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Phil. Trans. R. Soc. Lond. B 1992 **338**, 5-18
doi: 10.1098/rstb.1992.0124

Calcium and ABA-induced stomatal closure

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SUMMARY

Water loss from leaves is regulated by the state of stomatal pores, whose aperture is controlled by the level of potassium salt accumulation in guard cells. In water stress conditions abscisic acid (ABA), produced or imported into leaves, and acting on the outside of the guard cell induces net loss of potassium salts, and hence stomatal closure. The mechanism of ABA-induced closure and the role of calcium in the process are discussed. There are two questions at issue, whether Ca^{2+}-regulated fluxes of specific ions are an obligatory part of the signal cascade, and if this is the case, whether the necessary ABA-induced increase in cytoplasmic Ca^{2+} arises from Ca^{2+} influx at the plasmalemma, or by Ca^{2+} release from internal stores, or both.

Tracer flux studies establish that ABA-induced closure involves transient stimulation of both anion and cation fluxes at the plasmalemma, and stimulation of the transfer of both anions and cations from vacuole to cytoplasm. ABA-induced efflux transients can occur in very low external Ca^{2+}, but their reduction in the presence of La^{3+} suggests that Ca^{2+} influx is required for the response. The flux work can only be interpreted in terms of defined ion channels identified by electrical work, either whole-cell voltage clamping or patch clamp studies, and of the responses of these channels to Ca^{2+} and to ABA.

Electrical work identifies a number of ion channels in the plasmalemma; these include an inward K^+ channel open at negative membrane potentials, and inhibited by increase in cytoplasmic Ca^{2+}, an outward K^+ channel open at more positive membrane potentials, which is insensitive to Ca^{2+} but is more active at higher pH, a voltage-sensitive, Ca^{2+}-dependent anion channel, active only over a restricted range of potentials (about -100 mV to -50 mV), and some ill-defined conductances lumped together as the 'leak' or background conductance, which may include channels (selective or non-selective) allowing Ca^{2+} influx. The leak conductance is increased by increase in cytoplasmic Ca^{2+}.

Guard cells are capable of responding to inositol 1,4,5-trisphosphate released in the cytoplasm, by increasing cytoplasmic Ca^{2+}, by inhibition of the inward K^+ channel and by stimulation of the leak conductance (but without effect on the outward K^+ channel), and by stomatal closure. Recent work suggests that there is considerable turnover in the phosphoinositide cycle in guard cells, within 30 s of treatment with ABA.

Measurements by fluorescence techniques of cytoplasmic Ca^{2+} in guard cells following treatment with ABA give conflicting results. Some work shows increase in cytoplasmic Ca^{2+} in response to ABA, other studies show variable behaviour, with most cells closing in response to ABA, but without detectable changes in cytoplasmic Ca^{2+}. Nevertheless it seems likely that increases in cytoplasmic Ca^{2+}, at least locally, are a universal feature of the ABA response, but that they may be difficult to detect with present techniques. Fluorescence studies also show alkalinization of guard cell cytoplasm in response to ABA.

Whole cell electrical studies identify a number of ABA-induced changes. They show (i) depolarization of cells with very negative membrane potentials to potentials which are positive to E_K, and thus out of the activation range for the inward K^+ channel, and within the range for the outward K^+ channel, (ii) activation of an inward current, a voltage-insensitive component of the leak conductance, which is responsible for the depolarization, (iii) deactivation of the inward K^+ channel, (iv) activation of a voltage-sensitive channel carrying inward current, probably the Ca^{2+}-sensitive anion channel, and (v) the slower activation of the outward K^+ channel. The activation of the inward leak current seems to be the primary response, but its nature is not clearly established; a non-selective cation channel, which may allow Ca^{2+} influx, is perhaps most likely.

Thus the early events in the ABA-response include stimulation of tracer efflux, activation of an ill-defined component of the leak conductance, producing an inward current, and turnover in the phosphoinositide cycle. These occur within the first minute, but their time sequence and causal relationships are not yet clear. A plausible scheme for ABA-induced closure can be devised, involving Ca^{2+} influx through a non-selective cation channel as the first event, producing depolarization and increase (possibly local) in cytoplasmic Ca^{2+}. This may then be supplemented by release of Ca^{2+} from internal stores, triggered by inositol 1,4,5-trisphosphate produced by activation of phospholipase C. Increase in cytoplasmic Ca^{2+} will give deactivation of the inward K^+ channel, and activation of the Ca^{2+}-dependent anion channel, but some other trigger is required to explain the activation of the outward K^+ channel; increase in cytoplasmic pH (observed, but of mechanism unknown) is the most likely candidate. This is one scheme, but others can also be devised, and with the gaps still existing in our description of the events involved, and their time sequence, a definitive hypothesis is not yet available.

Phil. Trans. R. Soc. Lond. B (1992) **338,** 5–18
Printed in Great Britain

1. INTRODUCTION

Stomatal aperture is controlled by the solute content of stomatal guard cells, largely, but not exclusively, by their content of potassium salts, with K^+ balanced either by Cl^- or malate, dependent on the species and conditions. Thus stomatal movement is effected by accumulation of potassium salt in the process of opening, and by net loss of potassium salt during stomatal closure, and the changes in aperture reflect the regulation of processes of ion influx and ion efflux in guard cells, in response to a range of signals. It is important in considering stomatal movements to distinguish between the ability to open, and the ability to remain open, and to regard opening and closing as separate processes, with different fluxes involved in their triggering; closure is achieved by stimulation of ion efflux and not simply by cessation of the influx by which opening was achieved. There is now considerable evidence for Ca^{2+} as one of the regulating factors which determine stomatal aperture, and, in particular, for a role for Ca^{2+} in the signal transduction chain by which abscisic acid (ABA), a phytohormone produced in, or imported into, leaves in conditions of water deficit, and acting on the outside of the guard cell (Hartung 1983), induces stomatal closure. The detailed mechanisms involved in ABA-induced closure remain to be established, although plausible partial schemes may now be proposed, and evidence for their operation is gradually accumulating.

Effects of external Ca^{2+} on guard cells, in inhibiting stomatal opening and promoting stomatal closure, were first observed by Fujino (1967), Willmer & Mansfield (1969), and Fischer (1972). Schwartz *et al.* (1985, 1988) found that increased external Ca^{2+} was more effective in promoting closure than in inhibiting opening. MacRobbie (1986) showed that Rb^+ influx during the early stages of opening of newly isolated *Commelina* guard cells was inhibited by Ca^{2+}, and that the inhibition of influx in the dark was a Ca^{2+}-dependent process. De Silva *et al.* (1985*a*,*b*) showed that the inhibition of stomatal opening by ABA was dependent on external Ca^{2+}, and was sensitive both to Ca^{2+}-channel blockers (La^{3+}, verapamil, and nifedipine), and to calmodulin antagonists (trifluoperazine, W7, and compound 48/80), suggesting that Ca^{2+} influx at the plasmalemma was involved in the signal transduction chain by which ABA acts to inhibit opening. In contrast, Fitzsimons & Weyers (1987) found that ABA-induced shrinkage of guard cell protoplasts could occur in the absence of external Ca^{2+}. McAinsh *et al.* (1991) found only partial inhibition of ABA-induced stomatal closure in the presence of either EGTA or Ca^{2+}-channel blockers, and argued that release of internal Ca^{2+} may also be important.

Thus there is a need to identify specific ion transport systems in guard cell membranes which are sensitive to Ca^{2+}, and to establish whether such systems are involved in triggering stomatal closure in response to ABA. For this purpose it is necessary to establish very clearly the flux changes, and their time sequence, which follow application of ABA and which lead to net loss of K-salt. The question of whether Ca^{2+} is necessarily involved in the ABA signal transduction chain is still debated, and there are two questions at issue. The first is whether Ca^{2+}-regulated fluxes are an obligatory part of the signal cascade, and if so, what is their nature. If this is the case, the second question concerns the source of the increase in cytoplasmic Ca^{2+} which triggers subsequent responses, whether it arises from Ca^{2+} influx at the plasmalemma, or by Ca^{2+} release from internal stores, or both. The aim of this paper is to consider the evidence for the hypothesis that Ca^{2+} is necessarily involved in triggering ABA-induced stomatal closure, and to assess the likely sequence of events.

