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PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Signal transduction and ion channels in guard cells

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Our understanding of the signalling mechanisms involved in the process of stomatal closure is reviewed. Work has concentrated on the mechanisms by which abscisic acid (ABA) induces changes in specific ion channels at both the plasmalemma and the tonoplast, leading to efflux of both K^+ and anions at both membranes, requiring four essential changes. For each we need to identify the specific channels concerned, and the detailed signalling chains by which each is linked through signalling intermediates to ABA. There are two global changes that are identified following ABA treatment: an increase in cytoplasmic pH and an increase in cytoplasmic Ca^{2+}, although stomata can close without any measurable global increase in cytoplasmic Ca^{2+}. There is also evidence for the importance of several protein phosphatases and protein kinases in the regulation of channel activity.

At the plasmalemma, loss of K^+ requires depolarization of the membrane potential into the range at which the outward K^+ channel is open. ABA-induced activation of a non-specific cation channel, permeable to Ca^{2+}, may contribute to the necessary depolarization, together with ABA-induced activation of S-type anion channels in the plasmalemma, which are then responsible for the necessary anion efflux. The anion channels are activated by Ca^{2+} and by phosphorylation, but the precise mechanism of their activation by ABA is not yet clear. ABA also up-regulates the outward K^+ current at any given membrane potential; this activation is Ca^{2+}-independent and is attributed to the increase in cytoplasmic pH, perhaps through the marked pH-sensitivity of protein phosphatase type 2C.

Our understanding of mechanisms at the tonoplast is much less complete. A total of two channels, both Ca^{2+}-activated, have been identified which are capable of K^+ efflux; these are the voltage-independent VK channel specific to K^+, and the slow vacuolar (SV) channel which opens only at non-physiological tonoplast potentials (cytoplasm positive). The SV channel is permeable to K^+ and Ca^{2+}, and although it has been argued that it could be responsible for Ca^{2+}-induced Ca^{2+} release, it now seems likely that it opens only under conditions where Ca^{2+} will flow from cytoplasm to vacuole. Although tracer measurements show unequivocally that ABA does activate efflux of Cl^- from vacuole to cytoplasm, no vacuolar anion chnnel has yet been identified.

There is clear evidence that ABA activates release of Ca^{2+} from internal stores, but the source and trigger for ABA-induced increase in cytoplasmic Ca^{2+} are uncertain. The tonoplast and another membrane, probably ER, have IP_3-sensitive Ca^{2+} release channels, and the tonoplast has also cADPR-activated Ca^{2+} channels. Their relative contributions to ABA-induced release of Ca^{2+} from internal stores remain to be established. There is some evidence for activation of phospholipase C by ABA, by an unknown mechanism; plant phospholipase C may be activated by Ca^{2+} rather than by the G-proteins used in many animal cell signalling systems.

A further ABA-induced channel modulation is the inhibition of the inward K^+ channel, which is not essential for closing but will prevent opening. It is suggested that this is mediated through the Ca^{2+}-activated protein phosphatase, calcineurin.

The question of Ca^{2+}-independent stomatal closure remains controversial. At the plasmalemma the stimulation of K^+ efflux is Ca^{2+}-independent and, at least in *Arabidopsis*, activation of anion efflux by ABA may also be Ca^{2+}-independent. But there are no indications of Ca^{2+}-independent mechanisms for K^+ efflux at the tonoplast, and the appropriate anion channel at the tonoplast is still to be found.

There is also evidence that ABA interferes with a control system in the guard cell, resetting its set-point to lower contents, suggesting that stretch-activated channels also feature in the regulation of guard cell ion channels, perhaps through interactions with cytoskeletal proteins. There is evidence for involvement of actin in the control of guard cell ion channels, although possible mechanisms are still to be identified.

Stomatal closure involves net loss of vacuolar sugars as well as potassium salts, and there is an urgent need to address the question of the nature of the signalling chains linking transport and metabolism of sugars to the closing signal.

Keywords: guard cells; calcium; ABA; stretch-activated channels; phosphoinositols; signalling

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1. INTRODUCTION

Stomatal guard cells are among the most studied and best understood of signalling systems, yet there remain considerable gaps in our knowledge and in our attempts to construct a detailed description of the events and underlying signalling chains involved in the processes of stomatal opening and closing. The processes which are special to guard cells are those involved in stomatal closure, when in response to various environmental signals the guard cell loses the ability to maintain its high vacuolar solute content and volume, with consequential cell shrinkage, loss of turgor, shape change, and closure of the stomatal pore. This lability of vacuolar state is also a feature of motor cells of pulvini, which undergo large changes in vacuolar volume causing the associated leaf movements, either in sleep movements of leaves, or in mechanically generated movements in the sensitive plant, *Mimosa*, but such lability is uncommon as a feature of mature cells. This cycling between two stable states of the cell, between a state with a large central vacuole occupying nearly all the cell volume and one with many small vacuoles (or a fragmented vacuole) of relatively small volume, is the key to guard cell function, and the shrinkage phase in the closing response is the unexpected but characteristic pattern, the essential problem we need to understand. The aim of this paper is to consider the events involved in the process of stomatal closure and the extent to which the detailed signalling chains have been identified, and to highlight the areas in which the descriptions are incomplete. In some cases this concerns gaps in identified signalling chains leading to clearly identified end results, the activation and inactivation of specific ion channels, but in others neither the nature of the channel responsible nor the means of its modulation are clear. In some cases new elements, additional transport proteins and their modulating agents, are required to complete the overall description.

Stomatal opening is achieved by accumulation of high levels of solute, largely vacuolar. A large fraction of this solute is potassium salt, but sucrose is also involved. Recent studies (Amodeo *et al.* 1996; Talbott & Zeiger 1996) have emphasized the importance of sucrose in guard cells, and have shown that in the normal diurnal cycle stomatal opening in the morning is the result of accumulation of potassium salts, but that by afternoon a significant fraction of these have been replaced by sucrose, at more or less constant aperture and therefore constant total solute content. The process of stomatal closure is the result of massive loss of solute, largely from the vacuole. Again a large fraction of this solute is potassium salt, but sucrose must also be involved. Attention has focused on the mechanisms of regulation of the potassium salt content, and the means of regulation of ion channels in both the plasmalemma and tonoplast by which net loss of potassium and anions, from the vacuole and from the cell, can be achieved. Our understanding of the processes rests on the synthesis of information from a range of techniques, from electrophysiological studies (patch-clamping of the plasmalemma of guard cell protoplasts and of the tonoplast of isolated vacuoles, whole cell impalement, measuring electrical properties of the plasmalemma in intact cells), from the use of fluorescent dyes to measure changes in cytoplasmic Ca^{2+} and cytoplasmic pH, and from tracer flux studies providing evidence of flux changes at both the plasmalemma and tonoplast of isolated guard cells. There are two 'closing' signals that have been investigated, to varying degrees; the effects of abscisic acid (ABA, the drought hormone) have been investigated by the full range of techniques, in some detail, and more limited observations have been made on the effects of high CO_2.

2. CHANGES NECESSARY FOR STOMATAL CLOSURE

The end result is the loss of both K^+ and associated anion (Cl and/or malate depending on the plant and the conditions), both from the cell across the plasmalemma, and from the vacuole to the cytoplasm across the tonoplast. We therefore need to understand how the closing signal (ABA or high CO_2) is transduced via an identified signalling chain, into up-regulation of the outward fluxes of both potassium and anions at both the plasmalemma and the tonoplast. Thus four changes are necessary, two at each membrane. In each case we need to identify an appropriate channel and the agent for its activation, but it is also necessary, for channels which are voltage-dependent, that the voltage across the membrane concerned is within the range for opening of that channel; if the resting voltage is outside the required range, then we need to identify other ion currents whose activation can alter the membrane voltage, to bring it within the range required for ion efflux through the K^+-permeable and anion-permeable channels of the plasmalemma and tonoplast. Down-regulation of the inward fluxes of K^+ and anions is not essential for stomatal closure, but will speed the process driven by stimulation of the efflux processes.

There is clear evidence from tracer flux studies that all of these changes do follow the application of ABA to guard cells, that for both anions and cations there is stimulation of efflux at the plasmalemma, and of the flux from the vacuole to the cytoplasm.

