Signalling mechanisms in the regulation of vacuolar ion release in guard cells

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Summary

• Pharmacological agents were used to investigate the possible involvement of actin in signalling chains associated with abscisic acid (ABA)-induced ion release from the guard cell vacuole, a process which is absolutely essential for stomatal closure.

• Effects on the ABA-induced transient stimulation of tonoplast efflux were measured, using ^{86}Rb in isolated guard cells of *Commelina communis*, together with effects on stomatal apertures.

• In the response to 10 μM ABA (triggered by Ca^{2+} influx rather than internal Ca^{2+} release), jasplakinolide (stabilizing actin filaments) and latrunculin B (depolymerizing actin filaments) had opposite effects. Both closure and the vacuolar efflux transient were inhibited by jasplakinolide but enhanced by latrunculin B. At 10 μM ABA prevention of mitogen-activated protein (MAP) kinase activation by PD98059 partially inhibited closure and reduced the efflux transient. By contrast, latrunculin B inhibited the efflux transient at 0.1 μM ABA (involving internal Ca^{2+} release rather than Ca^{2+} influx).

• The results suggest that 10 μM ABA activates Ca^{2+}-dependent vacuolar ion efflux via a Ca^{2+}-permeable influx channel which is maintained closed by interaction with F-actin. A MAP kinase is also involved, in a chain similar to that postulated for Ca^{2+}-dependent gene expression in cold acclimation.

Key words: actin, cytoplasmic Ca^{2+}, guard cells, jasplakinolide, latrunculin B, mitogen-activated protein (MAP) kinase, tonoplast ion channels.

Introduction

Stomatal closure, essential for plant survival in dry conditions, requires net efflux of potassium salt at both plasmalemma and tonoplast of the guard cell, resulting in guard cell shrinkage, loss of turgor and reduction in stomatal aperture. In water stress conditions, the 'drought' hormone, abscisic acid (ABA), produced in the roots and transferred to the leaves, initiates the changes in guard cell ion transport which are responsible for cell shrinkage and stomatal closure. Stomatal opening is associated with increase in guard cell volume by 40–50%, almost entirely vacuolar volume, since the cytoplasm occupies only a small fraction of the total cell volume (< 10% in open stomata). ABA-induced stomatal closure is a much-studied process, but attention has focused strongly on changes in plasmalemma ion channels, and as yet we have only a very limited understanding of the mechanisms of control of the absolutely essential process of vacuolar ion release, and of the associated signalling chains (see reviews by Hetherington, 2001; Schroeder *et al.*, 2001; Fan *et al.*, 2004; Hetherington & Brownlee, 2004; Hetherington & Woodward, 2003; Roelfsema & Hedrich, 2005). This imbalance in our understanding arises because the tonoplast is not accessible for electrophysiological measurements in its normal physiological state, since patch clamping of isolated vacuoles investigates a system lacking most of its signalling components. Tracer flux measurements on isolated guard cells are, as yet, the only way to study processes at the tonoplast in the intact cell. Detailed

analysis of the kinetics of tracer exchange, using $^{86}Rb^+$ as an analogue for K^+, yields measurement of tonoplast fluxes in the steady state and allows the large ABA-induced net efflux of vacuolar K^+/Rb^+ to be followed (MacRobbie, 1995, 2000, 2002, 2006). The importance of the tonoplast in guard cell ionic relations is quite clearly demonstrated in the results of such analyses. The cytoplasmic content is a small fraction (10–20%) of the total cell content, the flux at the plasmalemma is some four to 40 times the flux at the tonoplast and although the cytoplasm exchanges rapidly, the half-time for vacuolar exchange is 3–17 h. Thus stomata will not close to the extent and at the rates observed unless ion release across the tonoplast is triggered. Such work also makes clear that, although ABA stimulates ion efflux at both the plasmalemma and the tonoplast, the effect at the tonoplast is much greater than that at the plasmalemma. In the original comparison of the relative effects of ABA at the two membranes in the same tissue (MacRobbie, 1995), the efflux of $^{86}Rb^+$ at the tonoplast was stimulated by twofold to 6.7-fold, whereas the plasmalemma efflux was stimulated up to twofold, similar to the effect on the outward K^+ channel observed by Blatt (1990). In several later experiments, the stimulation of the tonoplast efflux was greater, with one experiment in which the ABA stimulated the tonoplast efflux by more than 100-fold (MacRobbie, 2006). Thus the most dramatic effect of ABA is not at the plasmalemma, and it is vital that we understand the mechanisms and signalling chains involved in the major ABA-induced response, the triggering of vacuolar ion release. This priority is further reinforced by the finding that the decrease in cell content after ABA treatment is vacuolar, and the cytoplasmic content does not change (MacRobbie, 2006).