2. ABA-INDUCED FLUX CHANGES IN GUARD CELLS

Tracer flux work on isolated guard cells of *Commelina* (MacRobbie 1981, 1990) has identified the nature of the changes in ion fluxes following ABA treatment, but only semi-quantitatively (because of undefined changes in internal specific activities once fluxes change), and with poor time resolution. It is clear that the effects of ABA on efflux are dramatic, whereas influx is little affected. For efflux measurements, isolated guard cells are loaded with tracer overnight, to a steady state of tracer content and aperture (with $^{86}Rb^+$ as an analogue of K^+, and with either $^{36}Cl^-$, or $^{82}Br^-$ as an analogue for Cl^-). Steady-state efflux of tracer is followed for a period, and then ABA is added, producing a marked efflux transient, with a stimulation of ion efflux at the plasmalemma, lasting for about 20–30 min. The amount of extra tracer lost during this transient is greater than the estimated cytoplasmic tracer content at the time of ABA addition, and hence it is clear that ion fluxes from vacuole to cytoplasm are also increased by ABA, though the extent of this stimulation cannot be quantified. Nevertheless, the tracer flux studies make it clear that four fluxes are stimulated during the efflux transient, namely the effluxes of both anions and cations across the plasmalemma, and the fluxes of both anions and cations from vacuole to cytoplasm.

Later work (MacRobbie 1990) showed that the full efflux response is biphasic, and that one or more second messengers must be involved in its development. It was found that the response to a two minute pulse of ABA was indistinguishable from that produced by continuous ABA treatment, and that the efflux could continue to rise after the removal of ABA from the external solution. Because the occupancy of the ABA receptor sites cannot increase after the removal of ABA, this means that a sequence of events must be initiated by ABA on the receptor sites, but that the response is then self-sustaining, and no longer requires the continuous presence of ABA on its receptors. There was also evidence that short application of ABA could leave guard cells in some sort of desensitized state in which they were unable to respond to reapplication of ABA.

The other point to make about the changes in efflux at the plasmalemma is that the stimulations of both

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anion and cation are transient, and do not represent maintained increases in ion permeability in the presence of ABA. Rather the changes look more like enhanced permeability in cells which are above a changed 'set-point' for ion accumulation, and may share mechanisms with the changes seen in hypotonic turgor regulation in the brackish water Characean, *Lamprothamnium* (see review by Okazaki & Tazawa (1990)). In this instance the readjustment of high cell turgor, and restoration to the normal value, is achieved by net loss of KCl, a process which requires Ca^{2+} in the bathing medium, and the response involves a transient increase in cytoplasmic Ca^{2+}, presumably responsible for the activation of Ca^{2+}-sensitive ion channels. In this connection it may also be worth noting that ABA-induced shrinkage of *Commelina* guard cell protoplasts was observed only in protoplasts which had been pre-swollen by incubation in KCl, with accumulation of extra K-salt (Fitzsimons & Weyers 1987). After the initial shrinkage the volume and K^+ content stabilized at a lower basal level (of about 108 mM K^+), with ABA still present, again suggesting some sort of regulatory response.

In interpreting the effects of ABA in guard cells our description of the signal transduction mechanisms needs to account for all four changes. Each flux change needs to be attributed to a specific ion channel in plasmalemma or tonoplast, with properties defined by electrical studies, either by whole-cell voltage-clamp studies or by patch clamping, and the voltage- and Ca^{2+}-sensitivities of the channels identified are crucial for the construction of plausible reaction sequences.

3. ION TRANSPORT SYSTEMS IN GUARD CELL PLASMA MEMBRANES

The characteristics of particular ion transport systems in the plasma membrane are established by electrophysiological studies of two kinds, by whole cell voltage clamp studies, and by patch clamping. In general, in the patch clamp work the whole cell configuration has been used, in which, after forming a tight membrane seal, the patch is broken, allowing access of the pipette solution to the cell interior. Patch clamping has the advantage of allowing control of the solution bathing both sides of the membrane, but the disadvantage that cytoplasmic factors are lost to the pipette, to greater or lesser extent, and some channels may therefore show 'run-down' or loss of activity. The other disadvantage is that in preparation and isolation of protoplasts there are likely to be changes in the state of the guard cell and in membrane activities. The state of guard cell protoplasts is more akin to near closed guard cells than to fully open guard cells, and protoplasts may not show the full range and extent of transport processes of which the intact cell is capable.

(*a*) *Whole-cell current–voltage analysis*

Whole-cell current–voltage analysis, in which membrane currents are measured with the membrane potential clamped to a range of fixed values, provides a powerful method for the identification and characterization of different charge-carrying transport processes in the membrane. The steady state analysis involves two profiles, the current–voltage (I–V) profile, of membrane current against clamped membrane voltage, and the conductance-voltage (G–V) profile, obtained by differentiating the I–V profile, to show membrane conductance as a function of membrane voltage. The dissection of the full current–voltage profile into its component parts is made in two ways. The first is by the comparison of steady state I–V curves in solutions of different ionic composition, and in the presence and absence of various 'specific' inhibitors or ion channel blockers. The second uses the differences in the time courses of activation and deactivation of different transport systems in response to a voltage step, to distinguish slowly activating or deactivating K^+ channels from other systems which change 'instantaneously'. Thus changes in 'instantaneous' current are measured 4 ms after a voltage step, whereas new steady state currents may take some tens or hundreds of ms to stabilize, and the difference between these two values can be attributed to a slowly activating- and deactivating channel.

Work using this technique (Blatt 1987, 1988*a*,*b*, 1990; Blatt & Clint 1989; Clint & Blatt 1989; Thiel *et al.* 1992) has shown that a number of different transport systems contribute to guard cell currents, with their relative contributions dependent on conditions and on the particular voltage range. The systems contributing to guard cell currents include the following.

(1) The electrogenic ion pump, the proton-translocating ATPase characteristic of all plant and fungal cells. This is capable of driving the resting membrane potential very negative, well beyond any diffusion potential, and is also seen as an increased conductance negative of −150 mV, with a local maximum around −230 mV.

(2) An inward K^+ channel, activating or deactivating slowly after a voltage step, opening at membrane potentials more negative than about −120 mV (independent of external K^+), in millimolar K^+ and above; this channel is active at external pH 6.1, but very little active at pH 7.4. The inward K^+ channel is responsible for K^+ influx to the cell in conditions where the pump generates a sufficiently negative membrane potential at suitable external K^+ and pH, but at high external pH and low external K^+ some other transport system must be invoked for the influx of K^+.

(3) An outward K^+ channel, again responding slowly after a voltage step, opening at a potential close to and positive of the potassium equilibrium potential (E_K), and carrying K^+ out of the cell. The activation voltage for opening of this channel shifts with external K^+, thus ensuring that over a wide range of external K^+ the channel opens only positive of E_K, and mediates K^+ efflux only.

(4) An ill-defined 'leak' or 'background' conductance, which includes more than one charge-carrying current. This responds 'instantaneously' to a voltage step, allowing its contribution to be distinguished from

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those of the K^+ channels. This conductance, together with the pump, is responsible for the current in the voltage range between the activation voltages of the inward and outward K^+ channels i.e. between about -120 mV and -50 mV in 10 mM external K^+, but also contributes in the range in which one or other K^+ channel is active. Various transport systems will be included in this ill-defined background conductance, with their relative importance dependent on the conditions, but not known; it will include an anion channel, and any channel carrying Ca^{2+} into the cell, whether specific for Ca^{2+} or not.

(*b*) *Patch clamping*

More detailed information on factors controlling guard cell ion channels is available from patch clamp work, in which the composition of the solution bathing the inside of the plasmalemma can be modified. The inward and outward K^+ channels have both been demonstrated in the whole-cell configuration of patch clamping (Schroeder *et al.* 1984, 1987; Schroeder & Hagiwara 1989). Of particular interest is the finding that the inward K^+ channel is inhibited by increasing cytoplasmic Ca^{2+} from 0.1 μM to 1.5 μM, whereas the outward K^+ channel is Ca^{2+}-insensitive (Schroeder & Hagiwara 1989). Recently Fairley–Grenot & Assmann (1992*b*) found evidence for Ca^{2+} entry through the inward K^+ channels in *Vicia* guard cells, indicated by the existence of tail currents when K^+ channels activated by a voltage step to -180 mV were deactivated by stepping to -52 mV, the potassium equilibrium potential, in solutions in which only Ca^{2+} could carry the observed tail currents. They suggest that such channels may play a role in internal Ca^{2+} homeostasis, and also point out that the relative permeabilities to K^+ and Ca^{2+} may be subject to physiological control.

