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New Openings into Stomata

Guard cells are a favorite system for studying how external signals are transduced to alter cell behavior. This is partly because guard cells respond to an array of stimuli, including light (red and blue), humidity, abscisic acid (ABA), and auxin; partly because the response to these stimuli—rapid changes in ion transport and in the production of organic osmotica—can be detected easily as a change in guard cell volume and, therefore, stomatal aperture; and partly because the guard cells of many plants (particularly fava bean and *Commelina communis*) are readily accessible in epidermal peels and, once their walls are removed, make excellent subjects for electrophysiological studies of ion fluxes (for reviews, see Assmann, 1993; Ward et al., 1995).

These studies have revealed the basic outlines of the events that occur during both stomatal opening and stomatal closing. Opening involves hyperpolarization of the plasma membrane by a P-type H^+-ATPase, which drives uptake of K^+ through voltage-activated inward K^+ channels; additional osmoticum is provided by the conversion of starch to malate. Closing involves the depolarization of the membrane and the activation of voltage-dependent outward K^+ channels; these events are accompanied by the elevation (in some cases) of cytosolic free Ca^{2+} levels, by anion efflux, by an increase in cytosolic pH, and by glycolytic removal of organic osmotica.

Although many of the ion fluxes that occur during stomatal opening and closing have been identified, a full understanding of the mechanisms by which various stimuli initiate changes in stomatal aperture is not in hand. In the case of the closing stimulus ABA, a rapid response (within 2 sec) is often the elevation of cytosolic Ca^{2+} (e.g., McAinsh et al., 1990, 1992; Gilroy et al., 1991), which appears to play a pivotal role in stomatal closing. Indeed, release of Ca^{2+} from a caged form in the cytosol is sufficient for stomatal closure (Gilroy et al., 1990). (It is important to note, however, that under certain conditions, ABA can induce stomatal closing via a Ca^{2+}-independent mechanism [Allan et al., 1994].) Patch clamp studies have identified a number of possible mechanisms by which Ca^{2+} may contribute to the membrane depolarization necessary for K^+ efflux. Uptake of Ca^{2+} itself produces a slight depolarization of the membrane. In addition, Ca^{2+} activates voltage-dependent anion channels (Schroeder and Hagiwara, 1989; Hedrich et al., 1990). The resulting passive efflux of anions down the electrochemical gradient produces the sustained depolarization that is necessary for K^+ efflux; indeed, inhibitors of one anion channel, the S-type channel, prevent ABA-induced stomatal closure (Schwartz et al., 1995). On pages 1333–1342 of this issue, Kinoshita and colleagues provide evidence for an additional mechanism of Ca^{2+}-induced depolarization: increased cytosolic Ca^{2+} may inhibit the plasma membrane H^+-ATPase.

The possibility that inhibition of the plasma membrane proton pump might lead to membrane depolarization and stomatal closing has long been recognized, although this would require that the pump be involved in maintaining stomata in the open state as well as in opening them. The results of Cousson et al. (1995) suggest that this is indeed the case: treatment of open stomata with vanadate, which inhibits the H^+-ATPase, induces stomatal closing. Thus, inhibition of the H^+-ATPase could play a role in the closing process in vivo. Several studies (e.g., Nejidat et al., 1986) detected Ca^{2+} inhibition of a plasma membrane H^+-ATPase, but because the Ca^{2+} concentration required for significant inhibition was far above physiological levels, the relevance of this observation for stomatal closing was unclear.

To reinvestigate the question of Ca^{2+}'s effect on the proton pump, Kinoshita and coworkers assayed proton pumping activity (as measured by both a decrease in the absorbance of a reporter dye and the generation of electrical potential in the presence of ATP) as well as H^+-ATPase activity in microsomal membrane preparations from fava bean guard cells. Both proton pumping and H^+-ATPase activity were rapidly inhibited by Ca^{2+}; the inhibition of both activities rules out the possibility that the effect on proton pumping was due to activation of a Ca^{2+}/H^+ antiport rather than to inhibition of the pump itself. The Ca^{2+} concentration required for half inhibition of both activities was 0.3 μM, which is well within the physiological range; inhibition of both activities was reversed upon addition of the Ca^{2+} chelator BAPTA. The inhibitory effect was specific to Ca^{2+}: other divalent cations, including Ba^{2+}, Mn^{2+}, and Sr^{2+}, had no effect on proton pumping. The effect was not specific to guard cells, however: Ca^{2+} inhibited proton pumping and ATP hydrolysis by plasma membranes purified from mesophyll cells and did so at the same concentration at which it inhibited the guard cell ATPase.

