 1975; Grantz, 1990; Assmann, 1993). MacRobbie (1995) provides evidence that guard cells can "sense" their osmotic condition (solute content) and hydroactively regulate cell volume. Elevation of ABA levels upon osmotic stress may reset this internal regulatory system. In other words, the ABA-dependent and ABA-independent pathways may work together to regulate guard cell turgor in response to osmotic stress conditions. Most previous studies have been directed to the ABA-dependent signaling pathway (Schwartz et al., 1994; MacRobbie, 1995, 1997; Blatt and Grabov, 1997; Esser et al., 1997; Pei et al., 1997), but little is known about the nature of the ABA-independent osmosensing pathway for stomatal regulation.

Changes in guard cell turgor can be translated into a secondary mechanical signal imposed on the membrane—a membrane-stretching force caused by shrinking or swelling of the cell. Two classes of mechanosensitive channels have been identified in animal cells. One is the stretch-activated channel (SAC), which is operated by the application of pressure but not by membrane potential or voltage (Kullberg, 1987; Morris, 1990; Sachs, 1991). The other class consists of osmosensitive, voltage-dependent channels. These voltage-gated channels are strongly regulated by extracellular osmolarity and

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play a critical role in volume regulation of cells under osmotic stress (Morris, 1990; Gosling et al., 1995; Schoenmakers et al., 1995; Moran et al., 1996; Baraban et al., 1997; Cossins and Gibson, 1997; Sachs, 1997). Although SACs have been found in higher plant cells (Cosgrove and Hedrich, 1991; Pickard and Ding, 1993; Ramahaleo et al., 1996), there is no evidence that plant cells contain osmosensitive, voltage-dependent channels.

In guard cells, SACs have been identified in both the plasma membrane and tonoplast (Alexandre and Lassalles, 1991; Cosgrove and Hedrich, 1991). However, the physiological significance of these channels in stomatal regulation is unclear. In this study, we found that voltage-dependent rectifying K^+ channels are regulated by the osmogradient across the plasma membrane of guard cells. This finding reveals a mechanism for an ABA-independent osmosensing pathway and a positive feedback loop that accelerates stomatal movements.

RESULTS

Osmosensitive, Voltage-Dependent K^+ Currents in Guard Cells

In most of the patch–clamp procedures previously described, the osmolarity of the pipette solution is always slightly higher than that in the bath solution. This small "outward" osmogradient (10%) facilitates the formation of a high seal between the plasma membrane and the pipette (Schroeder, 1988). Under such osmotic conditions (450 milliosmoles [mOsM] in the bath and 500 mOsM in the pipette), we consistently observed an increase in the inward K^+ current (I_{Kin}) and a decrease in the outward K^+ current (I_{Kout}) after the whole-cell configuration was established. Typically, a guard cell protoplast would have a large I_{Kin} and a small I_{Kout} under such conditions (with a holding potential from −180 to 80 mV). This phenomenon is generally accepted as a change related to the "stabilization" of the patch–clamp recording, and its possible physiological significance has been neglected.

In conjunction with our interest in osmosensing in guard cells, the above-mentioned observation prompted the idea that I_{Kin} and I_{Kout} in guard cells may be regulated by the osmogradient between the cell and the bath solution, a state that mimics the osmotic stress conditions occurring in guard cells in their natural setting.

To test this idea, we kept the osmolarity of the bath solution at 500 mOsM and created both an inward and an outward osmogradient by changing the osmolarity in the pipette solution. With the bath solution at 500 mOsM, an inward osmogradient was formed by using a pipette solution of 450 mOsM, whereas an outward osmogradient was formed by using a pipette solution of 550 mOsM. As shown in Figure 1A, the patterns of I_{Kin} and I_{Kout} differed dramatically under opposite osmogradients. When exposed to an inward osmogradient (hypertonic condition), the cells produced a large I_{Kout} and small I_{Kin}. In contrast, an outward osmogradient (hypotonic condition) produced a large I_{Kin} and small I_{Kout} (Figure 1A). Comparing the channel activities under the two conditions, I_{Kin} decreased from −559.4 ± 55.4 pA per cell (hypotonic, $n = 7$) to −159.1 ± 21.7 pA per cell (hypertonic, $n = 10$) at −180 mV. In contrast, I_{Kout} increased from 64.1 ± 6.5 pA per cell ($n = 9$) to 232.2 ± 33 pA per cell ($n = 9$) at 80 mV.

To confirm that this change was induced by an osmogradient and not by intracellular changes resulting from different pipette solutions, we created similar osmogradients by adjusting the osmolarity in the bath solution while keeping the pipette solution constant (500 mOsM). As shown in Figure 1B, a hypotonic bath solution (400 mOsM) increased I_{Kin} by 294.4% ($n = 9$) compared with I_{Kin} in the hypertonic bath solution (550 mOsM, $n = 9$) at −180 mV. I_{Kout} was regulated in an opposing manner, with a 65.9% decrease in the hypotonic bath solution compared with that in the hypertonic solution (80 mV, $n = 9$).

These results show that I_{Kin} and I_{Kout} are sensitive to an osmogradient across the plasma membrane. Whereas I_{Kin} is activated in the hypotonic and inhibited in the hypertonic condition, I_{Kout} responds to the osmogradient in an opposite manner.

Osmogradients Modulate the Channel Opening Probability of I_{Kin} and I_{Kout}

To further investigate the osmosensing mechanism of K^+ channels, we studied the effects of osmogradients on single-channel activities. In both cell-attached and outside-out configurations, we found that the single-channel properties of I_{Kin} and I_{Kout}, including voltage dependence, reversal potential, and channel conductance, were similar to those reported previously (Ilan et al., 1995; Wu and Assmann, 1995). In the cell-attached configuration, a hypertonic solution in the bath significantly decreased the opening frequency of I_{Kin} but did not change other properties of the channel, which is consistent with the decrease in the whole-cell I_{Kin} current under the same osmotic conditions. As shown in Figure 2A, changing the extracellular osmolarity from 400 to 600 mOsM significantly reduced the opening probability of I_{Kin} (P_o, $n = 7$). This effect was only partially reversible when the bath solution was changed back to 400 mOsM (Figure 2A). Under the same configuration, I_{Kout} reduced its opening probability when the osmolarity in the bath solution was changed from 500 to 400 mOsM (Figure 3A).

Under the outside-out configuration ($n = 6$), osmolarity changes in the bath solutions did not affect the single-channel current of either I_{Kin} (Figure 2B) or I_{Kout} (Figure 3B). The conductance of I_{Kin} and I_{Kout} was not altered by osmotic conditions (Figures 2C and 3C). The conductance of I_{Kout} under the cell-attached configuration was smaller compared with

that under the outside-out configuration, because the pipette solutions contained different K^+ concentrations.

These results show that regulation of I_{Kin} and I_{Kout} by osmogradients is caused, at least in part, by the modulation of opening frequency of the channels, and this modulation requires cell integrity. Our findings suggest that osmoregulation of ion channels in guard cells involves changes in the cytoplasmic components rather than a simple hydraulic effect caused by an osmogradient across the membrane. This is not only supported by the loss of regulation in cell-free patches (Figures 2B and 3B) but is further argued by the results under the cell-attached configuration. Under such conditions, the membrane patch (attached to the pipette) is exposed to an osmogradient (cell versus pipette) that is opposite to that across the rest of the cell membrane (cell versus bath). But the regulation of channel activity follows the osmogradient between the cell and bath rather than the osmogradient across the small membrane patch (Figures 2A and 3A).