In the electrophysiological studies at the plasmalemma, either by impalement of intact guard cells or by patch clamping of guard cell protoplasts, current-voltage analysis has allowed the contributions and characteristics of specific ion channels to be distinguished. Thus a detailed picture of the responses to ABA of well-defined ion channels in the plasmalemma has been built up, and a large number of signalling intermediates identified (see reviews already cited). This has progressed in three stages: pharmacological studies to identify involvement of specific signalling chains; generation and study of mutants to demonstrate the role of specific genes in specific responses; and development of network models to integrate this information. In spite of its critical importance, work on regulation of ion fluxes at the tonoplast is still in the first stage of pharmacological study, and better knowledge of the signalling intermediates is urgently required.

The neglect of the tonoplast is most clearly seen in the recent attempt to identify essential components of signal transduction networks involved in ABA-induced stomatal closure (Li *et al.*, 2006). In their modelling, potassium release at the vacuole does not appear as an essential prerequisite of stomatal closing, but only as a factor said to contribute to membrane depolarization at the plasmalemma. In fact, ion

release at the vacuole will not lead to depolarization at the plasmalemma, but will instead contribute to hyperpolarization at that membrane. In the intact cell, the tonoplast potential is not clamped and is set by the requirement for charge balance in the membrane fluxes. Activation of potassium release from the vacuole will drive the cytoplasm positive with respect to the vacuole, increasing anion efflux at the tonoplast; the potential will stabilize at the value at which there is no net charge transfer across the tonoplast, with equal efflux of anion and cation, leading to increase in cytoplasmic K-salt. Increased cytoplasmic K^+, the ion to which the plasmalemma is most permeable, leads to an increase in K^+ efflux across the plasmalemma, driving the membrane potential more negative, not more positive. The condition of charge balance at the tonoplast ensures that activation of either K^+ or anion efflux at the tonoplast will change that membrane potential, increasing the driving force for efflux of the oppositely charged ion, balancing efflux of anion and cation, and achieving a net efflux of K-salt.

The aim of this paper is to extend our knowledge of signalling intermediates in pathways linking ABA to the vacuolar ion release which allows stomatal closure. There is evidence that release of vacuolar (K^+/Rb^+) in response to abscisic acid (ABA) is triggered by an increase in cytoplasmic Ca^{2+}, with both influx of Ca^{2+} from outside and its release from internal stores contributing to the increase, in different relative proportions in different conditions (MacRobbie, 2000). There remains uncertainty about intermediates upstream of Ca^{2+} and about the target for Ca^{2+}, and there is no unequivocal identification of the tonoplast channel involved.

One of the ABA-induced changes in guard cells is a reorganization of actin filaments, for which the mechanism, significance and consequences remain unknown. Differences in the state and distribution of actin filaments in guard cells in open and closed stomata have been observed by a number of authors (Kim *et al.*, 1995; Eun & Lee, 1997; Hwang *et al.*, 2000; Eun *et al.*, 2001; Hwang & Lee, 2001), with thick radial actin cables in open stomata, but more random filament orientation in closed stomata. Lemichez *et al.* (2001) showed an association between the activity of a small Rho-related GTPase in *Arabidopsis* (AtRac1) and actin organization, with active Rac1 and organized actin cables in open stomata, but inactive Rac1 and disorganized actin in closed stomata. There is also evidence that actin organization in guard cells and stomatal function are under the control of ADF (actin-depolymerizing factor) proteins (Dong *et al.*, 2001). However, it is not known whether actin reorganization and aperture change are causally related or parallel phenomena, and the role of actin in the control of any specific ion channels is not understood.