Fairley–Grenot & Assmann (1992*a*) have recently patch-clamped guard cell protoplasts from *Zea mays*, and compared their channel properties with those of *Vicia faba*. There are basic similarities, but also differences in kinetics. The inward rectifying channels operate faster in *Zea* than in *Vicia*, but the outward rectifying channels operate more slowly. More important is the observation that both inward and outward K^+ currents in *Zea* are reduced by increase in internal Ca^{2+} from 2 nM to 180 nM (by 80% inward and 84% outward); measurements in *Vicia* under the same conditions gave reductions of 63% inward and 59% outward. This contrasts with earlier measurements in *Vicia* (Schroeder & Hagiwara 1989) showing no change in outward K^+ current on increasing Ca, from 0.1 μM to 1.5 μM. However, the measured values of cytoplasmic Ca^{2+} in *Vicia* or *Commelina* are closer to the higher of the two values used by Fairley–Grenot & Assmann (1992*a*), but the lower of those used by Schroeder & Hagiwara (1989). The threshold value for inducing stomatal closure was said to be above about 500 nM (Gilroy *et al.* 1991), and hence assessment of the role of increases in internal Ca^{2+} in inducing stomatal closure in *Zea* will require electrical measurements at higher levels of cytoplasmic Ca^{2+} than are yet available.

Patch clamping has also demonstrated a voltage-sensitive anion channel in guard cells (Keller *et al.* 1989; Schroeder & Hagiwara 1989, 1990*a*; Hedrich *et al.* 1990). This channel is active only over a very limited voltage range, between about -100 mV and -50 mV, and shows inactivation with a half-time of 10–12 s. It is Ca^{2+}-activated, but also requires nucleotides in the cytoplasm, suggesting direct or indirect control through phosphorylation or nucleotide binding (Hedrich *et al.* 1990).

Thus the patch-clamp work identifies the consequences of an increase in cytoplasmic Ca^{2+}, as the inhibition of the inward K^+ channel and the activation, if the membrane voltage is not too negative, of the depolarizing anion channel. If the increase in cytoplasmic Ca^{2+} was the result of stimulated Ca^{2+} influx, then the associated depolarization is likely to allow anion channel activation, which will produce further depolarization; this in turn will stimulate further Ca^{2+} entry, as Ca^{2+} influx is voltage-sensitive (MacRobbie 1989). If, however, the initial increase in cytoplasmic Ca^{2+} was the result of release of Ca^{2+} from internal stores, this may, or may not, activate the anion channel (to give depolarization and consequent stimulated Ca^{2+} entry), depending on the value of the resting membrane potential. It may therefore be difficult to sort out cause and effect, and to distinguish the primary response to a stimulus from subsequent consequences.

The number of ion channels identified in guard cell membranes has been considerably increased recently, by the discovery of three different stretch-activated channels by Cosgrove & Hedrich (1991). These are specific for Cl^-, K^+ and Ca^{2+} respectively, and are distinguishable from the non-stretch-activated channels for these respective ions, on the basis of conductance, kinetics and voltage-dependence. They also identified a further non-stretch-activated channel which is permeable to Ca^{2+}, but with lower selectivity for Ca^{2+} over K^+. One interesting feature of these results, apart from the unusual richness of stretch-activated channels in guard cells, is that the guard cell channels have lower conductance, shorter open times and greater selectivity than the stretch-activated channels observed in other walled cells (tobacco, Falke *et al.* 1988; yeast, Gustin *et al.* 1988; *E. coli*, Martinac *et al.* 1987, Delcour *et al.* 1989), and are more similar to the stretch-activated channels found in animal cells (review by Morris 1990). It is possible that this is of significance, and is related to observations which suggest that guard cells may regulate volume (as do animal cells), rather than turgor (see discussion in MacRobbie (1988*a*)). Cosgrove & Hedrich (1991) also point out that ion flux through either the stretch-activated Cl^- or Ca^{2+} channels will produce secondary effects, depolarization in both cases, and also increase in cytoplasmic Ca^{2+} for the Ca^{2+} channel, which will in turn activate non-stretch-activated channels, the normal anion channel, and the outward K^+ channel. Thus activation of stretch-activated channels might well act as trigger for net salt loss in inducing stomatal closure.

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4. EFFECT OF ABA ON CYTOPLASMIC Ca^{2+}

There is still argument about the nature and generality of ABA-induced changes in the level of free cytoplasmic Ca^{2+} in guard cells, and the experimental evidence is conflicting. The extent to which this arises from technical problems in the measurements rather than real differences in the pattern of behaviour is not yet clear.

McAinsh *et al.* (1990) measured cytoplasmic Ca^{2+} by photometric measurements of fura-2 in *Commelina* guard cells. They found that, in eight out of ten cells examined, cytoplasmic Ca^{2+} increased after treatment with 1 μM ABA, by between two- and ten-fold over a period of about 10 min, from a resting level of 70–250 nM (mean 115 ± 26 nM ($n = 10$)). The increase in cytoplasmic Ca^{2+} preceded stomatal closure. The time for the first detectable increase in cytoplasmic Ca^{2+} corresponds well with the time for first rise of the efflux transient or for the appearance of ABA-induced electrical changes, although the full rise in cytoplasmic Ca^{2+} is somewhat slower than the full development of flux or current changes.

However, other workers find different patterns of ABA-induced changes in cytoplasmic Ca^{2+}. In a very thorough study of changes in cytoplasmic Ca^{2+} in a range of conditions, Gilroy *et al.* (1991) found very variable responses to ABA, also in *Commelina*; a minority of cells showed increases in cytoplasmic Ca^{2+} with time courses of the form described, but in other cells the response was much less dramatic, or there was no detectable change in response to ABA. Nevertheless, ABA induced stomatal closure, with or without detectable changes in cytoplasmic Ca^{2+}. In photometric measurements (using indo-1) on 38 guard cells, only four showed dramatic increases in cytoplasmic Ca^{2+} similar to those reported by McAinsh *et al.*, and a further ten cells showed some response. Fluorescence ratio imaging of guard cells showed the complexity of the distribution of cytoplasmic Ca^{2+}, with marked spatial inhomogeneity and local hot spots, particularly in the increases in cytoplasmic Ca^{2+} in response to stimuli. Although Gilroy *et al.* (1991) (see also Fricker *et al.* 1991) found clear localized increases in Ca^{2+} in response to increased external Ca^{2+}, or reduction in external K^+ from 50 mM to 25 mM, with associated stomatal closing, similar changes in Ca^{2+} were not observed after treatment with ABA. When increases were observed they were in the region of cytosol in which endomembranes were concentrated, around the nucleus and vacuole; this, together with observations that increases could be observed even with La^{3+}-treated cells, suggested that internal release of Ca^{2+}, rather than influx from the outside, was capable of effecting the changes. The authors argue that, although increased cytoplasmic Ca^{2+} (above about 500 nM) necessarily induces stomatal closure, the coupling between cytoplasmic Ca^{2+} changes and ABA-induced closure is not obligatory, and that ABA can induce closure through Ca^{2+}-independent pathways. This raises serious problems because, although plausible hypotheses for the variety of observed flux and electrical responses can be devised on the basis of Ca^{2+} changes, in their absence we have no entry into a signal transduction scheme, and would have to start again from scratch, for each flux change, having lost Ca^{2+} as the connecting link. The alternative is that the problem lies in the insensitivity of the Ca^{2+} measurements. Gilroy *et al.* (1991) point out that the fluorescence signal from the thin layer of cytoplasm between the vacuole and the plasmalemma is extremely weak, below the threshold set for accurate data collection in the imaging measurements. Localized increases in Ca^{2+} in the region of cytoplasm immediately inside the plasmalemma might well be adequate to trigger flux changes, but not detectable with present methods of measurement. For the time being, this remains a possibility.

A very different pattern of ABA-induced changes in cytoplasmic Ca^{2+} was found by Schroeder & Hagiwara (1990*b*), who made photometric measurements of cytoplasmic Ca^{2+} by introducing fura-2 in the patch pipette, and recording membrane current and fluorescence simultaneously. They found repetitive transient increases in cytoplasmic Ca^{2+} in response to 1 μM ABA, each lasting only a few seconds, and each associated with an inward current; the authors argue that this reflects Ca^{2+} influx through a non-selective channel, on the basis of its reversal potential of -11 mV, between E_K and E_{Ca} and well negative of the equilibrium potentials for other ions present (Mg^{2+}, Cl^-, H^+). They do not exclude a contribution from release of Ca^{2+} from internal stores in the intact cell, but argue that this is not the primary response initiating the sequence of events leading to stomatal closure in response to ABA.