Whereas Ca^{2+} produced a sustained inhibition of proton pumping in guard cells, ATPase activity recovered partially within 30 min after addition of Ca^{2+}; this may explain the low Ca^{2+} sensitivity observed in earlier studies, which used relatively long assay times. The apparent "recovery" of ATPase activity was probably not due to the activation of a Ca^{2+}-ATPase, because the authors included erythrosin B, an inhibitor of Ca^{2+}-ATPase, in their assays. Thus, Ca^{2+} may somehow decouple the pump and ATPase activities of the proton pump.

The finding that physiological Ca^{2+} increases inhibit proton pumping indicates that guard cells may possess redundant mechanisms for Ca^{2+}-dependent

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membrane depolarization: Ca^{2+} influx itself, Ca^{2+} activation of anion channels, and Ca^{2+} inhibition of H^+-ATPase activity. This is not surprising, given the importance to the plant of accurate control of stomatal aperture. It is also possible that these different depolarization effects work together, reinforcing one another to produce a strong depolarization. The anion channels necessary for stomatal closure are maximally active at membrane potentials that are far less negative than those typical of the guard cells of open stomata, and their activation may therefore require an initial depolarization of the guard cell membrane (Assmann, 1993). Ca^{2+} influx has been suggested to initiate this depolarization, and an interesting possibility raised by the results of Kinoshita and coworkers is that proton pump inhibition contributes significantly to it.

Although it is clear that increased cytosolic Ca^{2+}, whether through its inhibition of H^+-ATPase or through other activities, can initiate stomatal closure, a simple model in which increased Ca^{2+} is both necessary and sufficient for stomatal closure is incorrect. Not only can stomata apparently close in the absence of a Ca^{2+} increase (Allan et al., 1994), but increases in Ca^{2+} are sometimes associated with stomatal opening, for example, in response to auxin (Irving et al., 1992). The resolution of the paradox of Ca^{2+}'s ability to serve as a second messenger for opposing processes may lie in differences in the spatiotemporal nature of Ca^{2+} increases (Bush, 1995). In animal cells, increases in Ca^{2+} levels often occur as a series of oscillatory rises and falls in Ca^{2+} level, and waves of Ca^{2+} increases have been observed to move across stimulated cells. For some agonists, the frequency of the oscillations depends on the agonist concentration and is reflected in the strength of the response (Fewtrell, 1993). Oscillatory increases in Ca^{2+} may also serve to protect cells from the damage that would be caused by large, sustained increases in Ca^{2+} concentration; if the increase is transient, the cell can accommodate a larger (and more readily detectable over background Ca^{2+} "noise") increase than it could if the increase were sustained.

Oscillations in Ca^{2+} levels have been detected occasionally in plant cells stimulated by auxin (Felle, 1988) and ABA (McAinsh et al., 1990; Schroeder and Hagiwara, 1990; Gilroy et al., 1991), but the significance of these has never been clear. In this issue, McAinsh and coworkers (pages 1207–1219) show that a closing stimulus results in the production of oscillations in cytosolic Ca^{2+} whose pattern varies with the strength of the stimulus. These results raise the interesting possibility that the nature (i.e., frequency, amplitude, and perhaps shape) of oscillations in cytosolic Ca^{2+} may allow the plant cell to use Ca^{2+} as a second messenger for many different processes.