Effects of actin-modifying agents on stomatal aperture, and on the inward K^+ current in the plasmalemma, have been observed previously. Stomatal opening was stimulated by the actin depolymerizing agents, cytochalasin B or D, and inhibited by phalloidin (stabilizing actin filaments), whereas ABA-induced stomatal closure was inhibited by phalloidin,

although not affected by cytochalasin (Kim *et al.*, 1995; Hwang *et al.*, 1997). Inward K⁺ currents were increased by cytochalasin and decreased by phalloidin (Hwang *et al.*, 1997). Liu & Luan (1998) also observed stimulation of stomatal opening and of inward K⁺ current by cytochalasin. While the changes in the inward K⁺ currents predict the effects on stomatal opening, they do not explain any effect on stomatal closing. The effect of phalloidin on ABA-induced stomatal closure means that actin must be involved in some other process. There is therefore a need to investigate the effects of actin-modifying agents on specific processes critical for stomatal closure, most obviously on the key determining process in ABA-induced closure, the ion release from the vacuole.

Possible involvement of actin in stomatal function is also suggested by some results in *Mimosa*. The changes in guard cells associated with stomatal closure are similar to the changes in pulvinar motor cells associated with the bending of the touch-sensitive petiole in *Mimosa pudica*, namely massive ion release from the vacuole and from the cell as a whole, with consequent changes in cell volume and turgor. It seems likely that similar mechanisms and signalling chains are involved in the two processes. Petiole bending has been shown to be associated with fragmentation of actin filaments in the motor cells and with a reduction in the degree of tyrosine phosphorylation of actin in the petiole. Both the bending in response to touch and the associated tyrosine dephosphorylation of actin are inhibited by phenylarsine oxide (PAO), an inhibitor of protein tyrosine phosphatases (Kameyama *et al.*, 2000; Kanzawa *et al.*, 2006). PAO also has very marked effects on stomatal aperture, locking stomata open, preventing stomatal closure in response to a number of different closing stimuli (ABA, transfer from light to dark, hydrogen peroxide, and high external Ca²⁺), and inducing reopening when applied to closed stomata. Measurement of the effect of PAO on tracer fluxes in isolated guard cells showed that PAO prevents stomatal closing by blocking ion (K⁺/Rb⁺) efflux at the tonoplast, the release from vacuole to cytoplasm, with no effect on the efflux at the plasmalemma (MacRobbie, 2002).

Taken together, the evidence suggests that the effect of actin-modifying agents on aperture and on tonoplast ion fluxes should be investigated. In this paper two agents were tested: jasplakinolide, which stabilizes actin filaments (Bubb *et al.*, 1994), and latrunculin B, which depolymerizes actin filaments (Spector *et al.*, 1989, 1999). The results suggest that actin is involved in the regulation of tonoplast ion fluxes, but through modulation of the fluxes determining cytoplasmic Ca²⁺, and not as the target for PAO.

Actin has been implicated in the signalling chains associated with cold acclimation in plant cells and expression of cold-induced genes. In this process, cold induces Ca²⁺ influx and also the activation of a specific MAP kinase (Örvar *et al.*, 2000; Sangwan *et al.*, 2001; Sangwan *et al.*, 2002). A role for MAP kinase in ABA signalling in guard cells was proposed by Burnett *et al.* (2000), who showed by in-gel kinase assay that

ABA activated a myelin basic protein kinase activity, with the characteristics of a MAP kinase, in epidermal tissue of the Argenteum mutant *Pisum sativum* L. They also showed that this activation was blocked by PD98059, which blocks activation of MAP kinase, and that PD98059 partially inhibited ABA-induced stomatal closure. Jiang *et al.* (2003) showed that PD98059 prevented the ABA-induced generation of H₂O₂ in guard cells. A role for the MAP kinase MPK3 in guard cell signalling was further demonstrated by interference with stomatal responses to ABA and H₂O₂ in transgenic *Arabidopsis* plants with guard cell-specific expression of antisense *MPK3* (Gudesblat *et al.*, 2007). The effect of PD98059 on ion release from the vacuole was therefore tested. The results show that PD98059 inhibits both dark-induced and ABA-induced stomatal closure, and reduces the ABA-triggered release of vacuolar ions, suggesting that activation of MAP kinase is involved in the signalling chain linking ABA to tonoplast ion fluxes, highlighting similarities between this chain and that involved in cold acclimation.