Effects of ABA on both cytoplasmic Ca^{2+} and cytoplasmic pH have been observed in a range of plant tissues, in cells of corn roots, corn coleoptiles and parsley hypocotyls (Gehring *et al.* 1990). Curiously both ABA and auxin produce increases in cytoplasmic Ca^{2+}, but with opposite effects on cytoplasmic pH, which increases in response to ABA but decreases in response to auxin. Irving *et al.* (1992) show that guard cells given ABA show the same pattern of changes. Neither the cause of the pH change nor its consequences are understood, and it is clearly important to establish the relative timing of the pH- and Ca^{2+}-responses. Blatt (1992) has shown sensitivity of guard cell ion channels to cytoplasmic pH, but the integration of the various components of the ABA-response into a coherent description of the overall process remains to be done.

5. CAPACITY OF GUARD CELLS TO RESPOND TO IP_3

The importance of inositol 1,4,5-trisphosphate (IP_3) as a second messenger in animal cells is well-established, and the question of its role in plants is much debated. It is now clear that guard cells have the capacity to respond to IP_3, and it is therefore likely that this signal transduction pathway is involved in physiological responses. In two separate studies IP_3 was produced in guard cells by photolytic release from injected 'caged' IP_3. Gilroy *et al.* (1990) showed that such release

increased cytoplasmic Ca^{2+} and closed stomata, as did the release within the guard cell of Ca^{2+} from its 'caged' compound; the threshold for initiation of closure seemed to be about 600 nM Ca^{2+}. Blatt *et al.* (1990) introduced 'caged' IP_3 in the electrode in whole cell current–voltage studies, and measured the electrical changes following its photolysis to IP_3 within the cytoplasm. They observed the activation of an 'instantaneous' inward current, a component of the 'leak' or 'background' conductance, which will depolarize the cell, and the deactivation of the inward K^+ channels. There was, however, no effect on the outward K^+ channel, as to be expected from its insensitivity to Ca^{2+} shown in patch clamp work. Thus, generation of cytoplasmic IP_3 releases Ca^{2+} from internal stores, has the electrical effects to be expected from increased cytoplasmic Ca^{2+}, and induces stomatal closure. Guard cells are therefore capable of responding to this second messenger, but it remains to be established whether IP_3 has a role to play in ABA-induced stomatal closure. Preliminary results (Parmar 1991) suggest that the turnover of inositol phospholipids increases after application of ABA to *Commelina* guard cells, which would be consistent with a role for IP_3 as second messenger.

There are still questions about the nature of the IP_3-sensitive Ca^{2+} stores in plant cells. The vacuole is by far the largest store of Ca^{2+} in the plant cell, but there are also significant cytoplasmic stores, whose relative importance is not yet clear; their content may be small compared with that in the vacuole (say only about 5%), but they represent cytoplasmic concentrations in the millimolar range, rather than the sub-micromolar concentrations of free Ca^{2+}. It is clearly established that IP_3 can release Ca^{2+} from plant cell vacuoles (Ranjeva *et al.* 1988) and from tonoplast (Schumaker & Sze 1987) or microsomal vesicles (Brosnan & Sanders 1990; Canut *et al.* 1989; Drobak & Ferguson 1985; Reddy & Poovaiah 1987). A tonoplast Ca^{2+} channel which is sensitive to IP_3 has been observed in patch clamp work (Alexandre *et al.* 1990). It remains possible, however, that there is also an IP_3-sensitive Ca^{2+} channel in the endoplasmic reticulum (ER), as in animal cells. There is in addition an IP_3-insensitive Ca^{2+} channel in the tonoplast, which is voltage-sensitive over the physiological voltage range (Johannes *et al.* 1991), and the significance and role of two Ca^{2+} channels in the tonoplast remain to be clarified. In animal cells some of the subtleties of Ca^{2+}- signalling mediated through IP_3 rest on the interplay between emptying and refilling of the limited Ca^{2+} stores in ER (Berridge & Irvine 1989; Irvine 1990; Taylor & Richardson 1991). If plant cells use IP_3-sensitive channels in the tonoplast, accessing the near-infinite vacuolar store of Ca^{2+}, then such interplay will not be possible; it will be even more important to limit the extent of Ca^{2+} release by metabolic removal of the IP_3 message.

6. RESTING STATE OF GUARD CELL PLASMA MEMBRANE

Recent whole-cell electrical work on guard cells of *Vicia* (Thiel *et al.* 1992) shows that there are two distinct states of the guard cell, differing in the relative importance of the various transport systems discussed in the previous section. There is seasonal variation in the state of the cells, with cells during the summer showing much more negative membrane potentials than those in autumn and winter. The total of 43 cells examined in this study fell into two clear groups. In the low potential state cells bathed in 0.1 mM external K^+, pH 7.4, had a mean membrane potential ($\pm$ s.e.m) of -143 ± 4 mV ($n = 14$); this is positive of E_K, the potassium equilibrium potential, and the cells showed little evidence of pump activity, with the membrane dominated by diffusive processes. In contrast, most summer cells had strong pump activity, dominating the membrane properties, and the mean membrane potential in 0.1 mM external K^+ was -237 ± 10 mV ($n = 29$), well negative of E_K.

The differences between the two states were highlighted in comparisons of the I–V and G–V profiles of cells in the two states. This was most clearly seen in occasional cells which switched spontaneously between the two states, in both directions. The two features which clearly differentiated the two states, and which changed in concert, were the pump activity, active in the high potential state and little evident in the low potential state, and a voltage-insensitive leak conductance, producing an inward current at physiological potentials, which was activated in the low potential cells, but much reduced in the high potential cells. The transition to the low potential state was also associated with inhibition of the inward K^+ channel, and, at least in some cells, with activation of a voltage-sensitive channel just positive of -150 mV, likely to be the anion channel identified in patch-clamp studies. These changes resemble the effects of increased cytoplasmic Ca^{2+} in the patch-clamp work, and it is therefore tempting to speculate that the enhanced linear leak current in the low potential state involves influx of Ca^{2+}, but this remains to be established.

7. EFFECTS OF ABA ON ELECTRICAL PROPERTIES

The first demonstration of ABA-induced changes in the electrical properties of guard cells was by Blatt (1990), using whole-cell current–voltage analysis, in cells with little pump activity, whose membrane potentials before application of 10 μM ABA were already positive of E_K. The results showed an ABA-induced increase in the capacity of the outward K^+ channel to pass current, without any effect on its kinetics or voltage-dependence for activation or deactivation. The increase in steady state K^+ conductance, at voltages sufficiently positive for the channel to be active, was two- to threefold, and developed with a half-time of 1.1 ± 0.1 min ($n = 6$). There was also an increase in the leak current after treatment with ABA, and a decrease in an inward current at negative voltages, attributable to the inward K^+ channel seen previously in patch-clamp work (though at external pH 7.4 this was a less obvious component of the cell

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conductance than it would have been at lower pH). The decrease in inward current developed somewhat faster than the increase in outward K^+ current at positive voltages, with a half-time of about 0.6 min.

A more recent study (Thiel *et al.* 1992) examined the effects of ABA on the whole cell electrical properties of both high and low potential cells, and established a much more detailed description of the changes observed, and of their timecourses. The results in the rest of this section detail their findings.

In high potential cells, in which the resting membrane potential was well negative of E_K, ABA (10 μM) produced marked, prolonged depolarizations to membrane potentials which were positive of E_K; in low potential cells the ABA-induced depolarizations were small and transient. The mean time lag for ABA-induced depolarization was 83 ± 13 s ($n = 16$) (range 10–206 s). The effects of ABA were assessed in two ways, by comparison of steady state *I–V* curves before and after ABA, and by analysis of the kinetics of activation and deactivation of currents in response to suitably chosen voltage steps, allowing the separation of the slowly activating–deactivating K^+ channels from the instantaneous leak currents. Both K^+ channels were affected by ABA, with a decrease in the inward K^+ channel but stimulation of the outward rectifier. The primary cause of the ABA-induced depolarization was identified as the activation of a leak conductance, which at the pre-ABA resting potential produced an inward current, upsetting the previous balance between outward pump current and inward K^+ current. In some cells the steady state *I–V* profiles also revealed the activation by ABA of a voltage-dependent current, active in the region between about −150 mV and +50 mV. This is likely to be the voltage-sensitive anion channel identified in patch-clamp work (Hedrich *et al.* 1990), as discussed earlier.