3. ABA-INDUCED CHANGES IN GUARD CELLS

We need to identify both specific ion channels which respond to ABA, and potential signalling intermediates. There are indications of the importance of Ca^{2+} and of control of protein phosphorylation–dephosphorylation in these signalling chains, involving protein kinases and all types of protein phosphatase, PP1–2A, PP2B, and PP2C. Such evidence is derived from work using inhibitors of protein kinases or specific protein phosphatases, but also from characterization of two ABA-insensitive mutants of *Arabidopsis*, *abi-1* and *abi-2*, shown to be deficient in type 2C protein phosphatase (Leung *et al.* 1994, 1997; Meyer *et al.* 1994; Bertauche *et al.* 1996; see also review by Merlot & Giraudet 1997). Our understanding of changes at the tonoplast, and associated signalling chains, is much weaker, in respect of either the nature of the channels or of their modulation than our understanding of plasmalemma processes.

(a) *Cytoplasmic Ca^{2+} and pH*

There is good evidence that ABA can produce a measurable increase in cytoplasmic Ca^{2+} (McAinsh *et al.* 1990, 1992; Gilroy *et al.* 1991), and that increase in cytoplasmic

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Ca^{2+} above 500 nM produces stomatal closure, but equally that closure can be observed in guard cells in which no change in cytoplasmic Ca^{2+} can be detected. Allan *et al.* (1994) found large ABA-induced increases in Ca^{2+} in guard cells from *Commelina* grown at 25 °C and above, but none in plants grown at 15 °C and below, although these stomata still closed in response to ABA. Thus a global increase in cytoplasmic Ca^{2+} is not an essential feature of the ABA response. There are then two possibilities. The first, as suggested by Allan *et al.* (1994), is that there are Ca^{2+}-dependent and Ca^{2+}-independent signalling pathways for ABA-induced stomatal closure, that the same end results can be reached through different signalling chains. While it is quite clear that not all ABA-induced changes in ion channels are Ca^{2+}-mediated, and probable that some of the changes produced can be achieved through parallel signalling chains, only one of which features Ca^{2+}, it seems less probable that this applies to all the changes induced by ABA. The alternative view is that local increases in Ca^{2+}, close to the membrane, can act to alter activity of neighbouring Ca^{2+}-sensitive channels without translation into a global increase which is detectable by current techniques.

The other global change detectable in response to ABA is a change in cytoplasmic pH, with increase from a resting value of about 7.3 by about 0.2 pH units (Irving *et al.* 1992; Blatt & Armstrong 1993; Grabov & Blatt 1997). The source of this is not established, but activation of decarboxylation mechanisms, such as malic enzyme, is a possible cause, or activation of tonoplast proton pumping.

The challenge is to link the two global changes which have been observed, and the protein kinases and protein phosphatases known to be important in regulation of ion channels in guard cells, to the essential changes necessary to produce closure, using these processes, signalling intermediates, and effectors to identify defined signalling chains linking ABA to each of the end processes.

4. ABA-INDUCED CHANGES IN ION CHANNELS

(a) *Background conductance at the plasmalemma*

At the plasmalemma the fastest electrical response to ABA is the development, in cells whose resting membrane voltage is well-negative of the potassium equilibrium potential E_K, of an inward current, an increase in the instantaneous 'leak' or 'background' current seen in current–voltage curves, producing significant depolarization (Thiel *et al.* 1992). While this inward current may include a contribution from anion efflux, via anion channels discussed here, it was argued that the prime mechanism was activation of a non-selective cation-permeable channel carrying K^+ and Ca^{2+}; this argument was based on the observation that the reversal potential for the inward current shifted positive with an increase in external KCl, but was significantly positive of the E_K. Activation of a non-selective cation channel was also suggested by Schroeder & Hagiwara (1990), to explain associated spikes of inward current and increased cytoplasmic Ca^{2+} in response to ABA. There are no clear indications of the nature of the mechanisms by which ABA leads to activation of the leak conductance, although its activation by okadaic acid (Thiel & Blatt 1994) may reflect activation of the non-specific cation channel by phosphorylation on a site which is a target for protein phosphatase types 1 or 2A (PP1/2A).

(b) *Inward K^+ channel at the plasmalemma*

Best understood is the inward potassium channel responsible for K^+ influx at strongly negative membrane potentials, open only at voltages more negative than about −120 mV. This is down-regulated by ABA (Blatt 1990), which prevents stomatal opening, a change which would speed up but would not be essential for closure. This channel is inhibited by high cytoplasmic Ca^{2+} (Schroeder & Hagiwara 1989), and also by injection of inositol 1,4,5-trisphosphate (IP_3), which is also shown to increase cytoplasmic Ca^{2+} and induce stomatal closure (Blatt *et al.* 1990; Gilroy *et al.* 1990). Taken together, these results suggest that Ca^{2+} is an intermediate in the signalling chain leading to inhibition of the inward K^+ channel by ABA. The presence of Ca^{2+} chelator in the patch pipette prevents the ABA-induced inhibition, whereas external Ca^{2+} chelator does not (Lemtiri Chlieh & MacRobbie 1994), suggesting that high cytoplasmic Ca^{2+} is essential for the ABA effect, but that release of Ca^{2+} from internal stores can produce the necessary increase, without a requirement for Ca^{2+} influx from outside the cell. This does not rule out an ABA-induced Ca^{2+} influx when Ca^{2+} is present outside, but does provide clear evidence for ABA-induced release of Ca^{2+} from internal stores. The identification of the Ca^{2+} release channels is currently one of the key unsolved questions.

Downstream from Ca^{2+} the likely effector is calcineurin-mediated protein dephosphorylation; this view rests on the work of Luan *et al.* (1993) showing that the inhibition of the inward K^+ channel by Ca^{2+} was abolished by immunosuppressants, but produced by the introduction of a constitutively active fragment of bovine calcineurin. Upstream from Ca^{2+} the signalling chain is uncertain. Involvement of G-proteins is suggested (Fairley-Grenot & Assmann 1991), and inhibition of the inward K^+ channel by mas-7, the synthetic analogue of mastoparan from wasp venom, suggested involvement of a seven-trans-membrane span receptor (Armstrong & Blatt 1995). Together with the evidence already discussed, that guard cells have the capacity to respond to IP_3 by increases in cytoplasmic Ca^{2+}, such results raise the question of a signalling chain akin to the many well-characterized hormone-initiated signalling chains in animal cells, in which a G-protein couples the hormone receptor to activation of phosphoinositide-specific phospholipase C (PI-PLC) to produce IP_3. However, more recent evidence suggests that this may not be an appropriate model, following cloning of plant phospholipase Cs and comparison of their sequences with those of animal families. Kopka *et al.* (1998) have cloned three PI-PLCs from potato, all of which form a distinct group within the whole family of eukaryotic PLCs, but are closely related to the mammalian PLCδ family (Irvine 1996), which are neither activated by receptor tyrosine kinase nor G-protein coupled. Kopka *et al.* show that the potato enzymes are Ca^{2+}-activated, as also suggested for previously cloned plant PLCs (Hiroyama 1995; Shi 1995). On this basis, if IP_3 is involved in the ABA response it would be downstream of an ABA-induced increase in cytoplasmic Ca^{2+}, although further Ca^{2+}-induced Ca^{2+}

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release (CICR) may then follow, if Ca^{2+} release by IP_3-triggered Ca^{2+} channels leads to activation of other Ca^{2+}-permeable channels.

An alternative mode of control of the inward K^+ channel is indicated by the observation that the ABA-insensitive mutant *abi-1* discussed earlier, which is deficient in protein phosphatase type 2C, has lost the ABA-induced inhibition of the inward K^+ channel, but that this can be restored by protein kinase antagonists, H7 or staurosporine (Armstrong *et al.* 1995). Thus the inhibition of the inward K^+ current by ABA may need dephosphorylation by both PP2C and calcyneurin (PP2B), of either the channel itself or a regulatory agent. In contrast to this, inhibitors of PP1–2A (okadaic acid or calyculin) inhibit the inward K^+ channel, suggesting a site which must be dephosphoryated for the active channel, and give inhibition in the phosphorylated state. Thus several phosphorylation–dephosphorylation events are involved in the control, and the sequence of events in the cell following ABA application is still not clear.