Osmoregulation of Guard Cell Volume

To examine cell volume change and its correlation with ion channel regulation, we monitored guard cell volume under both hypotonic and hypertonic conditions. As shown in Figure 4, a hypotonic bath solution caused rapid cell swelling that occurred immediately after the change of the bath solution and reached a plateau in 10 min. Guard cells maintained maximal volume, and no significant recovery occurred within 40 min, in contrast to mammalian cells, which recover rapidly from a swelling state (Moran et al., 1996). The net

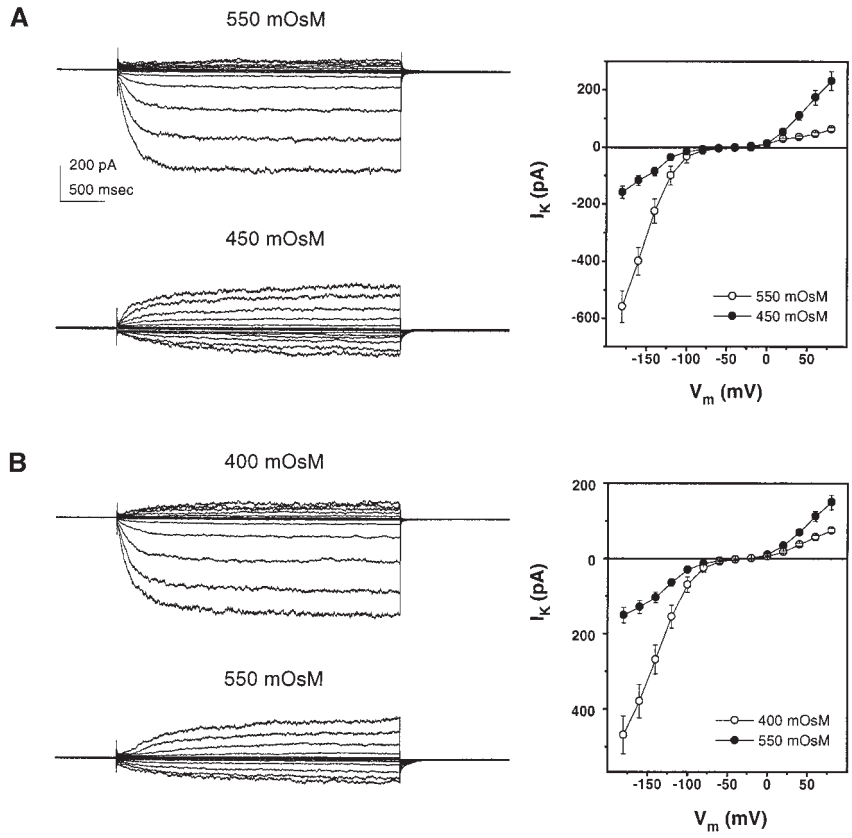

Figure 1. Osmogradients across the Plasma Membrane Regulate I_{Kin} and I_{Kout} in an Opposing Manner.

(A) Modulation of I_{Kin} and I_{Kout} (I_K) by changes in osmolarity of the pipette solution. Typical current traces of I_{Kin} and I_{Kout} were recorded when the osmolarity in the pipette solution was changed from 550 to 450 mOsM (the osmolarity in the bath solution was 500 mOsM). On the right side, the current–voltage (I–V) curves summarized the data from seven to 10 cells under each osmotic condition.

(B) Modulation of I_{Kin} and I_{Kout} by changes in osmolarity of the bath solution. The pattern of I_{Kin} and I_{Kout} was dramatically changed when cells were bathed in solutions of different osmolarity (400 and 550 mOsM). The osmolarity in the pipette solution was 500 mOsM. The I–V curves represent data compiled from nine cells under each osmotic condition. Error bars indicate ±SE.

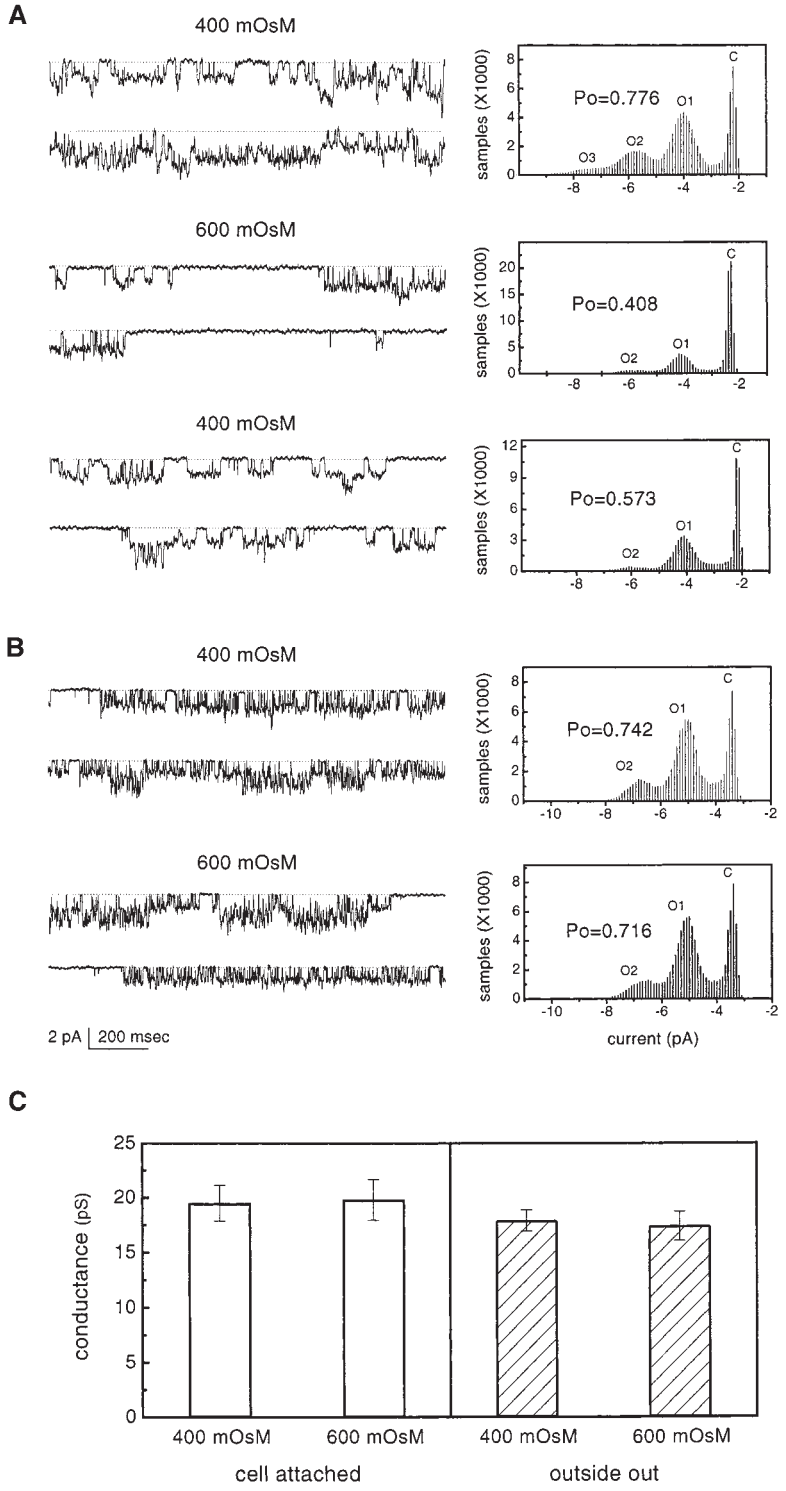

Figure 2. Single-Channel Current of I_{Kin} under Different Osmotic Conditions.