Materials and Methods

Inhibitors

Actin-modifying agents, jasplakinolide and latrunculin B, and PD98059 were obtained from Calbiochem (CN Biosciences, Nottingham, UK), and were dissolved in dimethyl sulphoxide (DMSO) before dilution into the incubation medium. Final concentrations of DMSO were in the range 0.1–1%, and solvent controls were tested.

Aperture measurements

Leaves of *Commelina communis* L. were submerged in water and incubated for at least 1 h, either in the light to open stomata or in the dark to close them. Abaxial epidermal strips were removed and floated on 30 mM KCl, 0.1 mM CaCl₂, 10 mM Pipes, pH 6, in a thermostated cabinet at 25°C, either in the light provided by a bank of fluorescent tubes (PAR 140 μmol m⁻² s⁻¹) or in the dark. Strips were transferred to a slide for aperture measurements with an eyepiece micrometer. Apertures were measured on the same strips throughout each experiment, initially before incubation with or without inhibitors present, and again at intervals during the incubation. In general, apertures were measured over 5–6 h, but in some experiments they were measured again after overnight incubation in closing conditions, with or without an inhibitor.

Efflux measurements

Flux measurements were as described previously (MacRobbie, 2002). Isolated guard cells of *C. communis* were prepared by treatment of epidermal strips at low pH, to kill all cells other than guard cells. Cells were loaded by incubation overnight (*c.* 16 h)

in solutions containing 2 mM RbCl, 0.1 mM $CaCl_2$ and 10 mM Pipes, pH 6, labelled with $^{86}Rb^+$ (Amersham International, Little Chalfont, UK). (Thus Rb^+ is used as an analogue for K^+, not as a tracer.) Effluxes were measured by transferring individual strips to successive portions of nonradioactive solutions of the same chemical composition, in the wells of plastic culture chambers on a vibrating shaker, and counting both these washout solutions and the residue left in the tissue at the end of the efflux, using standard scintillation methods. In general, 0.75 ml portions were used, but for experiments with jasplakinolide this was reduced to 0.5 ml, because of cost.

Tracer was expressed as pmol mm^{-2}, on the basis of area of each individual strip, and the rate of loss was calculated for each time interval. During the washout the total tracer content in the tissue (Q^*) will be represented by the sum of two exponentials, reflecting exchanges in the cytoplasm (fast component) and vacuole (slow component), respectively. Cytoplasmic and vacuolar contents were calculated from the intercepts and rate constants of the two exponentials. To reduce the variability between different strips arising from difference in number and size of guard cells, the rate constant for exchange (h^{-1}) was calculated as rate of loss of tracer/tracer content, and plotted against time. In constant conditions, this rate constant falls with time as the cytoplasmic efflux proceeds, to reach a steady value equal to the rate constant for vacuolar exchange when the slow phase is reached. The addition of an inhibitor during the slow phase of exchange allows its effect on the vacuolar flux to be assessed.

Imaging of actin filament organization

Arabidopsis thaliana (L.) Heynh., ecotype Landsberg *erecta*, lines expressing the green fluorescent protein (GFP)-mTalin (Kost *et al.*, 1998) were used in this study. *Arabidopsis* seeds were surface-sterilized in 20% (v/v) Parazone (commercial bleach) for 15 min, washed twice with sterile water and plated on media plates containing 1/2 strength MS basal salts + Gamborg's B5 vitamins (Sigma M-0404), 0.5 g l^{-1} MES, 0.7% agar (Sigma A-1296), pH 5.6, and sealed with gas-permeable tape. Seedlings were grown under continuous light at 18–22°C.

Whole 14-d-old seedlings were removed from the agar and incubated in 2 ml portions of solution in plastic culture dishes, in appropriate conditions as indicated. Individual leaves were then removed, and the adaxial surfaces of leaves were imaged using a Leica TCS-SP confocal microscope (Leica, Milton Keynes, UK). Images were acquired as z-series with approx. 1 μm intervals using the ×63 water immersion lens. To prevent movement during confocal microscopy, leaf samples were mounted between a microscope slide and a #0 coverslip in warm (35°C) 1% low-melting-point agarose, which was then allowed to set before imaging. GFP was excited using the 488 nm argon laser, and the fluorescence emission was collected between 505 and 530 nm for GFP, and between 600 and 700 nm for chlorophyll autofluorescence.