(c) *Outward* K^+ *channel in the plasmalemma*

In contrast to the inward K^+ channel, the second K^+ channel in the plasmalemma opens only at voltages more positive than the E_K, and is responsible for K^+ efflux from the cell. Fast ABA-induced K^+ efflux therefore requires both activation of this channel, and shift of the membrane potential positive to a value within its opening range, and ABA does both (Blatt 1990; Thiel *et al.* 1992). Activation of the postulated non-specific cation channel, or of a Ca^{2+} channel, or of an anion channel, would achieve the necessary voltage shift, but their relative contributions *in vivo* are not yet clear. The outward K^+ channel is not Ca^{2+}-sensitive (Schroeder & Hagiwara 1989), nor is it activated by the introduction of IP_3 (Blatt *et al.* 1990). Blatt & Armstrong (1993) showed that acidification of the cytoplasm by external sodium butyrate inhibited the outward K^+ channel, prevented the ABA-induced alkalinization of the cytoplasm, and blocked the ABA-induced activation of the outward K^+ channel, arguing for regulation by cytoplasmic pH, and positioning pH in the signalling chain leading from ABA to this channel. The *abi-1* mutant has much reduced activity of both the K^+ channels in the absence of ABA, and both currents are insensitive to ABA (Armstrong *et al.* 1995), the sensitivity being restored by protein kinase antagonists. The *abi-1* mutants had normal ABA-induced pH changes, which leaves the relation between the two signalling intermediates, cytoplasmic pH and PP2C, uncertain. However, the marked pH-dependence of PP2C activity (activated 2.5-fold by an increase in pH from 7.2 to 7.5; Grill *et al.*, this issue) may provide a means of coupling an ABA-induced cytoplasmic alkalinization to activation of an outward K^+ channel which requires dephosphorylation by PP2C for activity.

(d) *Anion channels in the plasmalemma*

Activation of anion efflux at the plasmalemma is one of the changes essential for stomatal closure. There are two types of anion channel behaviour that have been identified in patch-clamped protoplasts, but these may in fact reflect different states of a single channel protein (Schroeder *et al.* 1993; Dietrich & Hedrich 1994). The slow S-type (Linder & Raschke 1992; Schroeder & Keller 1992) activates over tens of seconds, is active over a wide voltage range, from −200 to +60 mV, and does not inactivate; it is therefore a candidate for the sustained anion efflux required for stomatal closing. This channel is Ca^{2+}-activated, and phosphorylation is required for its activation (Schmidt *et al.* 1995). There is clear evidence for activation of slow anion channels by ABA, in *Arabidopsis* (Pei *et al.* 1997) and in tobacco (Grabov *et al.* 1997), but identification of signalling chains is complicated by differences in behaviour in different species. In particular, the ABA-response of anion channels in the *abi-1* mutant (deficient in PP2C) differs in the two studies. Transgenic tobacco expressing the mutant *abi-1* did show activation of anion channel activity by ABA (Grabov *et al.* 1997), whereas in *Arabidopsis* the activation of anion channel activity by ABA was suppressed in the mutant (Pei *et al.* 1997). The effects of inhibitors of type PP1–2A protein phosphatases, okadaic acid and calyculin, also differ in different species. In *Vicia* and *Commelina* okadaic acid enhanced ABA-induced closing (Schmidt *et al.* 1995), and calyculin sensitized tobacco to ABA, increasing the activation of anion channel activity (Grabov *et al.* 1997). In contrast, the reverse was true in *Arabidopsis*, where okadaic acid reduced ABA-induced stomatal closing and activation of anion channels (Pei *et al.* 1997). The *Arabidopsis* work measured tenfold higher anion currents and showed that acetate, present at 200 mM in the electrodes in the whole cell impalement of the tobacco work, inhibits the anion channels in *Arabidopsis*. Pei *et al.* (1997) suggest that the two studies may measure different populations of anion channels, with different patterns of regulatory phosphorylation sites, but the tobacco work is consistent with the effects on aperture in *Vicia* and *Commelina*, so that genuine differences in regulation in different species seem to be indicated.

Interpretaion is further complicated by observations of the behaviour of a second ABA-insensitive mutant *abi-2*. The ABI-1 and ABI-2 gene products are both type 2C protein phosphatases (Bertauche *et al.* 1996; Leung *et al.* 1997), and the mutants both have single amino-acid changes (gly- to -asp) at equivalent positions in the phosphatase domains. In both mutants in *Arabidopsis*, Pei *et al.* (1997) found loss of ABA-induced activation of anion channels and of stomatal closure, but surprisingly the ABA effects were partly restored by protein kinase inhibitors only in *abi-1* and not in *abi-2*. Their working hypothesis is that in *Arabidopsis* ABA acts through ABI-1 to inhibit a negatively regulating protein kinase, with dephosphorylation of some substrate protein leading to anion channel activation. The site of action of ABI-2 is unspecified.

It is clear that protein kinases and protein phosphatases, of types 1–2A and 2C, can be involved in the signalling chains by which anion channels are modulated, but the patterns need to be clarified by further work. One aspect of the results in *Arabidopsis*, which is important, is that the anion channels were activated by ABA in conditions where the rise in cytoplasmic Ca^{2+} was buffered out by Ca^{2+} chelator in the patch pipette (to the extent that no ABA-induced inhibition of the inward K^+ channel was seen). Thus, even if the anion channels are Ca^{2+}-activated, the ABA-induced activation of anion efflux by activation of the S-type anion channels is,

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similar to the activation of K^+ efflux through the outward K^+ channels, a Ca^{2+}-independent process. Whether this is also true of the anion channel activation in *Vicia*, *Commelina* and tobacco remains to be seen. It remains to be established whether anion channel activation can be achieved by alternative mechanisms, one of which is Ca^{2+}-dependent and the other Ca^{2+}-independent, in the same cell.

(e) *Tonoplast ion channels*

Our understanding of the nature of ion channels in the tonoplast, and the means of their regulation, is much less satisfactory. It is clear from flux studies that flux of both K^+ and Cl^- from vacuole to cytoplasm is stimulated by ABA, and we need to identify appropriate channels to carry such fluxes. For K^+ the position is more satisfactory than for anions, where a channel capable of supporting anion efflux has not been identified, being located in a plausible signalling chain.

A total of two K^+-permeable channels have been identified, from patch-clamping of isolated guard cell vacuoles, with indications of potential control of their gating. These are the ubiquitous SV channels, first observed in sugar beet vacuoles by Hedrich & Neher (1987), but also extensively studied in guard cell vacuoles (Ward & Schroeder 1994; Allen & Sanders 1995, 1996; Schulz-Lessdorf & Hedrich 1995; Ward *et al.* 1995). The SV channel is Ca^{2+}-activated, opening above about 600 nM cytoplasmic Ca^{2+}, and is voltage-dependent, opening at cytoplasm positive potentials, at a voltage which shifts negative with increasing cytoplasmic Ca^{2+}. This polarity is the opposite of resting tonoplast membrane potentials (generally taken to be around -20 to -50 mV, cytoplasm negative), so that the SV channel is closed in physiological resting conditions. The ion selectivity has been hotly debated, but it now seems agreed that the channel is permeable to K^+, Ca^{2+}, and Mg^{2+}, with relative permeabilities dependent on ionic conditions, but not permeable to anions. If the SV channel is to carry the K^+ efflux from the vacuole, in response to ABA-induced increase in cytoplasmic Ca^{2+}, it will be necessary to bring the tonoplast potential into the appropriate range for channel opening. The necessary positive shift is proposed to arise from the operation of the second K^+-permeable channel identified in the tonoplast, the VK channel (Ward & Schroeder 1994). This is K^+-selective, but insensitive to voltage; it is also Ca^{2+}-activated, above about 100 nM cytoplasmic Ca^{2+}, a much lower level than is required by the SV channel. The current hypothesis is that an ABA-induced increase in cytoplasmic Ca^{2+} will first activate the VK channel, and the resultant K^+ flux will drive the cytoplasm sufficiently positive to activate the SV channel (whose activation voltage has also been shifted negative by the increase in Ca^{2+}), allowing further efflux of K^+. It was suggested that the SV channel would also carry efflux of Ca^{2+} from the vacuole, in a process of Ca^{2+}-induced Ca^{2+} release, but recent more detailed study of the voltage conditions for activation of the SV channel in barley mesophyll vacuoles argues against this. Pottosin *et al.* (1997) found that the open probability of the SV channel depended on the electrochemical gradient for Ca^{2+} across the tonoplast. At the equilibrium potential for Ca^{2+} the open probability was as low as 0.4%, and declined further in conditions favouring Ca^{2+} release from the vacuole; essentially the channel opened significantly only when the gradient was such as to drive Ca^{2+} into the vacuole. Thus a role in CICR seems to be ruled out, and indeed the activation voltages at millimolar concentrations of Ca^{2+} in the vacuole are so strongly positive that it remains uncertain whether in the ABA-treated cell the SV channel can be activated, even with cytoplasmic Ca^{2+} as high as 100 or 500 μM. If the SV channel is to play a role in K^+ release from the vacuole, some other mechanism for shift of its activation voltage seems to be required.