(A) Current traces and P_o charts under the cell-attached configuration. The single-channel currents were recorded in a bath solution with an osmolarity of 400 mOsM, which was perfused by a 600 mOsM solution and then switched back to 400 mOsM. The osmolarity in the pipette solution was 500 mOsM. The pipette potential applied was 120 mV. The P_o values were calculated from the ratio of total open time to total recording time.

volume increase reached 40% when cells were transferred from an isotonic (465 mOsM) to a hypotonic (400 mOsM) bath solution. When cells were transferred from an isotonic (465 mOsM) to a hypertonic (550 mOsM) bath solution, cell volume decreased by 27.5% during the first 20 min, and no further change took place during the next 20 min.

It is worth noting that the 40% volume increase for a 14% osmolarity change must involve a large change in solute content. As a major ion solute in the bath solution, K^+ may play an important role in the change of cellular solute. Indeed, when K^+ was replaced by Na^+, cell swelling was strongly inhibited, but hypertonic cell shrinking was not affected by this replacement. K^+ dependency of guard cell swelling suggested that the K^+ influx further increased the osmogradient and therefore the final cell volume. It appears that I_{Kin} was activated under the conditions used, which is consistent with the fact that the plasma membrane of plant cells normally retains a highly negative membrane potential (more than −100 mV) that is sufficient for activation of I_{Kin}. To the extent that K^+ is involved, there must be a balancing anion, and sugar may also be involved. More work is required to dissect the details of cell volume regulation by a given bath solution. The current experiment simply shows that osmotic conditions in the bath solution are rapidly converted into changes in guard cell volume.

Stretch-Activated K^+ Channels Are Not Responsible for Changes in I_{Kin} and I_{Kout}

SACs may be intimately involved in responses to osmotic shock because this type of channel is activated by a membrane stretch that could be induced by osmotic stress in both animal and plant cells (Sachs, 1991; Ramahaleo et al., 1996). The plasma membrane of guard cells also contains SACs (Cosgrove and Hedrich, 1991). To determine whether SAC activity may account for the osmosensitive regulation of K^+ currents, we recorded SAC single-channel currents in the outside-out patch configuration. Figure 5A shows that outward and inward SAC currents were activated by the application of negative pressure to the patch at 80 and −80 mV, respectively. When suction was held at 1 to 10 kPa, SAC currents were activated at various voltage steps (Figure 5B). Shown here are the inward SAC currents recorded at −80 to −120 mV and outward SAC currents recorded at +40 to +80 mV. This K^+ SAC exhibited different conductances for inward (55 ± 5 pS) and outward (95 ± 5 pS) currents, and its reversal potential (−5 mV) was close to E_K (0 mV; Figure 5C). Although the conductances differ from those reported previously (Cosgrove and Hedrich, 1991) due to differences in the solutions used, both studies show that outward SACs display larger conductance than do inward SACs.

Applying bath solutions with different osmolarities on outside-out patches did not significantly activate SACs. Only two of 11 patches showed transient activation of SACs when the patch was transferred from an isotonic (465 mOsM) to a hypotonic (400 mOsM) solution. In addition, no activation of SACs was observed under the cell-attached configuration when shifting the bath solution from hypotonic to hypertonic or vice versa (data not shown). The current–voltage (I–V) curves in Figure 5C show that negative pressure activates both inward and outward currents with conductances very different from those of voltage-dependent K^+ channels. Clearly, SAC currents differ from osmosensitive I_{Kin} and I_{Kout}.

Actin Filaments Are Involved in Osmosensing of I_{Kin} but Not I_{Kout}

As previously reported in animal cells, actin filament organization plays a role in the osmosensitivity of ion channels (Moran et al., 1996; Negulyaev et al., 1996; Tilly et al., 1996; Maximov et al., 1997; Xu et al., 1997; Yokoshiki et al., 1997). One study shows that in higher plants, cytoskeleton drugs modulate I_{Kin} in guard cells (Hwang et al., 1997). As shown in Figure 3, osmoregulation of I_{Kin} was observed only in the whole-cell or cell-attached configuration, implying that a "cellular context" was required for osmosensing in guard cells. We tested whether this cellular context reflects the organization of the cytoskeleton, as shown in animal cells.

When cytochalasin D (20 μM), a disrupter of the actin cytoskeleton, was perfused into the bath solution, the whole-cell current of I_{Kin} dramatically increased under hypertonic conditions (600 mOsM in the bath and 500 mOsM in the pipette; Figure 6A). The magnitude of I_{Kin} recovered to a level comparable to that under hypotonic conditions. However, cytochalasin D did not significantly affect the magnitude of I_{Kin} under hypotonic conditions (400 mOsM in the bath and 500 mOsM in the pipette; Figure 6B). The magnitude of I_{Kout} was not significantly affected by the presence of cytochalasin D

Figure 2. (continued).

(B) Single-channel traces recorded under outside-out configuration at −120 mV. The bath solution was switched from 400 to 600 mOsM by using the bath perfusion procedure with the same pipette solution as given in **(A)**. The P_o was not changed under this condition. The baseline of the channel currents in **(A)** and **(B)** is shown with dotted lines.

(C) Conductance of I_{Kin} was not significantly altered under all of the conditions described. Error bars indicate ±SE.