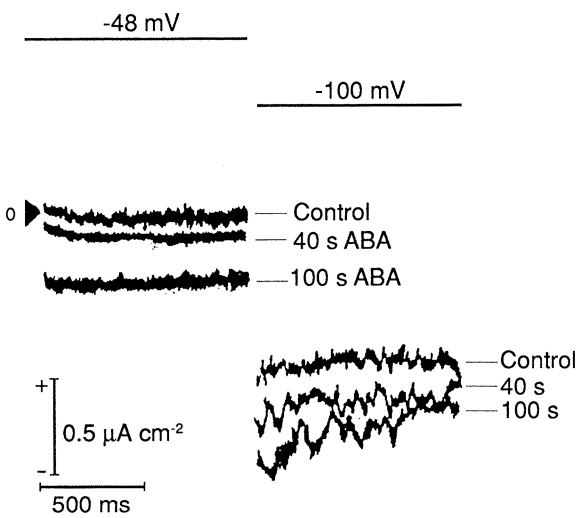

Figure 1. Effect of 10 μM ABA on the current activated by the voltage step from −48 mV (the resting potential before ABA) to −100 mV (a potential at which the inward K^+ channel does not activate). Clamp cycles before ABA and after 40 s and 100 s of ABA treatment. Guard cell of *Vicia faba* in 10 mM KCl, pH 6.1. Data obtained by Thiel *et al.* (1992).

The changes in the steady state *I–V* curves produced by ABA were in fact very similar to those seen in the transition from the high to the low potential state, and are likely to reflect very similar patterns of change in the transport systems. More insight into the nature of the changes is gained by looking at the changes in instantaneous and time-dependent current in response to voltage steps, and in particular, at the time course with which the distinguishable current components respond to ABA.

For example, the changes in instantaneous current can be seen from a clamp protocol like that shown in figure 1, in which a cell is clamped in a series of cycles before and after the application of ABA, in which it is held for 1 s at −48 mV (the pre-ABA resting potential) followed by 1 s at −100 mV, a potential which is still more positive than the activation voltage for the inward K^+ channel. Figure 1 shows the development of an inward current at −48 mV, the pre-ABA resting potential, and an increase in the instantaneous inward current at −100 mV, i.e. it shows the activation of an inward current, a component of the leak or background current. This change was also reflected in a depolarization of the resting potential by 5 mV after ABA treatment. It should also be noted from figure 1 that at longer times of ABA treatment there was slow inactivation of the current at −100 mV. The timecourse for the appearance of the inward current at −48 mV is shown in figure 2, and is identical to the timecourse for the increase in instantaneous current at −100 mV; in five experiments the time lags for appearance of this increased leak current were in the range 12 to 85 s after application of ABA to the guard cell.

The activation of the outward K^+ channel is slower than the response of the leak current, and is therefore a secondary rather than the primary response to ABA. This is shown in figure 3, the results of an experiment in which the cell was given a three-stage clamp sequence, from −35 mV (the pre-ABA resting potential), to −100 mV (to measure the leak current

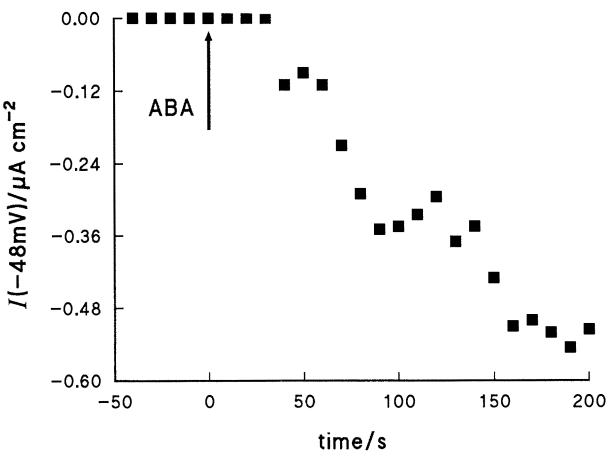

Figure 2. Timecourse for the development of ABA-activated inward current for the cell subjected to clamp cycles as in figure 3. Current at −48 mV, the resting potential before treatment with ABA, plotted against time. Data obtained by Thiel *et al.* (1992).

without activating the inward K^+ channels), and finally to 0 mV (when the response will show the instantaneous change in leak current, followed by the slow activation of the outward K^+ channels). Comparison of the currents before ABA and after 94 s of ABA treatment shows that the leak current (inward at -100 mV, outward at 0 mV) has increased, whereas the outward K^+ current (the slowly activating component at 0 mV) has not yet changed.

Thus five changes are identified in ABA: (i) depolarization of cells in the high potential state to potentials which are positive of E_K; (ii) activation of an inward current, a voltage-insensitive component of the leak conductance, which is responsible for the depolarization, (iii) deactivation of the inward K^+ channel; (iv) activation (in at least some cells) of a voltage-sensitive channel carrying inward current, probably the voltage-sensitive, Ca^{2+}-sensitive anion channel identified in patch-clamp experiments; and (v) slower activation of the outward K^+ channel, seen as an increase in its capacity to carry current, without any change in the voltage-dependence or kinetics of its activation–deactivation.

The changes in inward K^+ channel, in the leak conductance, and in the voltage-sensitive anion channel are also produced by increase in internal Ca^{2+}, as discussed in earlier sections, whether such increase is produced by increasing Ca^{2+} in the patch pipette, by large increases in external Ca^{2+}, or by IP_3-induced release of Ca^{2+} from internal stores. It seems likely therefore that these ABA-induced electrical changes are the consequence of an ABA-induced increase in cytoplasmic Ca^{2+}, and that such an increase is a universal feature of the ABA response. There remains, however, the activation of the outward K^+ channel, which is slower than the other changes, and which is not Ca^{2+}-sensitive. Irving *et al.* (1992) found increases in both cytoplasmic Ca^{2+} and cytoplasmic pH in guard cells of the orchid *Paphiopedilum*, in response to treatment with ABA. Typical changes were an increase from 0.24 μM to 0.53 μM for cytoplasmic Ca^{2+}, and an increase in cytoplasmic pH by 0.1–0.2 pH units. In view of the sensitivity of the outward K^+ channel to cytoplasmic pH (Blatt 1992), it is tempting to argue for a causal relationship, to attribute the activation of the outward K^+ channel to increase in cytoplasmic pH, in extended signal transduction mechanisms. It will be critical to compare the timecourses with which the various changes develop. Irving *et al.* (1992) show a lag of about 2 min before the cytoplasmic pH starts to rise after treatment with ABA, but it is important to define this more closely, in experiments in which the electrical changes are also measured. It is also necessary to identify the cause of the pH change; the electrical measurements suggest that stimulation of the pump is not responsible, and activation of some biochemical process which consumes H^+ (such as decarboxylation of malate) seems a more likely alternative. Possible mechanisms for linking whatever process is responsible to the ABA signal or its primary consequences remain to be considered.

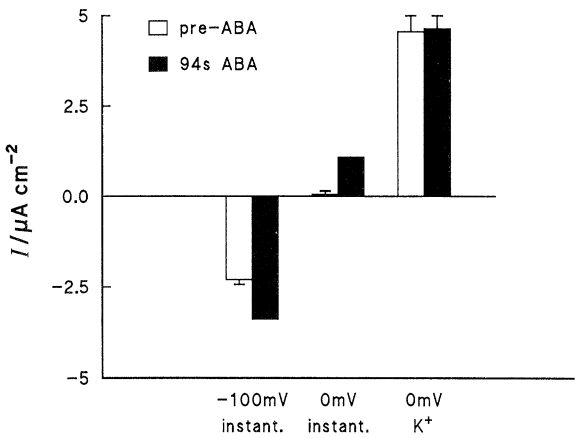

Figure 3. Differences in time course for development of different ABA-induced current changes. A *Vicia faba* guard cell in 10 mM KCl, pH 6.1, was subjected to a three stage clamp protocol, in which the voltage was held for 1 s at -35 mV, the pre-ABA resting potential, followed by 1 s at -100 mV, followed by 1 s at 0 mV. On transition to -100 mV only an instantaneous current changes, a component of the leak conductance, since the inward K^+ channels do not activate at this voltage. On stepping to 0 mV the current activation can be separated into a fast (instantaneous) change, the change in leak current, and a slowly activating K^+ current. The figure shows instantaneous currents i.e. leak currents, at -100 mV (inward) and 0 mV (outward), and outward K^+ current at 0 mV, before ABA treatment and after 94 s in ABA. Pre-ABA figures are the mean values of currents collected over a 120 s period before ABA was added, to be compared with the currents measured after 94 s of ABA treatment. At this time the leak current has increased, but the outward K^+ channel has not yet activated. Data obtained by Thiel *et al.* (1992).