There is also a question about the extent of the positive shift in tonoplast potential by efflux of K^+ through the VK channels, as proposed by Ward & Schroeder (1994). As ABA-induced anion efflux from the vacuole is also required for closure, and is shown to occur in tracer experiments, it would be expected that some anion-permeable channel, not yet identified, is also activated by ABA. The change in tonoplast potential will be determined by the balance of K^+ and anion effluxes, and in the end, because in the activated condition these two ions must be the principal charge carriers in the membrane, the two are likely to equalize to give salt efflux; the value of tonoplast potential at which this condition is reached is not known, but because the concentration gradient for anion efflux is likely to be higher than that for K^+ efflux, it may well not be positive. The part down-regulation of the VK channel by an ABA-induced increase in cytoplasmic pH will also affect this balance. There is an urgent need to identify an anion-permeable channel in the tonoplast capable of carrying the ABA-stimulated Cl^- efflux from vacuole to cytoplasm, and to establish its properties and agents of control.

(f) *Source of Ca^{2+} for ABA-induced increase in the cytoplasm*

We need to identify Ca^{2+}-permeable channels activated by ABA, and the mechanism of their activation. The patch-clamp results already discussed (Lemtiri Chlieh & MacRobbie 1994) show that the ABA-induced increase in cytoplasmic Ca^{2+} can be achieved without Ca^{2+} influx, by release from cytoplasmic stores, but do not rule out ABA-induced Ca^{2+} influx when external Ca^{2+} is present. There are three pieces of evidence that suggest activation of Ca^{2+} influx by ABA. Of these, two were discussed earlier as evidence for an ABA-sensitive non-specific cation channel, responsible for ABA-induced associated spikes of Ca^{2+} and inward current (Schroeder & Hagiwara 1990), and the stimulation of background current by ABA (Thiel *et al.* 1992) with a cation-sensitive reversal potential. The third comes from measurement of the efflux transients seen when ABA is added to $^{86}Rb^+$-labelled isolated guard cells in epidermal strips. If ABA is added during the washout, at a stage when the tracer remaining is almost entirely vacuolar, a transient stimulation of the rate of tracer loss is observed, reflecting stimulation of efflux from the vacuole. The form of this transient is strongly affected by conditions which would be expected to alter processes of internal Ca^{2+} release, and affect the time-course of changes in cytoplasmic Ca^{2+}. The addition of Ca^{2+} to a Ca^{2+}-free bathing medium in the absence of ABA reduces tracer efflux, but

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the addition of Ca^{2+} to a Ca^{2+}-free bathing medium during the vacuolar efflux transient increases the rate of tracer loss (MacRobbie 1990), suggesting that an increase in cytoplasmic Ca^{2+} by influx of Ca^{2+} from outside does occur.

Nevertheless, it is clear that ABA does initiate release of Ca^{2+} from internal stores, and the question of which stores and which channels is still being debated. The tonoplast has at least two ligand-gated Ca^{2+} channels and it has been argued that the vacuole is the main Ca^{2+} store in the cell active in signal transduction pathways (reviewed by Muir *et al.* (1997)). Most discussion has centred on the IP_3-sensitive Ca^{2+} release channel identified in the tonoplast by a variety of techniques. Its existence was first suggested by observation of IP_3-activated Ca^{2+} release from tonoplast vesicles or isolated vacuoles (Schumaker & Sze 1987; Ranjeva *et al.* 1988), but it has also been characterized in patch-clamped vacuoles (Alexandre *et al.* 1990; Alexandre & Lassalles 1992; Allen & Sanders 1994). These channels open at physiological tonoplast potentials, cytoplasm negative, so their activation by IP_3 is not dependent on manipulation of the tonoplast potential by some other ion flux. Specific IP_3 binding sites in red beet were characterized by Brosnan & Sanders (1993), and the IP_3 receptor was isolated, purified and reconstituted in liposomes by Biswas *et al.* (1995). Although it is present in the tonoplast, and it has been argued that the vacuole represents the main store of IP_3-releasable Ca^{2+} in plant cells, rather than the ER as in animal cells, there is no clear evidence for this, and both stores may be used in different responses. Muir & Sanders (1997) have shown that IP_3-triggered Ca^{2+} release is associated with a number of membrane fractions in cauliflower, and that only a small fraction of the total activity in the cell is associated with the tonoplast fractions; the great bulk of activity is associated with heavier fractions, with plasmalemma or an ER fraction which associates with the plasmalemma in the gradient separation procedures. Thus the involvement of ER as Ca^{2+} store for signalling chains remains possible, and, in my view, likely.

Although IP_3-sensitive Ca^{2+} channels are present in one or more membranes in the cell, and guard cells are capable of responding to IP_3 by release of Ca^{2+}, the question of whether this channel is involved in the ABA response is a separate one. It is not the only potential Ca^{2+} release channel in the tonoplast. Allen *et al.* (1995) showed that Ca^{2+} could also be mobilized from beet vacuoles by cyclic ADP-ribose (cADPR), that IP_3-gated and cADPR-gated Ca^{2+} release channels were present in the same individual vacuole, and that both open at physiological membrane potentials, cytoplasm negative. The relative contribution of either or both to the signal transduction chains in the ABA response is still to be established.

There is evidence for the involvement of cADPR in another ABA-initiated signalling chain, that responsible for ABA-regulated gene expression. Wu *et al.* (1998) used GUS-reporter gene constructs from two *Arabidopsis* genes, the desiccation-responsive gene *rd29A* and the cold-inducible gene *kin2*, both of which can be induced by ABA. Micro-injection of *rd29A-GUS* or *kin2*-GUS into etoliated hypocotyls of the phytochrome-deficient *aurea* mutant of tomato allowed signalling intermediates for gene expression to be identified. Both genes were activated by ABA, but treatment with the permeable Ca^{2+}-chelator EGTA-AM prevented activation, placing Ca^{2+} on the signalling chain, and this conclusion was confirmed by activation in the absence of ABA by co-injection of 160 μM Ca^{2+} with the GUS constructs. Co-injection of 1 μM cADPR (estimated internal concentration) also activated both genes in the absence of ABA, and again the activation was abolished by blocking any increase of cytoplasmic Ca^{2+} by the Ca^{2+}-chelator, showing that cADPR-induced Ca^{2+} release is responsible for the activation. This conclusion was strengthened by the demonstration of an increase in cADPR in the cells (as measured in a sea urchin bioassay for Ca^{2+} release) before changes in gene expression. Although co-injection of IP_3 also activated gene expression, in this case blocked by heparin, the ABA-response was not affected by heparin. Thus, the ABA-induced increase in cytoplasmic Ca^{2+} leading to gene expression is mediated by activation of a cADPR-sensitive Ca^{2+}-release channel and not through an IP_3-sensitive channel. The responses to cADPR, Ca^{2+}, and ABA were all abolished by K252a and diminished by staurosporine, implicating control by protein kinases–phosphatases. The stimulation of gene expression by okadaic acid in the absence of ABA suggested that PP1–2A may be upstream of the Ca^{2+} pools and the kinases.