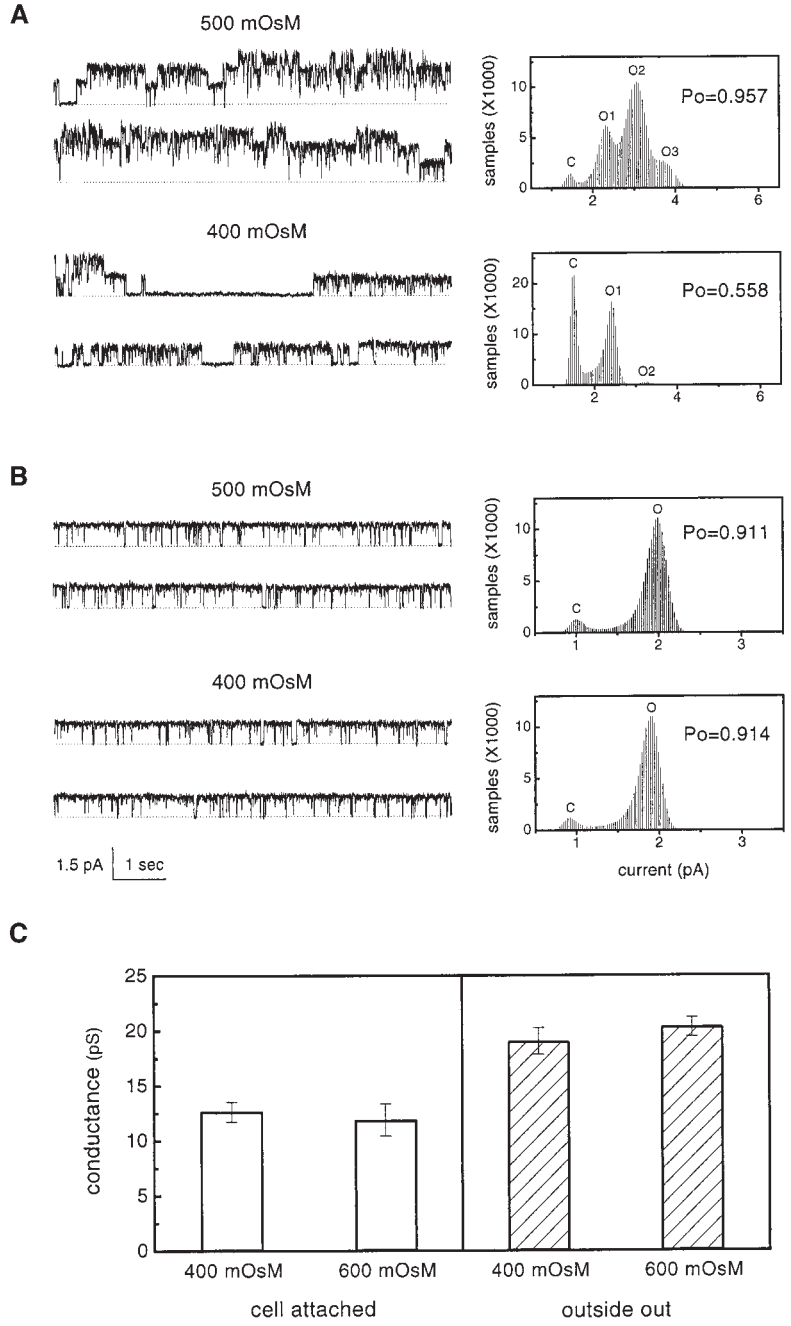

Figure 3. Single-Channel Current of I_{Kout} under Different Osmotic Conditions.

(A) Current traces and P_o charts under the cell-attached configuration. The single-channel currents were recorded in a bath solution with an osmolarity of 500 mOsM and 10 mM K^+, which was perfused by a 400 mOsM and 10 mM K^+ solution. The pipette solution had a 500 mOsM osmolarity and 10 mM K^+. The pipette potential applied was −80 mV. The P_o values were calculated from the ratio of total open time to total recording time.
(B) Current traces and P_o charts under the outside-out configuration at 60 mV. The bath solution was switched from 500 to 400 mOsM by using an external perfusion procedure. The K^+ concentration in the bath solution was 100 mM. The pipette solution had the same osmolarity as given in **(A)** but contained 100 mM K^+. The P_o value was not changed under these conditions. The baseline of the channel currents in **(A)** and **(B)** is shown with dotted lines.
(C) Conductance of I_{Kout} was not significantly altered under different osmotic conditions. Note the difference in conductance under the two configurations, which is caused by the difference in K^+ concentration in the pipette solutions described in **(A)** and **(B)**. Error bars indicate ±SE.

under hypotonic or hypertonic conditions ($P > 0.05$). Colchicine, a disrupter of microtubules, had no effect on I_{Kin} or I_{Kout} under all conditions described above (data not shown). These results show that hypertonic inhibition of I_{Kin} can be prevented by disrupting actin filaments, implicating the actin cytoskeleton in osmosensing of I_{Kin}.

Hypotonic Conditions Alter Actin Filament Organization

If actin filament organization plays a role in I_{Kin} regulation by osmotic stress, osmotic shock may change actin filament organization. To test this notion, we monitored the organization of actin filaments in guard cell protoplasts by staining with Oregon Green–conjugated phalloidin. By using this fluorescent label, we avoided the interference of chlorophyll autofluorescence from chloroplasts in guard cells. As shown in Figure 7, in cells bathed in a hypertonic solution (550 mOsM), filamentous actin appeared to be organized into a "network" that was distributed throughout the cell body (Figures 7A and 7B). Treatment with 20 μM cytochalasin D for 40 min disrupted the actin network, and the labeling was concentrated at the nucleus area (Figure 7C). When cells were bathed in a hypotonic solution (400 mOsM) for 40 min, their actin filament organization was similar to that of cytochalasin D–treated cells (Figure 7D) and differed from the actin "network" in cells under the hypertonic condition (Figures 7A and 7B). This experiment indicates that hypotonic conditions disrupted actin filaments as cytochalasin D did, providing a link between hypotonic activation of the inward K^+ channel and disruption of actin filaments.

Osmoregulation of Stomatal Aperture

To correlate the osmosensing of ion channels in guard cells with stomatal regulation, we examined the stomatal opening process in epidermal peels under different osmotic conditions. As shown in Figure 8A, a hypertonic solution (600 mOsM) significantly inhibited light-induced opening. After 2 hr of illumination, stomatal pores opened to 11.64 ± 0.69 μm in 600 mOsM compared with 17.69 ± 0.93 μm in 400 mOsM. The smaller stomatal aperture in the hypertonic solution may have resulted from a hydropassive (hydraulic) effect and/or the inhibition of I_{Kin} (a hydroactive effect).

As shown in Figure 6, hypertonic inhibition of I_{Kin} can be prevented by using cytochalasin D. If inhibition of stomatal opening under the hypertonic condition was a result of I_{Kin} inhibition, then cytochalasin D should overcome the inhibition and facilitate stomatal opening. On the other hand, a passive hydraulic effect would not be affected by cytochalasin D. As shown in Figure 8A, the addition of 20 μM cytochalasin D completely prevented the inhibitory effect of the hypertonic condition on the opening process. In addition, cytochalasin D induced a larger stomatal opening compared with that induced under the hypotonic condition. The addition of cytochalasin D to the hypotonic solution also had a stimulatory effect on the stomatal opening process (Figure 8A), which is consistent with a previous observation (Kim et al., 1995). These experiments suggest that I_{Kin} inhibition is a major component in hypertonic suppression of stomatal opening.

We further examined the effect of the hypertonic condition on induction of stomatal closure. After 2 hr of illumination to open the stomata, epidermal strips were incubated in hypotonic or hypertonic solutions under light. As shown in Figure 8B, the hypertonic condition (600 mOsM) reduced the stomatal aperture within 5 min. This was followed by a gradual recovery of the stomatal aperture to the control level within 2 hr. The addition of 20 μM cytochalasin D significantly increased stomatal aperture under both hypertonic and hypotonic conditions (400 mOsM), although it began to be effective 15 min after incubation. The delay in the effect of cytochalasin D may have resulted from the time required for cytochalasin D to penetrate the cells and reach an effective concentration. Interestingly, stomatal aperature under cytochalasin D treatment gradually reduced to control levels at 120 min, suggesting that cells may have recovered from actin filament disruption.