8. NATURE OF THE PRIMARY RESPONSE

The electrical measurements provide a description of the changes and their timecourses, but a major question that remains is the nature of the primary response, the activation of the inward current, and the identification of the channel involved. The current could be carried by anion efflux or by a channel capable of Ca^{2+} influx (whether specific for Ca^{2+} or not) and it should be stressed that it is not yet possible to distinguish clearly between these two possibilities, particularly since each will have similar secondary consequences in the cell.

Blatt (1991) discusses both possibilities, and comes down in favour of an anion channel, but one which is distinct from the Ca^{2+}- and voltage-sensitive anion channel identified by patch clamping. That channel is not a good candidate for the primary response, since its activation voltage of around -100 mV is much more positive than the resting membrane potential commonly observed in high potential cells. Opening of another channel capable of depolarizing the membrane potential into the range of the voltage-sensitive anion channel must precede its activation.

However the evidence indicates that a non-selective cation channel capable of carrying Ca^{2+}, as suggested by Schroeder & Hagiwara (1990*b*) is more likely. Their argument rested on the reversal potential of

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−11 mV for the inward current transient associated with the Ca^{2+} spike observed in the cytoplasm; this voltage is intermediate between E_{Ca} and E_K, but well negative of the anion equilibrium potential. The reversal potential for the primary leak current activated by ABA in whole cells would also suggest a non-selective cation channel rather than an anion channel (Thiel *et al.* 1992). Extrapolation of the linear leak current in the *I–V* profiles after ABA gave reversal potentials of −26 mV in 3 mM KCl and −4 mV in 10 mM KCl; essentially the same values were obtained by estimation of the reversal potential for the instantaneous current in response to voltage steps, clamping to voltages in the range −250 mV to +50 mV. The shift of the reversal potential to more positive voltages in KCl implies that the channel can carry K^+, but the siting of the reversal well positive of E_K shows the channel to be non-selective. Although the results are consistent with the opening of a channel carrying both K^+ and Ca^{2+}, producing both Ca^{2+} influx and depolarization out of the activation range of the inward K^+ channel, and into the range of the outward K^+ channel, the nature of the primary response is not yet conclusively established. Unstimulated Ca^{2+} influx is itself voltage-sensitive, activated by depolarization (MacRobbie 1989), and hence the primary depolarization, however produced, will induce Ca^{2+} influx, with further depolarization and an increase in cytoplasmic Ca^{2+}, further reinforcing the deactivation of the inward K^+ channel. Thus Ca^{2+} influx is likely to be a part of the ABA response, and may indeed be the primary initiating process, but nevertheless it may not be the source of the major increase in cytoplasmic Ca^{2+}. IP_3-induced release of Ca^{2+} from cytoplasmic stores may either supplement, or swamp, Ca^{2+} influx at the plasmalemma. Study of ABA effects in conditions of reduced Ca^{2+} influx should clarify this question, but have not yet provided a definitive answer.

9. ABA-INDUCED EFFLUX TRANSIENTS IN CONDITIONS OF REDUCED Ca^{2+} INFLUX

One way of trying to assess the relative importance of Ca^{2+} influx and internal Ca^{2+} release in the signal transduction chain is to measure ABA-induced efflux transients in conditions in which Ca^{2+} influx is impaired. This may be done in two ways, by drastically reducing external Ca^{2+} or by including La^{3+}, which will inhibit Ca^{2+} influx, in the external solution.

MacRobbie (1990) showed that a large ABA-induced efflux transient could be produced with very low external Ca^{2+}. Figure 4 shows the rates of $^{86}Rb^+$ efflux in one such experiment, in which the ABA-induced efflux transients were measured in replicate sets of tissue, in 0.1 mM external Ca^{2+} throughout, and in tissue transferred from 0.1 mM Ca^{2+} to 0.5 μM Ca^{2+} (BAPTA-buffered) for 4 min before adding ABA with 0.5 μM Ca^{2+}. In the absence of ABA the $^{86}Rb^+$ efflux was increased at low Ca^{2+} (2.4 fold), and there was then a further very large stimulation of efflux on adding ABA. The relative stimulation of efflux in the

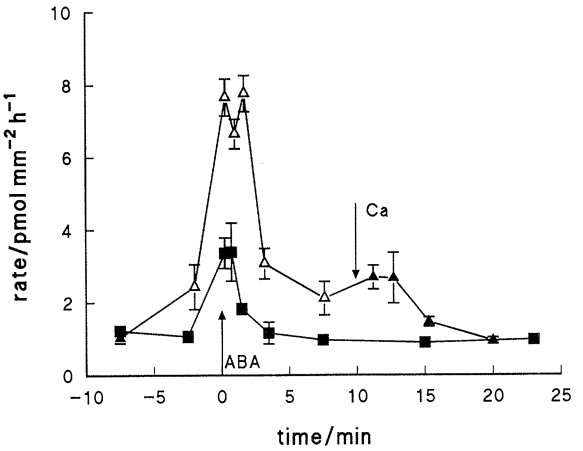

Figure 4. Comparison of efflux transients in guard cells of *Commelina communis* produced by 10 μM ABA in 0.1 mM external Ca^{2+} throughout, or in 0.5 μM Ca^{2+} increased to 0.1 mM after 10 min in ABA. Rate of $^{86}Rb^+$ efflux (on the basis of area of epidermal strip) plotted against time. Closed symbols, 0.1 mM external Ca^{2+}; open symbols, 0.5 μM Ca^{2+} (BAPTA-buffered). Each point shows the mean of four strips, and the standard error where this is larger than the symbol. Tissue was loaded overnight in ^{86}Rb-labelled solution containing: (in millimoles per litre) RbCl, 4; $MgCl_2$, 1; $CaCl_2$, 0.1; PIPES, 10; pH 6.8. Rate of loss of ^{86}Rb to successive portions of inactive solution of the same composition was then measured, before adding ABA after 40 min of wash-out (zero time on graph); for the low Ca^{2+} transient Ca^{2+} was reduced from 0.1 mM to 0.5 μM (BAPTA-buffered) 4 min before adding ABA.

ABA-peak (the maximum rate relative to the pre-ABA rate) was not significantly different at 0.1 mM Ca^{2+} and 0.5 μM Ca^{2+} (3.3 ± 0.3 (n = 4) and 3.6 ± 0.6 (n = 4) respectively). Thus the efflux transient can occur even at very low external Ca^{2+}, suggesting that Ca^{2+} influx is not the rate-limiting step in triggering the response. However it should also be noted from figure 4 that when Ca^{2+} was restored to 0.1 mM in the low Ca^{2+} transient, after 10 minutes in ABA, there was an increase in efflux (the opposite effect from that seen on changing Ca^{2+} in the absence of ABA); this suggests that Ca^{2+} influx can contribute to the events which are important in the efflux transient.