Curiously, although the phytochrome-regulated *cab* gene is also Ca^{2+}-controlled, and was expressed after injection of Ca^{2+}, its expression was not induced by either cADPR or IP_3, suggesting that the pattern of gene expression is determined by the nature of the trigger and the site of Ca^{2+} release. The importance of this paper, which must be a model for similar studies in guard cells in the future, is therefore twofold, that the signal transduction chain leading from ABA to Ca^{2+} to ABA-induced gene expression involves cADPR-induced Ca^{2+} release and not IP_3-induced Ca^{2+} release, but also that different pools of Ca^{2+} are involved in different signalling chains which lead to different Ca^{2+}-dependent responses.

The relative importance of the two identified potential internal Ca^{2+} release mechanisms in the ABA response in guard cells remains to be established. There is evidence suggesting IP_3 may be involved, but it is not conclusive and this channel may not be the main contributor. Parmar & Brearley (1993) showed that all the components of the phosphoinositide signalling pathway are present in guard cells, and Lee *et al.* (1996) showed ABA-induced phosphoinositide turnover in guard cell protoplasts of *Vicia*, from measurements of the pattern of ^{32}P label in both inositol lipids and inositol phosphates in the period up to 300 s of ABA treatment. Using the radioreceptor assay for measurement of guard cell IP_3, they showed an increase in the amount of ^{32}P label in a component which bound to the bovine $Ins(1,4,5)P_3$ receptor and was displaced by $Ins(1,4,5)P_3$; when cells were pretreated with Li^+ to inhibit inositol phosphatase activity, a 90% increase in label in putative $Ins(1,4,5)P_3$ was observed, suggesting ABA-induced activation of phospholipase C does occur. They also observed changes in the distribution of label in inositol lipids, but these are more problematic. They observed a 20% decrease in ^{32}P label in both PtdInsP and $PtdInsP_2$ at 10 s of ABA

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treatment, followed by a rise over 60–180 s and a second fall at 300 s. The interpretaion is complicated by the finding of Parmar & Brearley (1995) that guard cells also contain 3-phosphorylated inositol lipids and that these are present in larger amounts and turn over more rapidly than the 4-phosphorylated lipids PtdIns4P and PtdIns(4,5)P_2 of the conventional signalling pathway. It is therefore essential to establish the individual time-courses of change for each of the 3-phosphorylated and 4-phosphorylated lipids before signalling pathways from ABA can be identified.

(h) *Source of Ca^{2+}: evidence from tracer flux experiments*

These experiments involve comparison of the $^{86}Rb^+$ efflux transients in different conditions, and follow the pattern I used previously (see MacRobbie 1995*a*,*b*). Isolated guard cells are loaded with $^{86}Rb^+$ and the rate of loss of tracer to non-labelled solution is then measured. As already discussed, when ABA is added at a stage of washout when the tracer remaining is almost entirely vacuolar, the transient stimulation of the rate of tracer loss which is observed reflects stimulation of efflux from the vacuole. Because release of Rb^+ (as an analogue for K^+) is postulated to be triggered by an increase in cytoplasmic Ca^{2+} (whether release is through the VK or the SV channel), the form of this transient can be an indicator of the time-course of increase of cytoplasmic Ca^{2+}. A number of factors change the form of the efflux transient, and the working hypothesis is that such changes can arise from modulation of Ca^{2+} influx or of the processes of release of Ca^{2+} from internal stores. It was shown earlier that the form of the efflux transient was modified at low concentrations of ABA (MacRobbie 1995*a*), that the ABA-induced stimulation of efflux of vacuolar tracer was both delayed and reduced at sub-optimal concentrations of ABA (0.1 μM rather than 10 μM ABA), as shown in figure 1*a*; the same results are shown as a plot of tracer content against time in figure 1*b*. The lag period before induction of an increased rate of loss of vacuolar tracer was interpreted as a requirement to reach a threshold of some kind before activation of vacuolar channels is initiated, and the reduction in peak height suggested different degrees of activation of tonoplast ion channels at different concentrations of ABA. The pH sensitivity of the efflux transient suggested an internal ABA receptor at the head of the signalling chain for vacuolar release. While the dependence of the transient on cytoplasmic ABA could be direct, with a requirement for threshold concentration of ABA, it was argued that an indirect effect was perhaps more likely, with sub-optimal concentrations of ABA resulting in lesser increases in cytoplasmic Ca^{2+}, and the effective threshold being a required level of cytoplasmic Ca^{2+}, for activation of a specific K^+ channel. Subsequent results showing the manipulation of the form of the efflux transient in other ways lend support to the idea that the level of cytoplasmic Ca^{2+} is critical for the threshold.

The form of the efflux transient may also be modified by the addition of Ba^{2+} (for preliminary results, see MacRobbie (1997); further results, E. A. C. MacRobbie, unpublished data). Ba^{2+} has effects similar to those of low ABA, with a delayed, slowed and reduced peak (figure 2).

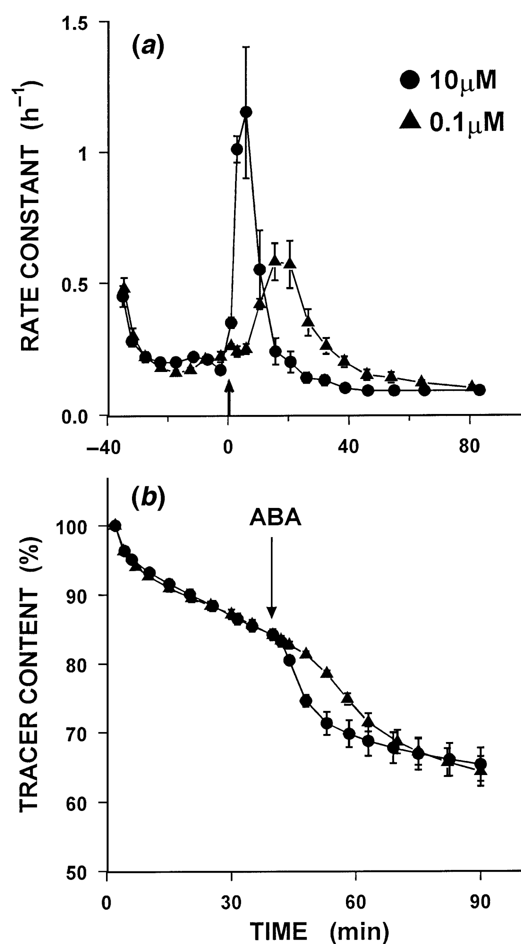

Figure 1. Comparison of the effect of 0.1 μM and 10 μM ABA on the efflux of $^{86}Rb^+$ from guard cells of *Commelina communis* L. Epidermal strips, after treatment at low pH to kill all cells other than guard cells, were loaded with tracer overnight, in solutions containing 2 mM ^{86}RbCl, 0.1 mM $CaCl_2$, and 10 mM Pipes buffer at pH 6. Rate of loss of tracer to non-radioactive solutions of the same composition was then measured, together with the amount of tracer remaining in the cells at the end of the experiment. ABA was added after 40 min washout in the absence of ABA. Each plot shows the mean ±s.e.m. of four replicate strips. (*a*) Rate constant for efflux (rate of loss of tracer/tracer content h^{-1}) plotted against time. ABA added at zero time on the graph. (*b*) Tracer content (percentage of content at the start of washout) plotted against time of washout.

Cd^{2+}, whose effects on the tracer efflux when added in the absence of ABA are similar to those of Ba^{2+}, does not change the time-course. The interpretation put on these effects is that Ba^{2+}, a K^+ channel blocker, inhibits the release of Ca^{2+} from internal stores. Such a release will be conditional on a charge-balancing flux of K^+, a requirement which has been demonstrated in two studies, in rat brain microsomal vesicles by Shah & Pant (1988), and in plant tissue, in *Acer* microsomal vesicles, by Canut *et al.* (1989). Thus the most plausible explanation for the delayed efflux transient in the presence of Ba^{2+} is that Ba^{2+} interferes with release of Ca^{2+} from internal stores, and prolongs the time needed to reach the required threshold.