A rapid decline in stomatal aperture may suggest a hydraulic effect associated with water flow out of the cell. Cytochalasin D reversal of the hypertonic effect indicated a

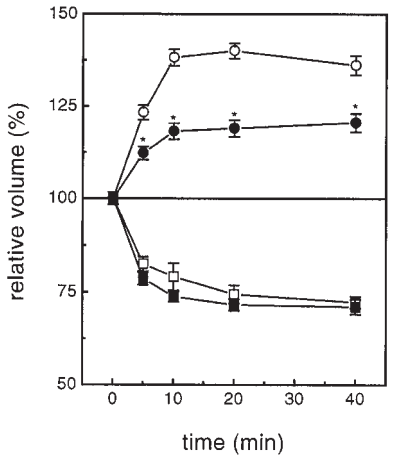

Figure 4. Osmoregulated Changes in Guard Cell Volume.

Taking the volume of guard cells in the isotonic solution (465 mOsM) as the control (100%), cell volume, measured at various time points after switching to different osmotic solutions, is presented as the percentage of the control. Open circles, volume at 400 mOsM in the presence of 10 mM K–glutamate; filled circles, volume at 400 mOsM with 10 mM Na–glutamate; open squares, volume at 550 mOsM with 10 K–glutamate; filled squares, volume at 550 mOsM with 10 mM Na–glutamate. The asterisks indicate significant differences in cell volume in Na-containing versus K-containing solutions. Error bars indicate ±SE.

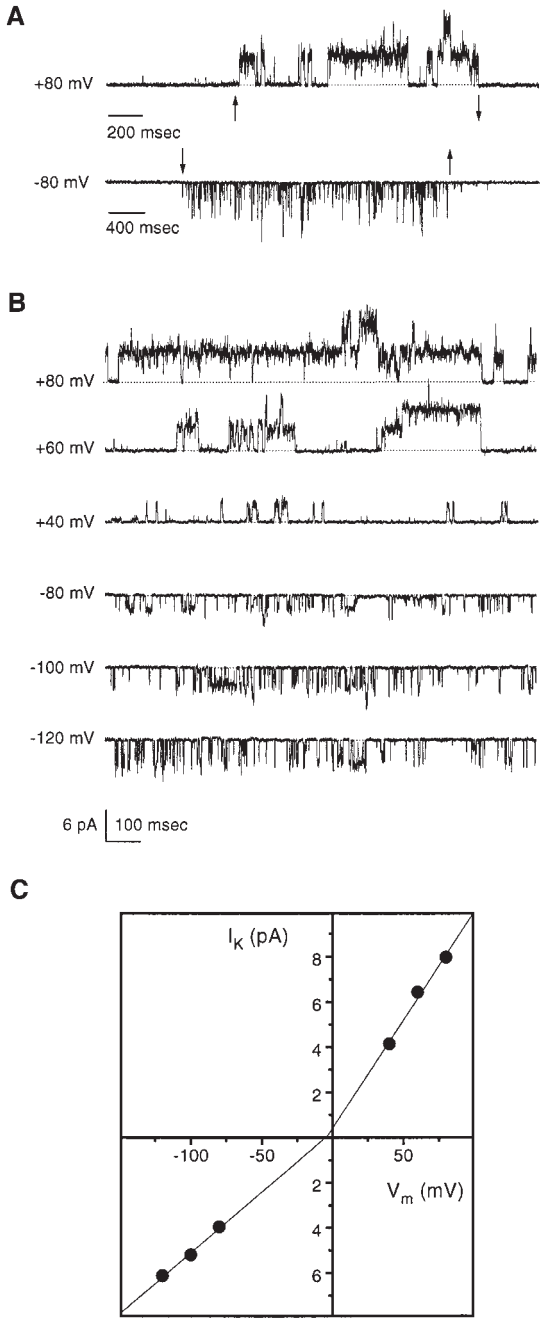

Figure 5. Stretch-Activated K^+ Channels in the Plasma Membrane of Guard Cells.

(A) Suction application activated both outward and inward SACs in an outside-out membrane patch. The upper trace shows an outward SAC recorded at +80 mV (the upward arrows indicate the onset of suction, and the downward arrows indicate removal of suction). The lower trace shows an inward SAC recorded at −80 mV (downward and upward arrows denote onset and removal of suction, respectively). The negative pressure was held at 1 to 10 kPa for the indicated time period. Baselines are shown as dotted lines. The current scale is the same as shown in **(B)**.

hydroactive involvement of I_{Kin} inhibition. It is possible that hypertonic conditions cause stomatal closure by both hydraulic and hydroactive processes, which may involve inhibition of I_{Kin} in the plasma membrane of guard cells. Actin filaments may be involved in this osmosensing process.

DISCUSSION

Guard cell swelling and shrinking control stomatal movements. In this report, we have shown that cell-swelling conditions activate K^+ influx through I_{Kin} and inhibit K^+ efflux through I_{Kout}, achieving a larger net influx of K^+ and higher cell turgor. Cell-shrinking conditions, in contrast, inhibit K^+ influx and increase K^+ efflux, resulting in a larger net efflux of K^+ and lower cell turgor. This finding has two implications: (1) it indicates that K^+ channels serve as an osmosensing target in guard cells, providing a system for dissecting osmotic stress signal transduction in a single cell; and (2) it provides evidence that a positive feedback mechanism accelerates stomatal opening and closing processes, which also may be responsible for stomatal oscillation (Cowan, 1972).

Ion Channels and Cell Volume Regulation under Osmotic Stress

In animals, cell volume regulation under osmotic stress is crucial for function and survival in many cell types. In particular, hypotonic stress may result in cell death due to swelling, because most animal cells lack the strong mechanical support of a plant cell wall. During evolution, animal cells have acquired an extensive population of membrane channels and pumps for osmoregulation (reviewed in Grinstein and Foskett, 1990). For example, osmotic swelling would rapidly activate K^+ and Cl^- efflux through voltage-dependent channels in intestine cells that experience frequent changes in their environmental osmolarity (Tilly et al., 1996). Loss of osmolytes reduces the osmogradient across the cell membrane, which prevents the cells from swelling further and eventually restores cellular volume.

In plants, cells are confined by the cell wall that restricts cell volume changes caused by an osmogradient across the cell membrane. Because the extracellular space in plants is almost always filled with a “hypotonic” solution, most plant cells swell, resulting in the development of turgor pressure

(B) SAC currents at various membrane potentials (shown at left) under an outside-out configuration.

(C) The I–V curves used to calculate the conductance of both inward and outward K^+ currents carried by SAC. The conductance is 55 and 95 pS for the inward and outward currents, respectively.