Results

Effects of actin-modifying agents on the organization of actin filaments in guard cells

The effects of the two actin-modifying agents on the organization of actin filaments in guard cells were checked, by confocal imaging of guard cells in *Arabidopsis* expressing the actin-binding domain of mouse talin tagged with GFP (Kost *et al.*, 1998). Cells were imaged after incubation in various conditions for a range of times. In control conditions (Fig. 1a), the arrangement is similar to that observed previously, with thick actin fibrils in a predominantly radial orientation. After treatment with 5 μM latrunculin B (Fig. 1b), fibrils disappeared, showing that this agent depolymerizes actin filaments in guard cells. In cells treated with ABA, in the absence of jasplakinolide (Fig. 1c), there are fewer filaments than in the control, giving a reduced fluorescent signal, as observed by previous authors. In contrast, Fig. 1(d) shows cells treated with ABA in the presence of 10 μM jasplakinolide in which actin fibrils persist, with short thick fibrils, similar to the findings of Sawitsky *et al.* (1999) and Bubb *et al.* (2000). Thus the two agents are effective in guard cells, with the expected results.

Effects of actin-modifying agents on stomatal aperture

Stomata in isolated epidermal strips respond less rapidly and less dramatically to changes in conditions than do stomata in intact leaves, and apertures may not be steady at the start of the incubation, after isolation and transfer of strips to the incubation medium. The starting apertures therefore vary between experiments, and comparisons of different treatments must be made between replicate sets of strips in the same experiment.

The two agents, jasplakinolide and latrunculin B, with opposite effects on the degree of polymerization of actin, also had opposite effects on stomatal closure induced by 10 μM ABA. Figure 2(a) shows an experiment in which epidermal strips were pretreated in 9 μM jasplakinolide, before adding 10 μM ABA at zero time on the graph; in each of three experiments jasplakinolide prevented or strongly inhibited ABA-induced closure (in 18 epidermal strips in all). A pretreatment period was required to observe the full effect. In two other experiments, jasplakinolide and ABA were added together, without pretreatment (eight strips). In this instance, stomata in jasplakinolide-treated strips closed or partially closed, but then reopened; in control strips treated with ABA alone, stomata remained closed. Figure 2(b) shows that 5 μM latrunculin B had the opposite effect, increasing the rate of ABA-induced stomatal closure; this effect was observed in eight strips.

The effect of jasplakinolide on dark-induced stomatal closure was also investigated. Figure 3(a) shows that jasplakinolide (after pretreatment) inhibited closure of open stomata on transfer from light to dark. Figure 3(b) shows that jasplakinolide
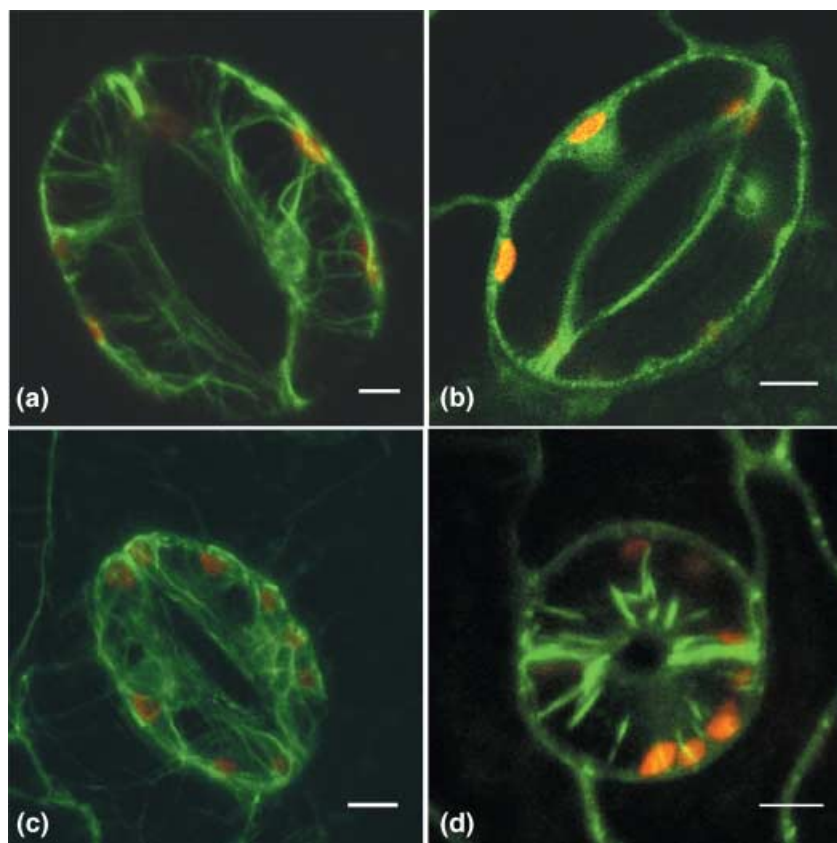
Fig. 1 Effects of jasplakinolide (JK) and latrunculin B (Lat B) on the organization of F-actin in *Arabidopsis* guard cells. Confocal imaging of guard cells expressing green fluorescent protein (GFP)-tagged mTalin; seedlings were in light at the start, followed by incubation in the light with or without an inhibitor. (a) Control leaves, stomata opened in light; (b) incubation in 5 μM Lat B for 2 h; (c) incubation in 10 μM abscisic acid (ABA) in the absence of JK for 1 h; (d) incubation in 10 μM ABA in the presence of 10 μM JK for 4 h. Bar, 4 μm.