The addition of La^{3+} to the external solution provides an alternative way to block Ca^{2+} influx, and results from an experiment of this kind are shown in figure 5. In this experiment the tissue was pre-treated with 0.4 mM La^{3+} before ABA was added, and the efflux transient in the presence of La^{3+} was compared with that without the addition of La^{3+}. Figure 5 shows the mean ± s.e.m. for four control epidermal strips, and the individual efflux curves for the four La^{3+}-treated strips. The effect of La^{3+} is variable, but only one strip has an efflux transient like that of the control; one other has an efflux peak similar to that in the control but the high rate is not then sustained for the normal period, and two strips have a very much smaller transient. Rates of efflux are plotted in figure 5, but it should be noted that the addition of La^{3+} produced a marked inhibition of $^{86}Rb^+$ efflux before

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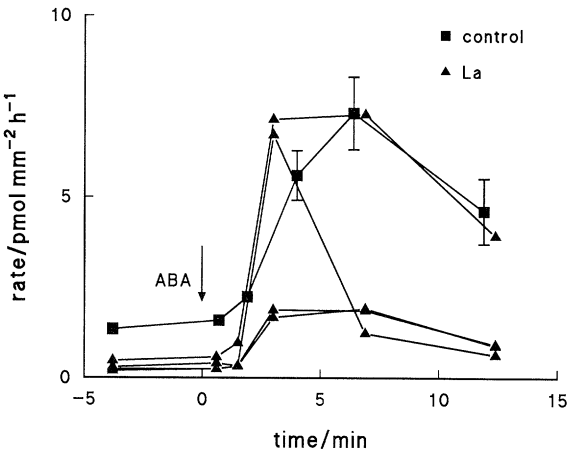

Figure 5. Effect of 0.4 mM La^{3+} on the ABA-induced $^{86}Rb^{+}$ efflux transient in isolated guard cells of *Commelina*. Rate of $^{86}Rb^{+}$ efflux against time. Tissue was loaded for 16.3–17.7 h in 2 mM $^{86}RbCl$, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM PIPES, pH 6, followed by wash-out in unlabelled solution of the same composition. In the control tissue 10 μM ABA was added after 40 min of wash-out (zero time on graph); for the La^{3+}-treatment 0.4 mM $LaCl_3$ was added after 34 min of wash-out, and 10 μM ABA 17 min later. The control shows the mean ± s.e.m. of four strips, but each of the four La^{3+}-treated strips is shown individually. The pre-La^{3+} rate was not significantly different from that in the control, but La^{3+} treatment reduced efflux, before ABA was added, by a factor of about 3.6.

ABA was added, and that the relative stimulation of the efflux by ABA in La^{3+} was therefore higher than that in the control, even in the smaller peaks.

Changes in the efflux transient were seen in three other experiments with La^{3+}, but the effects were variable, both between experiments and between individual strips within an experiment. These results will be presented fully in a future paper. Nevertheless, in spite of the variability, the experiments with La^{3+} suggest that influx of Ca^{2+} does play a role in initiating and maintaining the ABA-induced efflux transient, particularly as the inhibition of Ca^{2+} influx by La^{3+} is unlikely to be complete.

Thus the results of flux experiments designed to interfere with Ca^{2+} influx during the efflux transient do not provide a clear answer to the question of the role of Ca^{2+} influx; they suggest that Ca^{2+} influx makes a contribution to increasing cytoplasmic Ca^{2+}, but that release of internal Ca^{2+} must also play a role. Some cooperative interaction between the two may well be involved.

10. ROLE OF PHOSPHOINOSITIDE SIGNALLING IN THE ABA-RESPONSE

The work discussed in previous sections suggests that internal release of Ca^{2+} may be a part of the ABA-induced signal transduction chain in guard cells, and provides evidence that guard cells are competent to respond to cytoplasmic IP_3, by increasing cytoplasmic Ca^{2+}, by showing some (but not all) of the electrical changes seen in the ABA response, indicating modifications of the activity of several ion channels, and by stomatal closure. The question still remains as to whether IP_3 is involved in the physiological response to ABA, whether it triggers internal Ca^{2+} release during that response. Recent work suggests that the phosphoinositide signalling pathway is indeed involved, and that conversion of inositol phospholipids to soluble inositol phosphates occurs very early in the chain of ABA-induced events.

In this work Parmar (1991) labelled isolated guard cells with *myo*-[2n-^{3}H]inositol for 17–18 h, then extracted phospholipids and inositol phosphates, and used high-performance liquid chromatography (HPLC) to resolve and identify individual labelled compounds. The work shows that guard cells do contain the various components of the polyphosphoinositide signalling pathway, namely inositol phospholipids, phosphatidylinositol (PtdIns) and a number of its phosphorylated derivatives, isomers of PtdIns*P* and PtdInsP_2, and also soluble inositol phosphates, including inositol monophosphates, bisphosphates, trisphosphates, tetrakisphosphates, pentakisphosphates, and hexakisphosphate. Of particular interest is the finding that guard cells contain not only the components of the conventional phosphoinositide signalling pathway (PtdIns, PtdIns(4)*P*, PtdIns(4,5)P_2), but also the 3-phosphorylated inositol phospholipids PtdIns(3)*P*, PtdIns(3,4)P_2. This may suggest that another phosphoinositide signalling pathway involving the 3-phosphorylated inositol phospholipids also exists in guard cells. The 3-phosphorylated inositol phospholipids have been detected in a number of animal cells (see review by Majerus *et al.* (1990)), and PtdIns(3)*P* has been detected in plants in *Spirodela polyrhiza* (Brearley & Hanke 1992), but their role remains uncertain.

Parmar then looked for changes in the labelling pattern at short times of ABA treatment, to establish whether turnover in the phosphoinositide cycle was associated with the ABA response. In these experiments isolated guard cells were labelled for 17–18 h, and half the tissue was then treated with 10 μM ABA for short periods of time (30–130 s) before freezing the tissue, followed by extraction and separation of labelled compounds by HPLC. The distribution of label was then compared in the control and ABA-treated tissue. Because at the end of the labelling period the radioactivity and its distribution in different compounds should be identical in the two batches of tissue, any differences in the pattern of labelling can be attributed to events during the 30–130 s of ABA treatment. The tissue is very far from equilibrium labelling, which complicates the interpretation of the changes observed. In fact the initial specific activity (before ABA treatment) must have been much higher in the soluble inositol phosphates than in the membrane phospholipids (as judged from the size of the labelled pools relative to the likely total pools of lipids and solubles). This means that it is not possible to get quantitative estimates of any flow in the phosphoinositide cycle, since changes will reflect the balance of transfer of label at low specific activity from inositol phospholipid to soluble inositol phosphates, and at higher specific activity from soluble to lipid to replenish

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the lipid pools. Nevertheless qualitative indications of considerable turnover in the cycle following ABA treatment can be obtained from the results.

The results were assessed by comparing the fraction of label in phospholipids and in soluble inositol phosphates, in control and ABA-treated tissue, using the total label in inositol phospholipids plus inositol phosphates in the control tissue as the basal value for both sets of tissue. Thus the radioactivity in phospholipids and in inositol phosphates (d.p.m. per square millimetre of epidermal strip), in both control and ABA-treated tissue, were expressed as percentages of the total radioactivity (d.p.m. mm^{-2}) in the control tissue. The results were expressed in this way because in four out of five experiments there was a significant increase (by 40 to 50%) in the total label in the ABA treatment, relative to that in the control. It is argued that this is a consequence of turnover in the phosphoinositide cycle, involving flow of low specific activity label out of inositol phospholipids but of higher specific activity label from the *myo*-inositol pool to the inositol phospholipids. Thus once the return flow is established, turnover in the cycle will result in an increase in radioactivity in PtdIns, even if the net chemical flow involves hydrolysis of PtdInsP_2 to produce IP_3. This is borne out by examination of the time course of changes in radioactivity in inositol phospholipids and inositol phosphates. Figure 6 shows the label in inositol phospholipids in control and ABA-treated tissue, as a percentage of total label in the control, in five experiments, with ABA treatments of 30–130 s; for each time point, the control and ABA treated tissue are replicate samples of the same tissue, given the same labelling treatment. At 30 s there is a large increase in label in the ABA-treated tissue, but at 40 s this falls below the control value,and rises again at 80 s and 130 s. The corresponding changes in inositol phosphates are shown in figure 7 for the same experiments; this shows a delayed rise relative to the phospholoipid changes, with large increases in the percentage label in the ABA-treated tissue at 40 s (in one of the two experiments at that time), and at 80 and 130 s.

Given the relative specific activities in membrane phospholipids and soluble inositol phosphates and free *myo*-inositol in the tissue before ABA was added, these results are consistent with considerable flow in the phosphoinositide cycle following ABA treatment, starting before 30 s and continuing at a high rate in the period 30 to 80 s. Thus the evidence suggests that this signalling pathway is involved in the ABA response, and that IP_3 mobilization is an early event in the sequence.