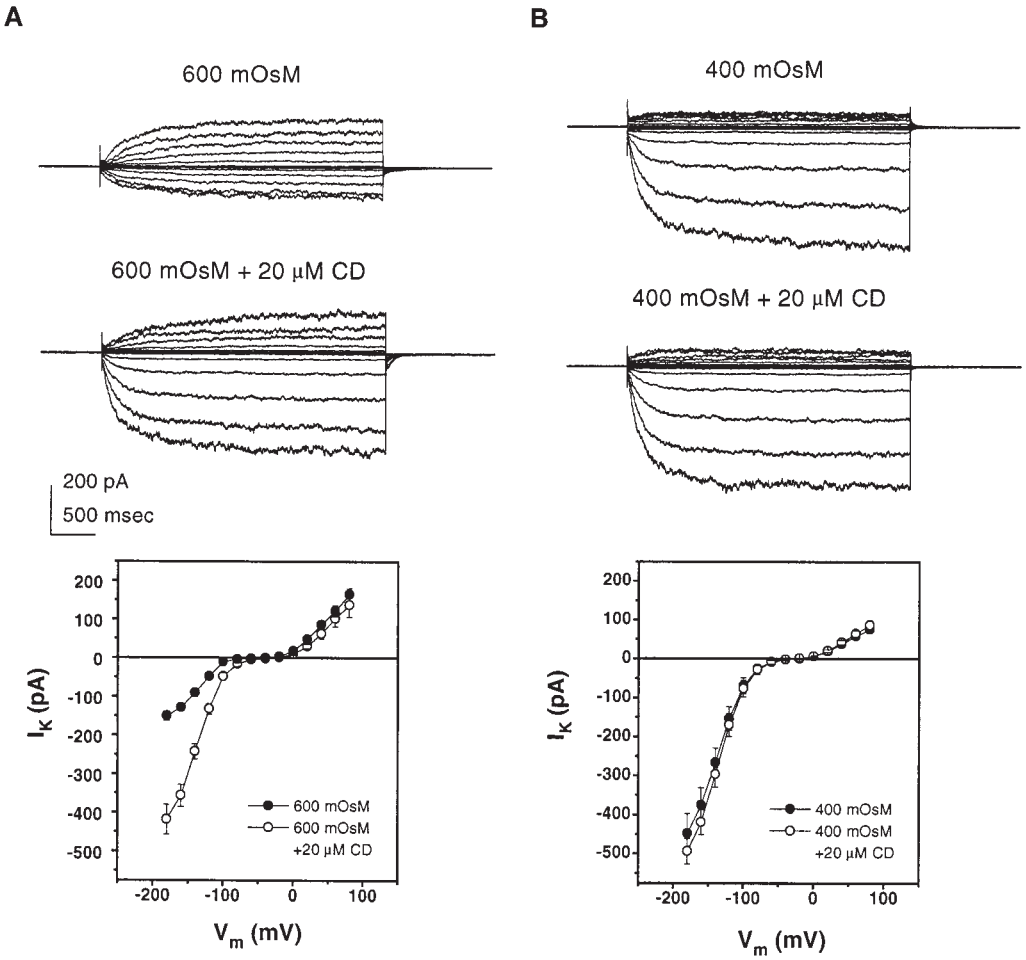

Figure 6. Cytochalasin D Restores I_{Kin} from Hypertonic Inhibition but Does Not Affect the Current under Hypotonic Conditions.

(A) The whole-cell I_{Kin} and I_{Kout} from the same protoplast perfused with a 600 mOsM solution or the same solution containing 20 μM cytochalasin D (CD). Data from nine cells were plotted in the I–V curves shown below the traces.

(B) The whole-cell current was recorded under an osmolarity of 400 mOsM in the perfusing solution. Data from seven cells are summarized in the I–V curves shown below the traces.

Error bars indicate ±SE.

imposed on the cell wall. For most living plant cells, this turgor pressure can be converted to a cell volume change, because the cell wall is extensible, permitting cell growth and, in cell types such as stomatal guard cells, cell "movement" (Assmann, 1993; Ward et al., 1995).

Due to the unique organization of guard cell walls, variations in turgor pressure are rapidly converted into changes in cell volume and stomatal aperture (Raschke, 1975). Similar to animal cells, guard cell turgor is also regulated by membrane channels and pumps. Studies have shown that I_{Kin} and I_{Kout}, which are responsible for K^+ fluxes across the plasma membrane, are among the most important channels in turgor regulation (Assmann, 1993; Schroeder et al., 1994; Blatt and Grabov, 1997; Maathuis et al., 1997). In this study, we have also shown that, similar to animal cells, osmotic stress regulates the activity of both I_{Kin} and I_{Kout} in guard cells, thereby controlling cell volume (or turgor). However, in contrast to animal cells, in which swelling activates outward ion channels for loss of osmolytes, our studies show that hypotonic swelling activates I_{Kin}, further increasing cell turgor and volume. This distinction reflects the difference in the structural organization of animal and plant cells, namely, the absence or presence of a cell wall. Perhaps more importantly, each regulation pattern seems to be designed according to the physiological function of the process. Animal cells respond with ion efflux to recover cell volume, because they cannot afford further swelling without the support of a cell wall (Mastrocola et al., 1993; Cossins and Gibson,

1997). Hypotonic swelling of guard cells is a way to open the stomata, and further activation of I_{Kin} speeds up this process under a water-sufficient condition.

For guard cells, hypertonic stress occurs under drought or low-humidity conditions (reviewed in Raschke, 1975; Zeiger, 1983; Grantz, 1990). Besides having a passive hydraulic effect, hypertonic stress affects cell turgor through a hydroactive pathway that involves an efflux of metabolites from guard cells. One such hydroactive process is initiated by the production of ABA, which induces stomatal closure by regulating activities of several ion channels in guard cells (Schwartz et al., 1994; Blatt and Grabov, 1997; Cowan et al., 1997; MacRobbie, 1997; Pei et al., 1997). Some studies have shown that ABA participation is not necessary for solute loss from guard cells after exposure to low water potentials (reviewed in Raschke, 1975; Assmann, 1993). Consistent with this notion, our studies show that hypertonic conditions facilitate the activation of I_{Kout}, leading to K^+ loss from guard cells. This finding may present initial molecular evidence for the ABA-independent hydroactive pathway that also targets voltage-dependent K^+ channels. This pathway, together with the hydropassive pathway, may provide a more rapid mechanism to close stomata under low-humidity or drought conditions. We propose that drought and low humidity impose a

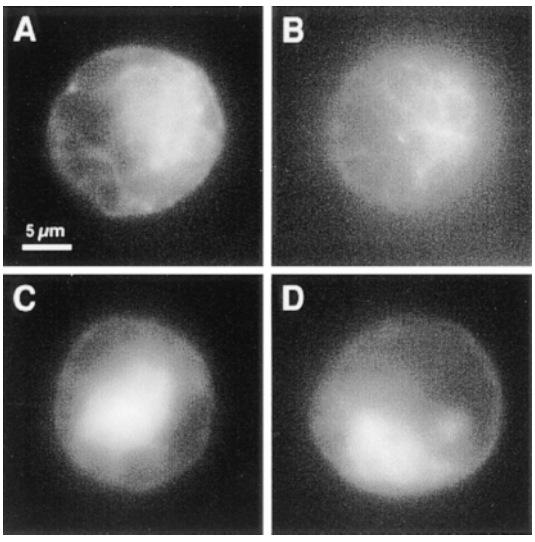

Figure 7. Actin Filament Organization in Guard Cell Protoplasts.

(A) A guard cell protoplast bathed in 550 mOsM solution. The nucleus of the cell served as the focal plane.
(B) The same protoplast as shown in **(A)**, focusing on the cortex of the protoplast.
(C) A protoplast bathed in 550 mOsM solution containing 20 μM cytochalasin D. The focal plane is the same as in **(A)**.
(D) A protoplast bathed in 400 mOsM hypotonic solution. The focal plane is the same as in **(A)** and **(C)**.
Bar in **(A)** = 5 μm for **(A)** to **(D)**.