did not inhibit stomatal opening on transfer of closed stomata from dark to light, but did block closing on re-transfer to the dark. Inhibition of dark-induced closure was observed in 19 strips, and lack of effect on light-induced opening in 17 strips. As with the ABA experiments, it appeared that a period of pretreatment with jasplakinolide was necessary before the inhibition of closure developed.

Effect of actin-modifying agents on the ABA-induced efflux transient at the tonoplast

In flux experiments, the most marked effect of ABA is a transient stimulation of efflux at the tonoplast (MacRobbie, 1995). The effect of inhibitors on tonoplast ion fluxes was determined by adding the inhibitor during the slow phase of exchange, when exchange of cytoplasmic tracer is complete and rate of tracer loss is limited by transfer from vacuole to cytoplasm. As the absolute values for tracer loss differ between experiments, comparisons of efflux transients must be made within each individual experiment, between replicate sets of strips in different treatments, rather than between different experiments.

The effects of jasplakinolide and latrunculin B on this efflux transient were established by pretreating with the inhibitor for 40 min, before adding ABA at 60–70 min of washout. The final concentration of DMSO was 1% in the

jasplakinolide experiments, and 0.1% in the experiments with latrunculin B. The ABA-induced efflux transient was not affected by 0.1% DMSO, but the peak was significantly increased by 1% DMSO. The effect of jasplakinolide or latrunculin B was determined by comparison with a control containing the same concentration of DMSO.

The two inhibitors have opposite effects on the efflux transient induced by 10 μM ABA. Figure 4 shows that the efflux transient induced by 10 μM ABA is inhibited by 7 μM jasplakinolide (Fig. 4a), but enhanced by 5 μM latrunculin B (Fig. 4b); neither inhibitor affected the rate constant for efflux before the addition of ABA. In two such experiments with jasplakinolide, the peak was inhibited to 66 and 43% of the control peak. In three experiments using 5 μM latrunculin B, the peak height was increased to $157 \pm 7\%$ of the control; the effects of 1 μM latrunculin B were very similar, with a peak of $141 \pm 10\%$ of the control in three experiments. The results suggest that a component of filamentous actin may act as a negative regulator of the response to 10 μM ABA. At this concentration of ABA, Ca^{2+} influx makes the major contribution to increase in cytoplasmic Ca^{2+}, whereas at suboptimal concentrations of ABA (0.1 μM) release of Ca^{2+} from internal stores plays the major role (MacRobbie, 2000).