Flux experiments also provide evidence that the IP_3 signalling pathway may contribute, but may not be the

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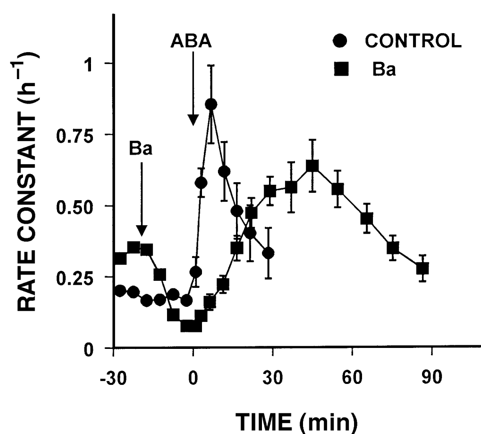

Figure 2. Effect of 1 mM Ba^{2+} on the ABA-induced efflux transient in *Commelina communis* L. Tissue was loaded and the washout started in the absence of Ba^{2+}. Ba^{2+} was added to one set of tissue 20 min before the addition of ABA (10 µM) at time zero on the graph. The graph shows the rate constant for efflux (h^{-1}) against time. Each plot shows the mean ±s.e.m. of four replicate strips.

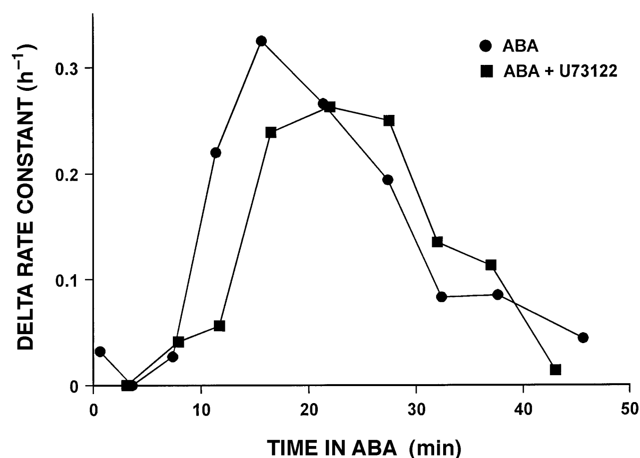

Figure 3. Effect of 1 µM U73122 on the ABA-induced efflux transient. Tissue was loaded and the washout measured in three sets of tissue in control conditions (2 mM RbCl, 0.1 mM $CaCl_2$, 10 mM Pipes buffer pH 6) for about 20 min. U73122 (1 µM) was then added to two sets of tissue, and after a further 6 minutes ABA (10 µM) was added to two sets, one with and one without U73122. Zero time on the graph is the time of ABA addition. Time plots of the rate of loss in tissue treated with ABA, with ABA in the presence of U73122, and with U73122 alone were measured. The plots show the difference in rate constants for efflux (delta rate constant h^{-1}) in ABA + U73122 and in U73122 alone, compared with the difference in rate constant produced by ABA in the absence of U73122. Each treatment had four replicate strips.

principal contributor, to ABA-induced increase in cytoplasmic Ca^{2+} (E. A. C. MacRobbie, unpublished data). If IP_3-sensitive Ca^{2+} release channels are involved, then it would be predicted that inhibition of phospholipase C would also delay the start of the efflux transient, and in preliminary experiments this has been observed. These experiments used the aminosteroid U73122, reported earlier to be a specific inhibitor of phopholipase C, with the inert U73343 as a control, and compared the form of the ABA-induced efflux transients in the presence and absence of an inhibitor. However, as U73122 can itself promote release of Ca^{2+} from internal stores (Mogami *et al.* 1997), it is important to measure the effects of ABA alone, of U73122 alone, and of ABA plus U73122 together. It was found that U73122 on its own had no effect on vacuolar release of $^{86}Rb^+$ for about 20 min, but did increase efflux to some degree after that time, suggesting that it is capable of mobilizing internal Ca^{2+}. However, the effect of ABA in the presence of U73122 can be assessed by subtracting the rate in the presence of ABA + U73122 from that in the presence of U73122, alone, and figure 3 shows the result of one such experiment, using 0.1 µM ABA and 1 µM U73122. From that figure it is clear that U73122 does affect the time-course of the ABA-induced vacuolar release of $^{86}Rb^+$, that it lengthens the lag period and reduces the peak height, but it does not abolish the transient. The results suggest that IP_3-triggered Ca^{2+} release does contribute to the increase in cytoplasmic Ca^{2+} following application of ABA, but that this channel is not the only and perhaps not the main contributor to the increase. Further experiments are required to test whether the cADPR-mediated Ca^{2+} release is the main effector, as in the signal pathways responsible for ABA-activated gene expression.

(i) *Summary of changes*

Figure 4 attempts to summarize the potential signalling chains by which the global changes and protein phosphorylation may be linked to the flux changes in the plasmalemma and tonoplast necessary for ABA-induced stomatal closure.

5. ABA RESETS THE SET-POINT OF A CONTROL SYSTEM: INVOLVEMENT OF STRETCH-ACTIVATED CHANNELS

Detailed comparison of the ABA-induced efflux transients in different conditions provides evidence that ABA acts to reset the set-point of a control system, and suggests that stretch-activated channels may be involved in guard cell signalling (MacRobbie 1995*a*). As shown earlier in figure 1*a*, ABA produces a transient stimulation of efflux of vacuolar tracer, but after a period the rate of loss returns to a value close to that measured before ABA was added—there is no indefinitely sustained increased efflux. At sub-optimal concentrations of ABA the peak of stimulated efflux is delayed, has a reduced peak height and is wider, but in both conditions the rate then returns to the pre-ABA level. If the tracer contents are plotted against time as in figure 1*b*, there is a period of greater slope, of high rate of loss, but the two curves at different ABA concentrations come together again when a given proportion of ion content has been lost. This suggests that the same end-state has been reached, but that different times are required to reach this end-state in the two conditions. Such behaviour suggests an ABA-induced change in a regulatory system, a change in the set-point of some control system. This hypothesis demands that the guard cell membrane system can in some way sense the cell volume, or turgor, or membrane stretch, that the efflux responds to the difference between the sensed content

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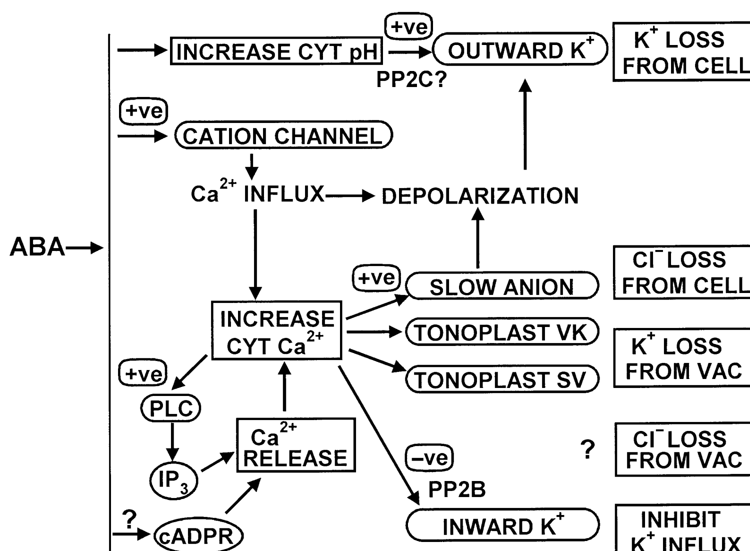

Figure 4. Proposed signalling pathways linking ABA to changes in specific ion channels in guard cells, and to the changes which contribute to stomatal closure. There is evidence for each of the links shown, but of variable weight, and the scheme must be regarded as a working hypothesis. Elements need to be added to this scheme to fill gaps, and some of the links proposed may later be amended.

and a set-point, and that ABA resets the set-point. This behaviour is more clearly seen if the rate is plotted against the amount lost during the transient (as a percentage of tracer content when ABA is added), when the peak and 'end' of the transient occur at the same content, but the absolute values of the rate in the two conditions are still different. If instead the relative excess rate is plotted against the amount lost (figure 5) then the curves become coincident. This suggests that different numbers of channels are activated at high and low amounts of ABA, but the behaviour of individual ion channels is sensitive to ion content (volume). A stretch-sensitive channel whose set-point is reset by ABA is suggested by these results, although the relation between such a channel and the tonoplast channels involved in K^+ (Rb^+) release remains to be established. It is commonly argued that stretch sensitivity arises from attachment of channel proteins to cytoskeletal components (or to components of the cell wall), and the set-point must be determined by the nature of this interaction. The set-point could therefore be reset by changes in membrane–cytoskeleton interactions, and this needs to be investigated in future work.