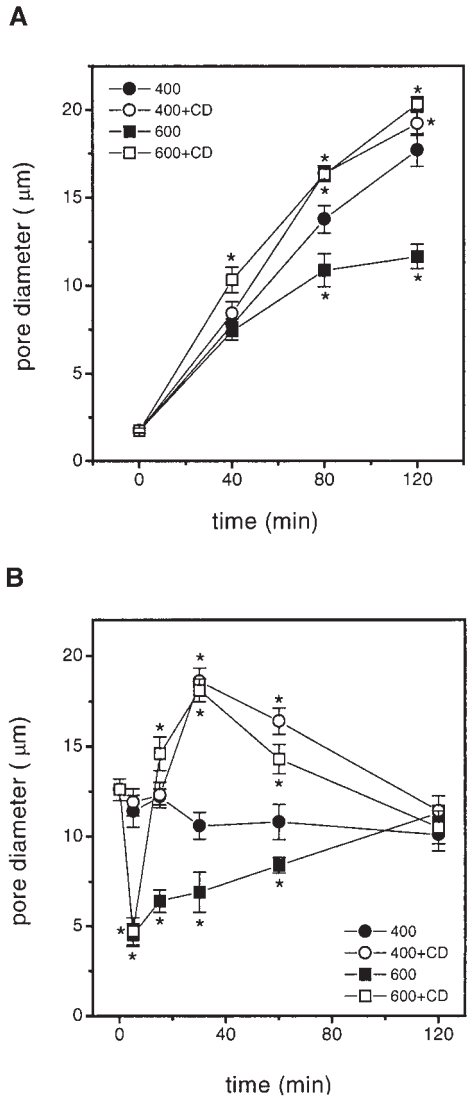

Figure 8. Osmoregulation of Stomatal Movements.

(A) The hypertonic condition inhibited light-induced stomatal opening. Solutions (400 and 600 mOsM) in the absence (400 and 600) or presence (400+CD and 600+CD) of 20 μM cytochalasin D were used to incubate epidermal peel strips. Treatment with 400 mOsM solution is defined as the control to conduct the Student's *t* test. Asterisks indicate significant differences ($P < 0.05$).
(B) The hypertonic condition induced transient stomatal closure. Epidermal peels were incubated in 400 mOsM (400) or 600 mOsM (600) solutions in the absence or presence of 20 μM cytochalasin D (400+CD and 600+CD) for various times before the stomatal aperture was measured. Asterisks indicate significant differences compared with the control (400 mOsM) ($P < 0.05$).
Error bars indicate ±SE.

negative osmogradient across the plasma membrane of guard cells that inhibits I_{Kin} and activates I_{Kout}, resulting in larger net efflux of K^+ and loss of guard cell turgor. This process is synergistic with ABA action on both K^+ channels (MacRobbie, 1997) and may function together to close stomata in response to water stress conditions.

A Positive Feedback Loop for Stomatal Movements and Oscillation

During the initial opening of illuminated stomata, the H^+-pump in the plasma membrane of guard cells is activated, resulting in a more negative membrane potential that activates I_{Kin}. K^+ influx takes place and drives water into the cell, making guard cells swell (Assmann, 1993). Cell swelling, as shown in this study, further activates I_{Kin} by increasing the opening frequency of the channel and therefore accelerates K^+ and water influx. This positively regulates the opening process. During stomatal closure induced by darkness, the plasma membrane becomes depolarized due to the activation of anion channels or inhibition of the H^+-pump (Assmann, 1993; Ward et al., 1995). The I_{Kout} is activated, and guard cells begin to shrink. Cell shrinking further activates I_{Kout} (and inhibits I_{Kin}), leading to a further decrease in guard cell turgor and more rapid closure of stomatal pores. This model (Figure 9) describes a positive feedback mechanism for accelerating stomatal opening or closing processes during the diurnal cycle.

This positive feedback loop may also explain stomatal oscillation under relatively constant environmental conditions. It is well known that leaf stomatal conductance is not necessarily stable but tends to oscillate (Barrs, 1971; Cowan, 1972). These oscillations occur in illuminated, transpiring leaves as well as in excised, fully turgid, darkened leaves. Although this phenomenon has been widely observed in a variety of plant species, its mechanism is not known (Cowan, 1972; Raschke, 1975). Some models have used CO_2 and H_2O feedback loops as the basis for oscillation, although none has all of the components corresponding to those in real plants (Karmanov et al., 1966; Lang et al., 1969). In Cowan's hydraulic model, positive water feedback is the essential feature (Cowan, 1972). In this study, we demonstrate a positive feedback loop for the regulation of K^+ channels that control the water status of guard cells. This finding may provide a component for initiation of stomatal oscillations.

The Actin Cytoskeleton May Serve as an Osmosensor

Regarding the mechanism of osmoregulation of inward K^+ channels in guard cells, actin filament organization may play a vital role. In animal cells, hypotonic swelling induces a reorganization of the actin filaments and activates outward channels (Schwiebert et al., 1994; Moran et al., 1996; Cantiello, 1997). Although an inward (rather than outward) K^+ channel is activated by hypotonic swelling in guard cells, the mechanism for channel regulation may be similar to that in animal systems. Indeed, we have shown that osmotic swelling may reorganize the actin filament network in guard cells and cause activation of I_{Kin}.

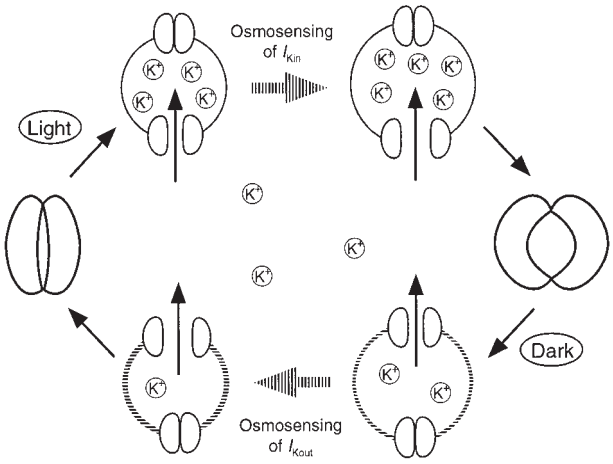

Figure 9. A Positive Feedback Mechanism for Stomatal Movements.

Light and dark switches are used as model signals that induce opening and closing of stomatal pores. Initial changes in guard cell turgor would regulate I_{Kin} or I_{Kout} through the osmosensing pathway described in this study, leading to acceleration of stomatal movement (for details, see Discussion).

In our study, the data derived by using an actin filament disrupter can be used to make an interesting correlation between actin filament organization and osmosensing of I_{Kin} and stomatal aperture. Under hypertonic conditions, actin filaments in protoplasts were organized in a network (Figures 7A and 7B), correlating with a small I_{Kin} (Figures 1 and 6) and a small stomatal aperture (Figure 8). The addition of cytochalasin D to the guard cells disrupted the actin network and induced activation of I_{Kin} and stomatal opening (Figures 6, 7C, and 8). Under hypotonic conditions, protoplasts increased their volume (Figure 4). The actin filament network in these cells was disrupted, and I_{Kin} was activated (Figures 6 and 7D). The addition of cytochalasin D to the hypotonic solution did not activate I_{Kin} to a higher level (Figure 6), because actin filaments were already disrupted. In contrast, hypotonic swelling of guard cells in the epidermal peel was limited by the cell wall. The addition of cytochalasin D, even under "hypotonic" conditions, would activate I_{Kin} further by additional actin filament disruption. This may explain why cytochalasin D enhances stomatal opening even under hypotonic conditions (Figure 8; Kim et al., 1995). We speculate that actin filaments may serve as an "osmosensor" and target inward K^+ channels in guard cells for turgor regulation.