The effects of latrunculin B on efflux transients at 0.1 μM ABA were also tested and, in contrast to those at 10 μM ABA,
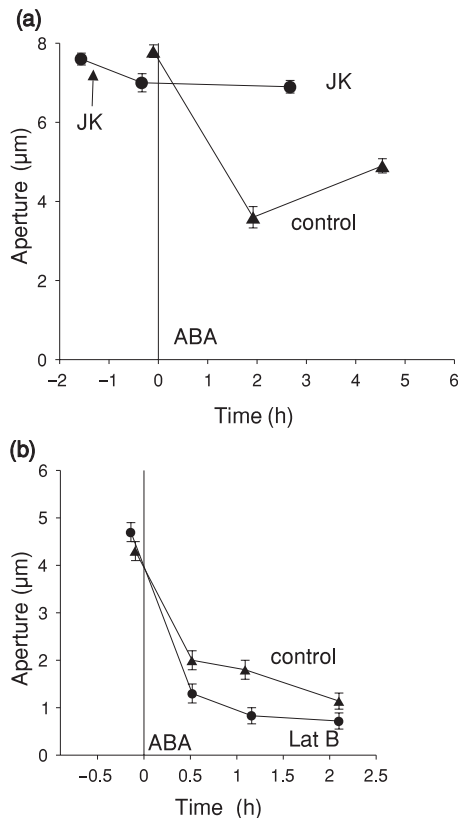
Fig. 2 Jasplakinolide (JK) inhibits and latrunculin B (Lat B) enhances abscisic acid (ABA)-induced stomatal closure. Stomatal aperture plotted against time. Each point is the mean and SEM of four to six strips (64–109 pores). (a) 10 μM ABA added at zero time, after pretreatment with 9 μM JK for 1.4 h; (b) 10 μM ABA added at zero time, after pretreatment with 5 μM Lat B for 1.2 h. Triangles, control; circles, treated.
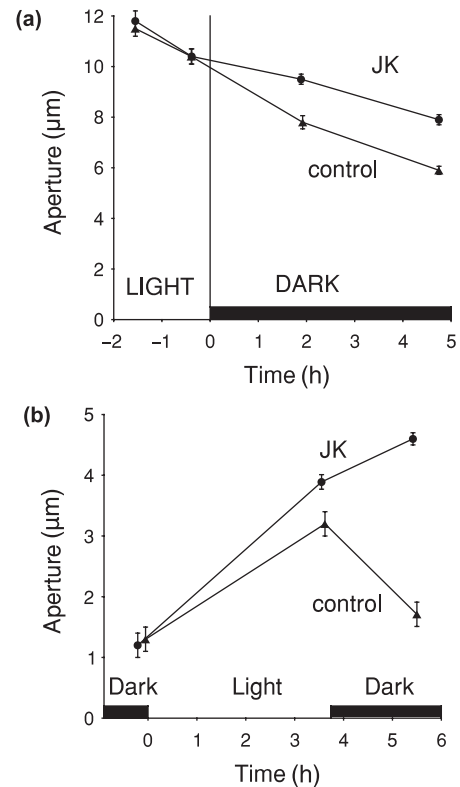

Fig. 3 Jasplakinolide (JK) inhibits dark-induced stomatal closure but has no effect on light-induced stomatal opening. Stomatal aperture plotted against time. Each point is the mean and SEM of four to six strips (64–96 pores). (a) Transfer from light to dark at zero time, after pretreatment with 7.5 μM JK for 1.4 h; (b) transfer from dark to light at zero time, after pretreatment with 5 μM JK for 1.6 h. Return to dark at 04:13 hours. Triangles, control; circles, treated.

were found to be inhibitory. Figure 5 compares the effect in the same tissue of 5 μM latrunculin B at 10 and 0.1 μM ABA; at 10 μM ABA the peak is increased by latrunculin B, to $160 \pm 38\%$ of the control, whereas the peak induced by 0.1 μM ABA is decreased by latrunculin B, to $70 \pm 12\%$ of the control. The mean in three experiments at 0.1 μM ABA was a reduction in peak height to $58 \pm 7\%$ of the control. Thus in conditions where the efflux transient is triggered by internal Ca^{2+} release, actin depolymerization inhibits the transient. The results suggest multiple roles for actin in the ABA signalling processes concerned with triggering ion release at the tonoplast. They suggest that components of filamentous actin are active in negative regulation of the response involving Ca^{2+} influx, but play a positive role in the response involving Ca^{2+} release from internal stores.