The stimulus used by the authors is high external Ca^{2+}, which is a potent closing stimulus. Although the physiological relevance of this stimulus remains to be established, in part because it is difficult to measure free Ca^{2+} levels in the apoplast, increases in rhizosphere Ca^{2+} have been found to increase the Ca^{2+} concentration in the xylem, and very high xylem Ca^{2+} concentrations in turn cause stomatal closure in *C. communis* (Ruiz et al., 1993). McAinsh and coworkers found that whereas addition of 0.01 mM Ca^{2+} to *C. communis* epidermal peels had no effect on open stomata, both 0.1 and 1.0 mM external Ca^{2+} caused stomata to close. The responses elicited by the two stimuli followed different kinetics, suggesting that the guard cells perceive a difference between the two external Ca^{2+} concentrations. Ratio photometry of fura-2–loaded guard cells showed that with both stimuli, cytosolic Ca^{2+} levels rose transiently and then began to oscillate in a stimulus-dependent fashion. The oscillations induced by 1.0 mM Ca^{2+} showed a lower frequency and were less symmetric and more broad than those induced by 0.1 mM Ca^{2+}, but the integrated total increase in Ca^{2+} was much greater. In both cases, the oscillations were maintained only as long as the stimulus was present.

To ask whether the Ca^{2+} involved in the rising phase of the oscillations comes from external sources, internal sources, or both, the authors examined the effect of both Ca^{2+} channel blockers and Mn^{2+} on the oscillations. The blocker verapamil reduced the amplitude of oscillations, ultimately abolishing them (in 0.1 mM Ca^{2+}) or altering their pattern (in 1.0 mM Ca^{2+}). Mn^{2+}, which is generally assumed to enter cells through the same channels that mediate Ca^{2+} uptake, quenches fura-2 fluorescence at all wavelengths, making it possible to examine Mn^{2+} uptake independent of Ca^{2+}. The addition of 1 mM Mn^{2+} to the medium of guard cells undergoing Ca^{2+} oscillations resulted in a reduction in the amplitude of each oscillation; in addition, the fura-2 fluorescence dropped in discrete steps, each occurring simultaneous with a remaining rising phase of cytosolic Ca^{2+}. Assuming that Mn^{2+} enters cells through Ca^{2+}-selective channels, this result implies that some fraction of the oscillating Ca^{2+} is taken up from external supplies.

Although these data implicate a membrane oscillator in Ca^{2+}-induced Ca^{2+} oscillations in guard cells, the finding that verapamil does not eliminate the oscillations elicited by the high Ca^{2+} concentration indicates that internal stores may be the source of some of the oscillating Ca^{2+}. Indeed, the authors show that release of caged Ca^{2+} into the cytoplasm of cells in low-Ca^{2+} medium resulted in several oscillations that damped rapidly, indicating the possible involvement of a cytosolic oscillator analogous to those found in the majority of animal cells showing Ca^{2+} oscillations (Berridge and Dupont, 1994).

Although the observation that external Ca^{2+} induces oscillations in cytoslic Ca^{2+} is intriguing, it has not been ruled out that these oscillations represent the cell's attempt to respond to Ca^{2+} overload by expelling excess Ca^{2+} after it enters the cell. Indeed, the fact that oscillations have not been seen consistently with other stimuli, in particular ABA, might indicate that oscillations are not a general feature of guard cell signaling. On the other hand, the evidence that some of the oscillating Ca^{2+} may be recruited from internal

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stores, as is the case in animal cells undergoing Ca^{2+} oscillations, suggests that the guard cell oscillations may have a role in signaling and do not simply represent the cell's attempt to reach Ca^{2+} homeostasis. Further detailed analysis of cytosolic Ca^{2+} levels in response to ABA and other stimuli may help to resolve this question.

The finding that Ca^{2+} is an important second messenger for stomatal closure was an important breakthrough, but the challenge now is to define exactly how it plays this role. It is clear that Ca^{2+} may have multiple functions, even in a single process (e.g., stomatal closing), and all of these functions and their relative importance to the process need to be determined. (Of course, it is possible that their relative importance may change, depending on the environmental conditions.) One way to do this might be by eliminating individual putative Ca^{2+} targets (or, in the case of the H^+-ATPase, transforming guard cells with a Ca^{2+}-insensitive form of the pump). In addition, as more functions for Ca^{2+} are unearthed, including some contradictory ones, the necessity for cells to possess some means of discriminating between the Ca^{2+} rises elicited by different stimuli has become increasingly evident. An important future challenge entails defining the spatial and temporal heterogeneities of the Ca^{2+} increases elicited by different stimuli and exploring the mechanisms by which these increases are achieved.

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