In summary, we provide strong evidence that some voltage-dependent ion channels in higher plants serve as targets for osmosensing pathways. Because plant cells are constantly exposed to osmotic stress conditions, finding osmosensitive ion channels may have revealed an important mechanism for osmoregulation. We also provide evidence for a possible link among osmosensing, actin filament organization, and ion channel regulation. Further studies will be directed toward understanding the molecular interactions between I_{Kin} and actin filaments. In addition, more studies are required to understand the molecular mechanism underlying I_{Kout} regulation by osmotic stress.

METHODS

Preparation of Guard Cell Protoplasts

Seeds (*Vicia faba*) were germinated in potting mix, and plants were grown in a growth chamber for 4 weeks under a 10-hr light/14-hr dark cycle (180 μmol m^{-2} sec^{-1}). Guard cell protoplasts were isolated as described by Kruse et al. (1989). The protoplasts were kept in 0.45 M sorbitol, 1 mM $CaCl_2$, and 5 mM Mes medium (465 milliosmoles [mOsM]) on ice in the dark for at least 1 hr before patch–clamp recording.

Whole-Cell Recordings

Whole-cell patch–clamp recordings were performed with isolated guard cell protoplasts by using an Axopatch 200B patch–clamp amplifier connected to a Dell Optiplex GL+5100 computer system via a DigiData-1200 interface (Axon Instruments, Foster City, CA). The procedures were essentially as described previously (Luan et al., 1993; Bei and Luan, 1998). Briefly, recording pipettes were made from borosilicate glass capillaries by using a vertical two-stage puller (model PB-7; Narishige, Tokyo, Japan) and fire-polished by a microforge (model MF90; Narishige) before use. The pipette solution contained 100 mM K–glutamate, 2 mM EGTA, 2 mM $MgCl_2$, 10 mM Hepes, and 2 mM Mg–ATP, pH 7.2. The osmolarity was adjusted with D-sorbitol to 500 mOsM, unless otherwise indicated. Protoplasts were bathed in a solution consisting of 10 mM K–glutamate, 1 mM $CaCl_2$, 4 mM $MgCl_2$, and 10 mM Mes, pH 5.7. The osmolarity was adjusted with D-sorbitol to 450 mOsM or other values, as indicated in the text. Voltage-clamping steps and data acquisitions were performed using pClamp 6.0 software (Axon Instruments). Whole-cell currents were low-pass filtered at 1 kHz during measurements. Liquid junction potentials were corrected in all of the experiments according to Neher (1992).

Single-Channel Recordings

For single-channel recording, pipettes were coated with Sylgard 184 (Dow Corning Co., Midland, MI) under the microforge. The cell-attached configuration was formed after the pipette was sealed to the membrane (resistance >5 Gohm). Outside-out patches were obtained by retracting the pipette from a cell in the whole-cell configuration. In the cell-attached configuration, the pipette and the bath were both filled with the same solution that was described as the bath solution for whole-cell recordings, except that the osmolarities indicated in context were modified for studies of the inward K^+ current (I_{Kin}) with 100 mM K–glutamate. In outside-out configuration, the same solutions as those described for whole-cell recording were used, except that they all contained 100 mM K–glutamate. Data acquisition was conducted during the subsequent 30 sec under each holding potential described specifically Figures 2 and 3. Data were filtered at 1 kHz, digitized at 0.25 msec per sample, and stored on computer disk. pClamp 6.0 software was used for data analyses. The opening probabilities (P_o) were calculated from the ratio of total open time to total recording time. For patches in which more than one channel was recorded, P_o was defined as the total sum of P_o values calculated from each of the open channels. Single-channel current amplitude was obtained from Gaussian fitting of open-state amplitude histograms derived from data recorded for 10 to 30 sec.

Volume Measurement of Guard Cell Protoplasts

Guard cell protoplasts were incubated in isotonic bath solution (465 mOsM) for 40 min and then transferred to 400 and 600 mOsM bath solutions, respectively. The isotonic, hypotonic, and hypertonic solutions contained either 10 mM K–glutamate or Na–glutamate. Cell diameter was measured under the microscope by using a calibrated ocular micrometer (Nikon, Tokyo, Japan). Cell volume was calculated, and the data were presented as relative volume change, with control as 100%.

Staining of Actin Filaments in Guard Cell Protoplasts

Freshly isolated guard cell protoplasts were resuspended in isotonic bath solution (465 mOsM) for 40 min before being transferred to 400 and 550 mOsM solutions, respectively. Cytochalasin D treatment was performed in 550 mOsM solution containing 20 μM cytochalasin D. After a 40-min incubation, protoplasts were fixed and permeabilized for 0.5 to 1 hr in 100 mM Pipes buffer, pH 6.9, containing 3% formaldehyde, 10 mM EGTA, 5 mM $MgSO_4$, 1 mM phenylmethylsulfonyl fluoride, 2 mg/mL aprotinin, 2 mg/mL leupeptin, 1 mg/mL pepstatin, and 10% DMSO. Fixed protoplasts were rinsed in 100 mM Pipes buffer, pH 6.9, and stained with 0.5 μM Oregon Green (Molecular Probes)–conjugated phalloidin for 40 min. Protoplasts were washed twice with PBS and transferred to the mounting medium containing 20 ng/mL Dabco (Sigma) in 0.1 M Tris, pH 6.9, and 90% glycerol. A Zeiss Axiophot epifluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) was used to observe the actin staining pattern in guard cell protoplasts. Digital images were processed using Adobe Photoshop (Adobe Systems Inc., Mountain View, CA).

Measurement of Stomatal Aperture

To test the effect of osmolarity on stomatal opening, we obtained the fully expanded leaves from 4-week-old fava bean plants before the initiation of the light period. The epidermis was peeled and floated in the patch–clamp bath solution (500 mOsM). After a 1-hr incubation in the dark, the peels were transferred to different Petri dishes with 400 mOsM, 600 mOsM, 400 mOsM plus 20 μM cytochalasin D, and 600 mOsM plus 20 μM cytochalasin D bath solutions, respectively, and incubated under white light (intensity at 300 mmol m^{-2} sec^{-1}). The

images of stomatal aperture in the peel strips were captured at 0, 40, 80, and 120 min with a TEA/CCD-1317K/2 camera (Princeton Instrument, Inc., Trenton, NJ) under a Zeiss Axiophot microscope controlled by IPLab (Signal Analytics, Vienna, VA). The digitized data were stored on computer disk (Power Macintosh 8100; Apple Inc., Cupertino, CA). The maximum diameter of the stomata was measured on a computer screen by using NIH Image software (National Institutes of Health, Bethesda, MD).

To examine the effects of osmolarity on inducing stomatal closure, we obtained leaves 2 hr after the beginning of the light period. The peels were floated in a 400 mOsM bath solution under white light (300 mmol m^{-2} sec^{-1}) for 2 hr and then treated with the same solutions used in the stomatal opening experiments. The stomatal aperture images were captured, stored, and processed as described above. At least four images in each treatment with 15 to 20 stomata in each image were processed. The experiments were repeated three times. The data were presented as the mean ±SE, and the Student's *t* test was used to determine the significant difference between control and treatment.

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