The stretch sensitivity may reside in a plasmalemma channel allowing Ca^{2+} influx (perhaps the non-specific cation channel already discussed), or it may instead be a tonoplast channel. A total of three different stretch-activated channels were identified in the guard cell plasmalemma by Cosgrove & Hedrich (1991), permeable to K^+, Ca^{2+} and Cl^-, respectively, and there are two reports of stretch-activated channels in tonoplasts, although not in guard cells (Alexandre & Lassalles 1991, in red beet; Badot *et al.* 1992, in onion). There may be parallels with two other systems. The first is the touch-sensitivity in higher plants, involving mechanically stimulated increase in cytoplasmic Ca^{2+} (Knight *et al.* 1991, 1992), perceived at the plasmalemma but leading to release of Ca^{2+} from internal stores (Haley *et al.* 1995). The second is the role of mechano-sensitive channels in volume regulation in *Fucus* rhizoids in hypoosmotic conditions, which again involves increase in cytoplasmic Ca^{2+} leading to activation of ion efflux channels (Taylor *et al.* 1996).

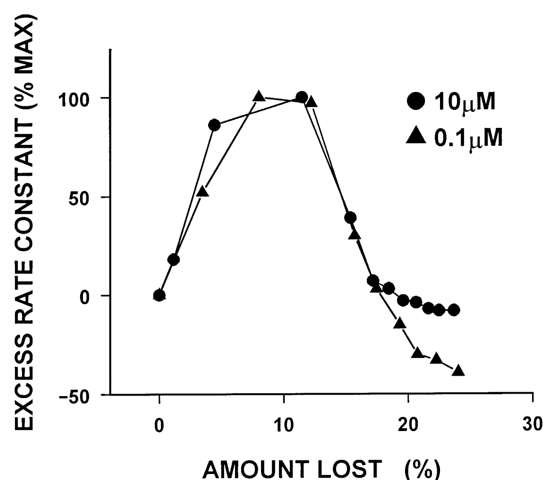

Figure 5. Relation between the rate constant for efflux and the amount of content lost during the transients produced by 0.1 μM and 10 mM ABA, as shown in figure 1. The rate constant for efflux (rate of loss of tracer/tracer content h^{-1}) was calculated for each time-interval. The excess rate constant was calculated, the rate constant during the ABA transient minus the rate constant before ABA was added. The excess rate constant was then expressed as a percntage of its maximum value at the peak of the transient, and is plotted against the amount of loss of tracer by the corresponding times during the transient (as a percentage of the tracer content at the start of the rise).

There is now evidence for involvement of cytoskeletal proteins, and in particular actin filaments, in regulation of guard cell channels and in guard cell function. Kim *et al.* (1995) showed that treatment of guard cells with phalloidin or cytochalasin D both altered the pattern of distribution of actin filaments within the guard cells and interfered with stomatal movement; phalloidin inhibited ABA-induced stomatal closing. These observations have since been extended to show that such agents affected inward K^+ channel activity (Hwang *et al.* 1997), and the postulated importance of actin interactions in guard cell function is greatly strengthened by the observation by Eun & Lee (1997) that the radial pattern of arrays of actin filaments in guard cells is disrupted by ABA.

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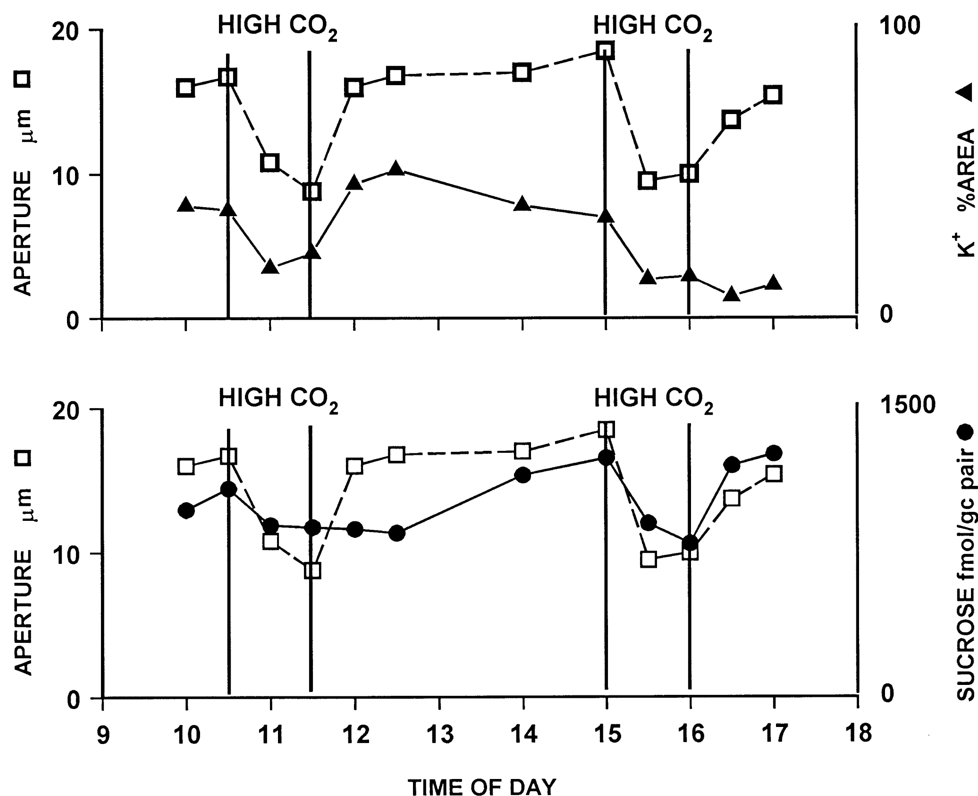

Figure 6. Changes in guard cell potassium (top) and sucrose (bottom) content correlated with aperture changes induced by high CO_2 (600 p.p.m.) in plants of *Vicia faba*. Data from Talbott & Zeiger (1996). Sucrose was measured by HPLC analysis of sonicated epidermal peels (in which only guard cells were alive). K^+ was estimated by the histochemical staining technique using sodium hexanitrocobaltate, and is therefore semi-quantitative only, not convertible to absolute units.

6. CO_2-INDUCED CHANGES IN GUARD CELLS

Stomatal closing induced by high CO_2 is a second process for which we have some indications of the signalling intermediates involved, although our knowledge is much less complete than for closure induced by ABA. Webb *et al.* (1996) showed high CO_2-induced increases in cytoplasmic Ca^{2+} (from a resting level of 80–200 nM) occurred in 73% of *Commelina* guard cells investigated, although by very variable amounts, with increases of 100–1000 nM in about half of the cells. Thus, as with ABA-induced closure, a significant number of cells without any detectable increase in cytoplasmic Ca^{2+} nevertheless close in response to the 'closing' signal. The response is prevented by the presence of external EGTA, suggesting that Ca^{2+} influx from the outside solution is required.

The effects of high CO_2 (1000 µl l^{-1} and 10 000 µl l^{-1}) on K^+ and anion channels in *Vicia* guard cells have been established by Brearley *et al.* (1996), and are similar to those of ABA, but not identical. There is a transient stimulation of the background current, an increase in the outward K^+ current, and an inhibition of the inward K^+ current. There is stimulation of the anion channels, measured in the presence of K^+ channel blockers, with changes to their kinetics which are similar to those produced by ABA. But a key difference is that there is no change in cytoplasmic pH at 1000 µl l^{-1} and an acidification by 0.1–0.2 pH units at 10 000 µl l^{-1}; thus the CO_2-induced activation of the outward K^+ channel cannot be attributed to alkalinization of the cytoplasm as was argued to be responsible for the ABA-induced activation of the outward K^+ channel. There are no suggestions yet of alternative signalling chains.