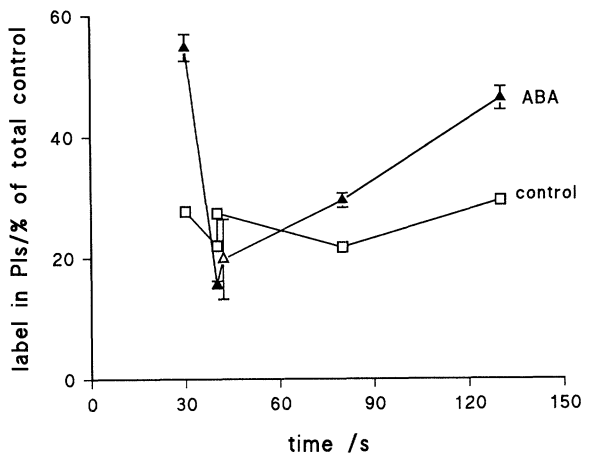

Figure 6. Changes in label in inositol phospholipids after treatment with 10 μM ABA for specified times. Epidermal strips containing isolated guard cells of *Commelina communis* were labelled with *myo*-[2n-^{3}H]inositol for 17–18 h, and washed to remove extracellular label. Half the tissue was then treated with 10 μM ABA for periods of 30 to 130 s before freezing the tissue, followed by extraction and separation by HPLC of labelled compounds. The radioactivity in inositol phospholipids and inositol phosphates was measured in control and ABA-treated tissue, and expressed as a percentage of the total label, in inositol phospholipids plus inositol phosphates, in the control tissue. This percentage is plotted against time of ABA treatment. Each time represents a separate experiment, with analysis of replicate samples of tissue for control and ABA-treatment. Errors are calculated from recovery of specific labelled compounds and from counting errors. Two experiments were done at 40 s, but are shown displaced from each other for clarity; in one of these a fraction of phospholipid extract was lost in the ABA treatment, and the range of possible values is shown rather than an exact figure.

11. DISCUSSION

We now have a partial understanding of the events involved in ABA-induced stomatal closure, and the role of Ca^{2+} in that process, but the full details are not yet established, and there are gaps in our knowledge. Most important, we do not yet have a clear picture of

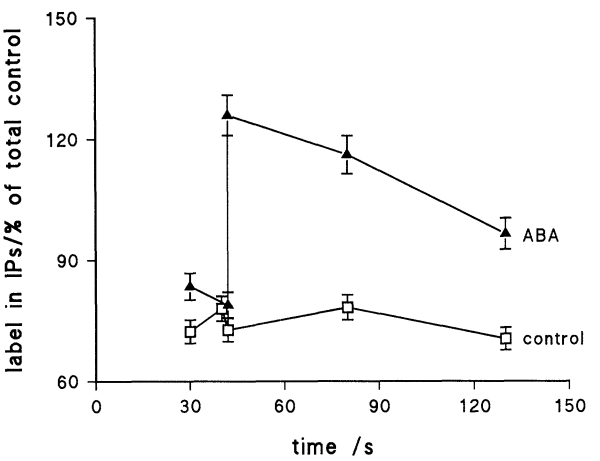

Figure 7. Changes in labelling in inositol phosphates after treatment with 10 μM ABA for specified times. Experimental procedure as in figure 8. The radioactivity in inositol phosphates was measured, in control and ABA-treated tissue, and expressed as a percentage of total label (in inositol phospholipids plus inositol phosphates), in the control tissue. This percentage is plotted against time of ABA treatment. Each time represents a single experiment, with analysis of replicate samples of tissue for control and ABA-treated tissue. Two experiments were done at 40 s, but are shown displaced from each other for clarity.

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the time sequence of the various ABA-induced events, involving different ion channels, and of their causal relationships.

In my view the evidence suggests that rise in cytoplasmic Ca^{2+} is a universal feature of the ABA signal transduction chain, and is responsible for some, but not all, of the observed electrical effects; thus it is responsible for the deactivation of the inward K^+ channel and for the activation of the voltage-sensitive anion channel. Ca^{2+} is not, however, responsible for the slower activation of the outward K^+ channel, for which some other trigger must be found. The increase in cytoplasmic pH is the most likely candidate for this activation, but the mechanism by which that pH change is produced remains unknown. The question of whether the early activation of some component of the leak conductance is a consequence of increase in cytoplasmic Ca^{2+}, or the cause of that increase (in whole or in part), is also uncertain.

With respect to the pH change it is interesting that there is an increase in intracellular pH associated with *Xenopus* egg activation, seen as a wave of pH increase spreading from the site of activation, which follows closely on a Ca^{2+} wave spreading from the same site (Grandin & Charbonneau 1992). In *Xenopus* the mechanism of the pH change, and its link to increase in cytoplasmic Ca^{2+}, are unknown. Taylor & Richardson (1991) point out that the interrelations of cytoplasmic pH and IP_3-induced Ca^{2+} release are complex, and not understood. In many animal cells alkalinization of the cytoplasm is attributable to activation of Na^+/H^+ countertransport following stimuli which activate protein kinase C (by diacylglycerol, the other messenger produced by breakdown of $PtdInsP_2$ to give IP_3). However in plant cells the Na^+/H^+ exchanger operates in the reverse direction, coupling influx of H^+ to efflux of Na^+, and hence could not be invoked as a cause of cytoplasmic alkalinization. Taylor & Richardson point out three ways in which cytoplasmic pH may influence IP_3-induced Ca^{2+} mobilization in animal cells: (i) binding of $Ins(1,4,5)P_3$ to its receptor is pH-sensitive, favoured at high pH; (ii) the size of the IP_3-releasable pool of Ca^{2+} may be sensitive to pH; and (iii) in aortic endothelial cells intracellular alkalinization can cause Ca^{2+} mobilization even at resting levels of $Ins(1,4,5)P_3$ (Danthuluri *et al.* 1990). However these effects would be relevant to a pH change which preceded the Ca^{2+} change, whereas Grandin & Charbonneau (1992) found that, at least in *Xenopus* eggs, the pH wave follows, rather than precedes the Ca^{2+} wave. In guard cells it is clearly critical to establish unequivocally the time sequence of pH, Ca^{2+}, and consequent electrical changes, and to identify the mechanisms producing alkalinization.

The results discussed earlier in this paper suggest that increase in cytoplasmic Ca^{2+} is inherent in the ABA-response, but they do not provide clear answers to two critical questions, namely the relative importance of influx of Ca^{2+} at the plasmalemma and release of Ca^{2+} from internal stores, and the nature of the primary response, the first identifiable change in response to ABA. Three early events are identified, the stimulation of $^{86}Rb^+$ efflux in tracer experiments, the activation of an unidentified component of the leak or background conductance producing an inward, depolarizing, current at the resting potential, and turnover in the phosphoinositide cycle. These occur within the first minute, and precede activation of the outward K^+ channel, but it is still unclear which occurs first. Nor is it clear which of these events are consequences, and which are causes, of the others.

A plausible scheme can be drawn up, in which the first event is the opening of a non-selective cation channel, allowing Ca^{2+} influx, thereby producing both depolarization and increase (possibly local) in cytoplasmic Ca^{2+}. Deactivation of the inward K^+ channel and activation of the voltage-dependent, Ca^{2+}-dependent anion channel will then follow. It is possible also to envisage that the initial increase in cytoplasmic Ca^{2+} may stimulate phospholipase C, with release of $Ins(1,4,5)P_3$ as second messenger triggering the release of internal Ca^{2+}, further reinforcing the increase in cytoplasmic Ca^{2+}. However an alternative scheme could also be devised, with the reverse time sequence, in which stimulation of phospholipase C is the earliest event, and increase of cytoplasmic Ca^{2+}, arising from the discharge of internal stores, is then the trigger for the electrical changes at the plasmalemma. The flux experiments show that ABA-induced efflux transients can occur at very low external Ca^{2+}, but the (variable) reduction of the efflux transient in the presence of La^{3+} could be taken as evidence for a critical role for Ca^{2+} influx in the response, and as an argument for the first of these schemes, in which Ca^{2+} influx initiates the sequence (even if it is not the only source for the increase in cytoplasmic Ca^{2+}). It must be hoped that future work will allow a clearer distinction between the possibilities, to allow firm conclusions to be drawn.

In spite of the diverse results in the studies in which changes in cytoplasmic Ca^{2+} in response to ABA were investigated by fluorescence techniques, some of which throw doubt on the universality of the role of Ca^{2+} in ABA-induced stomatal closure, the evidence of the electrical work seems strongly in favour of the view that Ca^{2+} has an integral role in the process, that ABA-induced stomatal closure necessarily involves increases in cytoplasmic Ca^{2+}, at least locally in the region of the plasmalemma. Nevertheless our description of the overall process and its component parts remains very incomplete, and a definitive time sequence, with understanding of the causal relationships, is not yet available.

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