7. CHANGES IN SUGARS

We have extensive investigation and discussion of the mechanisms for modulation of ion channels at the plasmalemma and tonoplast leading to a loss of potassium salts and stomatal closure, and partial understanding of the signalling processes. This attention ignores the fact that although potassium salts account for a large fraction of the osmotic changes associated with stomatal opening and closing they do not account for the full changes under all conditions, and that other solutes must also be involved. The osmotic requirements for changes in stomatal aperture may be compared with the osmotic contributions of the measured changes in potassium salts, estimated from measurements of K^+ and assuming the balancing anion is Cl^-. The results make it clear that some other solute must contribute significantly to the osmotic content in guard cells (MacRobbie 1980; MacRobbie & Lettau 1980*a*,*b*). Outlaw & Manchester (1979) found significant increases in sucrose in guard cells of *Vicia faba* as stomata open. Recent measurements from Zeiger's group show the complexity of the changes (Talbott & Zeiger 1996; Amodeo *et al.* 1996). In this work, aperture and changes in guard cell sucrose and K^+ were measured throughout the course of a day. K^+ measurements were made by the semi-quantitative potassium staining technique and cannot be converted to real concentrations, but nevertheless the pattern of change is clearly established. Stomata open after the light comes on in the morning, and accumulate potassium salt; but later in the day potassium salt is partly replaced by sucrose, at constant aperture. Both are then lost when stomata close. Figure 6 shows the effect of high CO_2 on open stomata of *Vicia* in both states of opening, the high potassium salt state and the high sucrose state. The aperture response to high CO_2 is very similar in the two states, but in one case this

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involves loss of potassium salt and in the other, loss of sucrose. Attention so far has focused on the signalling mechanisms involved in modulation of ion channels at the plasmalemma and tonoplast, leading to net efflux of K^+ and anions at both membranes. These results suggest that attention needs also to be given to the mechanisms by which sucrose is lost in response to the same closing signal. None of the mechanisms discussed earlier apply to sugar loss, and we need to start from the beginning to identify signalling chains. It may be that a more general mechanism of solute loss rather than ion loss is needed, but clearly this important problem needs to addressed.

There are two appparently unrelated observations that are noteworthy. In many ways the dramatic loss of vacuolar solute associated with stomatal closure may have a parallel in the processes involved with the loss of turgor in the motor cells of pulvini which are responsible for leaf movements, whether in sleep movements or in the much faster movement in the mechanically sensitive plant *Mimosa*. Here mechanical disturbance of the sensitive leaflets leads to propagation of an action potential in phloem; on arrival of the action potential in the primary pulvinus a slower flat-topped action potential is excited in pulvinar motor cells, leading to a massive loss of solute from the cell and from the vacuole, loss of turgor in the motor cells, and consequent leaf movement (Abe & Oda 1976; Samejima & Sibaoka 1982). The main loss is of potassium salt, and emphasis has focused on activation of K^+ and Cl^- channels associated with the action potential. However, measurements of distribution of ^{14}C label in stimulated and unstimulated *Mimosa* (Fromm & Eschrich 1988*a–c*) suggest that stimulation and excitation of the action potential in sieve tubes and motor cells involves release of both ions and sucrose; thus, before stimulation ^{14}C label (sucrose) is confined to phloem strands, but after stimulation the label has been unloaded to the apoplast in the pulvinar region of both tertiary and primary pulvini. The mechanism of such sucrose release is unknown, but may be shared with sucrose release in closing guard cells.

Mimosa may offer an even more speculative parallel, from some very old microscopic observations which have never been followed up. Weintraub (1951) observed the changes in appearance of *Mimosa* motor cells associated with stimulation, and described the appearance of vesicles in the cytoplasm as the vacuole shrinks dramatically, suggesting that these bud off the central vacuole and account for its decrease in volume. Such a process, of budding of vesicles from the tonoplast, would result in solute loss from the vacuole, salt and sugar, and offers an alternative mechanism to single ion movements through a number of specific ion channels activated by the various signalling intermediates generated from the closing signal. While highly speculative the idea may be worth further investigation.

8. CONCLUSIONS

It is clear that our understanding of the guard cell signalling processes involved in stomatal closing is very incomplete. Stomatal closing requires the up-regulation of four processes, the efflux of K^+ and anions at the plasmalemma and the release of K^+ and anions from the vacuole to the cytoplasm. The process is speeded up by the down-regulation of K^+ influx, but this is not essential. Some key signalling intermediates and effectors in ABA-induced stomatal closure have been identified in the ABA-induced increases in cytoplasmic Ca^{2+} and pH, and in modulation of protein kinases and phosphatases, both Ca^{2+}-dependent and Ca^{2+}-independent, but the challenge remains of linking these (and other) signalling intermediates upstream to ABA and downstream to each specific flux change, in defined signalling chains.

Least understood is the anion release at the tonoplast, where the problem has not yet advanced to the search for signalling intermediates but still requires the identification of an appropriate channel. Of the other three essential changes, two are Ca^{2+}-dependent, and may be achieved as a consequence of the ABA-induced increase in cytoplasmic Ca^{2+}. Thus both K^+-permeable tonoplast channels, the VK and the SV channels are activated by Ca^{2+}, and the plasmalemma S-type anion channel is also Ca^{2+}-activated (although in *Arabidopsis* it appears that its activation by ABA may be independent of increase in cytoplasmic Ca^{2+}). The detailed mechanisms by which Ca^{2+} acts to activate these channels are not yet fully understood. The SV channel is activated by Ca^{2+}-calmodulin (Schulz-Lessdorf & Hedrich 1995), but is inhibited by calcineurin in the presence of calmodulin (Allen & Sanders 1995), although low concentrations of calcineurin in the absence of calmodulin stimulate; *in vivo* it is likely that dephosphorylation (whether of the channel directly or indirectly) is inhibitory. The mechanism of Ca^{2+} activation of the VK channel has not yet been established. Thus, although Ca^{2+} is a plausible second messenger in these signalling chains, the details of its interactions with the specific channels remain uncertain.

The other essential change is up-regulation of the K^+ efflux at the plasmalemma. An absolute requirement for this is depolarization of the cell to give a membrane potential positive of the E_K, the potassium equilibrium potential, and faster loss will be achieved if the outward K^+ channel is also activated, to increase the outward current at any given voltage. ABA does activate the channel, in a Ca^{2+}-independent way, and this is attributed to activation by the ABA-induced increase in cytoplasmic pH: it may be that this reflects the pH-sensitivity of PP2C.

The change which aids closure but is not essential is inhibition of the inward K^+ channel at the plasmalemma; this is clearly Ca^{2+}-dependent and down-regulated by activation of Ca^{2+}-activated PP2B, but may also be modulated by the activity of PP2C.

The question of Ca^{2+}-independent closing remains controversial. For this to occur we need Ca^{2+}-independent signalling mechanisms for each of the essential changes. It may be that the plasmalemma anion channel can be activated in a Ca^{2+}-independent mechanism (as in *Arabidopsis*) as well as by increased cytoplasmic Ca^{2+}, but there are no indications of Ca^{2+}-independent activation of the tonoplast K^+-permeable channels. The activation of the anion channel will contribute to the necessary depolarization, but there is also evidence for activation of a Ca^{2+}-permeable channel, which would provide depolarization independently of the increase in cytoplasmic Ca^{2+}. Thus, it may be possible that the necessary changes at the

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plasmalemma can be achieved independently of the increase in cytoplasmic Ca^{2+}, but we need to have mechanisms for Ca^{2+}-independent release of vacuolar ions before Ca^{2+}-independent stomatal closure becomes a feasible hypothesis.

It is clear that ABA triggers release of Ca^{2+} from internal stores, but the source and nature of the gating agent are not yet clear; IP_3 and/or cADPR are candidates for gating agents, and vacuole and/or ER are candidates for the site of release. The processes of transport or metabolism which are responsible for the ABA-induced increase in cytoplasmic pH remain unknown.

Thus, there is only partial understanding of the signalling networks linking ABA to the identified changes in K^+, Cl^- and Ca^{2+} channels, with gaps to varying degrees in all the chains. More serious is that we have not begun to define signalling chains for two main systems of change contributing to stomatal closure. There is evidence for resetting of a control system by ABA, suggesting involvement of stretch-activated channels; the proteins involved in these events need to be integrated in our signalling schemes linking ABA to the transport proteins immediately responsible for closure. The second need is to recognize that net loss of vacuolar sugars as well as salts can be involved in stomatal closure, to identify the transport processes or metabolic changes responsible for initiating changes in sugar content, and to address the question of the nature of the signalling chains involved in linking these to the closing signal.

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