Involvement of MAP kinase in signalling chains

The effect of PD98059, which prevents activation of MAP kinase, was tested in both aperture and flux experiments, Fig. 6(a)

shows that PD98059 inhibited ABA-induced stomatal closure; a repeat experiment gave very similar results. PD98059 also inhibited dark-induced stomatal closure to a similar extent (data not shown). The effect of PD98059 on aperture in the absence of any closing signal was small, and could be either positive (as shown in Fig. 6a) or negative.

In flux experiments, PD98059 proved very difficult to use, since it tended to precipitate out with time, and when this happened no effect was observed. The problem was less at higher ionic strength (as was used in aperture experiments). In one experiment when treatments at 2 mM and 20 mM Rb^+ were compared, the inhibitor stayed in solution at 20 mM, to give the results shown in Fig. 6(b), but precipitated out at 2 mM and gave no effect. In the experiment shown, 100 μM PD 98059 delayed the ABA-induced efflux transient and reduced the rate of rise and the peak height. In three separate experiments (all at 20 mM RbCl), the rate of rise was reduced to 27 and 48% of the control by 100 μM PD98059, and to 64% of the control by 50 μM PD98059; in the last experiment 20 μM U0126, another inhibitor of MAP kinase
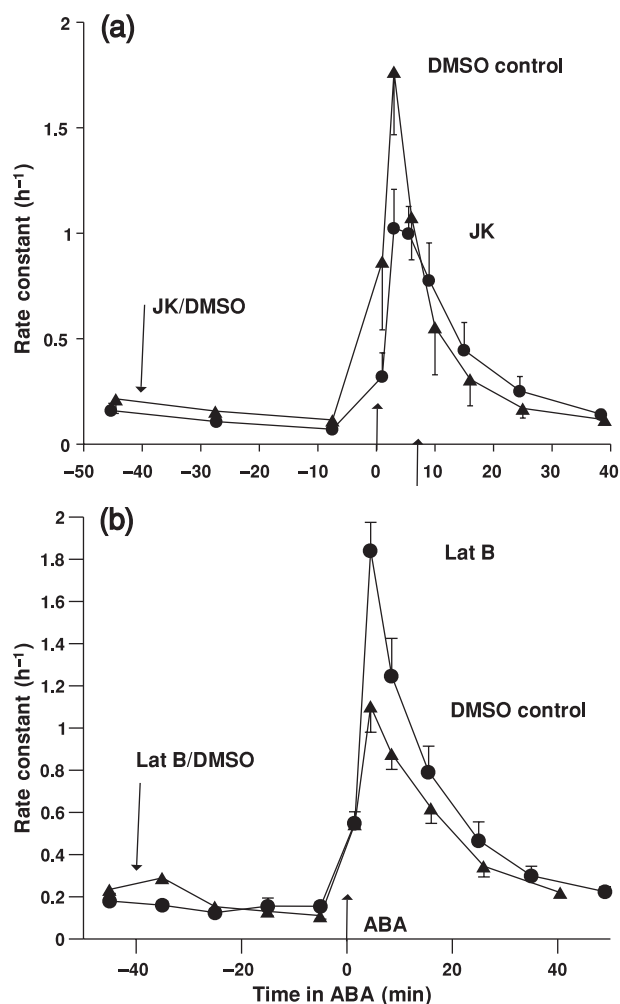
Fig. 4 Jasplakinolide (JK) inhibits and latrunculin B (Lat B) enhances the vacuolar efflux transient induced by 10 μM abscisic acid (ABA). Rate constant for $^{86}Rb^+$ efflux plotted against time. Each point shows mean and SEM of four strips. Pretreatment with 7 μM JK or 5 μM Lat B (or dimethyl sulphoxide (DMSO) in controls) for 40 min before adding 10 μM ABA at zero time on the graph, at, respectively, 70 or 60 min of washout. Triangles, control; circles, treated.

Fig. 5 Latrunculin B (Lat B) enhances the vacuolar efflux transient in response to 10 μM abscisic acid but inhibits that induced by 0.1 μM ABA. Rate constant for $^{86}Rb^+$ efflux plotted against time. Each point shows mean and SEM of four strips. Pretreatment with 5 μM Lat B (or dimethyl sulphoxide (DMSO) in controls) for 40 min before adding 10 or 0.1 μM ABA at zero time on the graph, at 60 min of washout. Control 10 μM ABA, upward triangles; 10 μM ABA/Lat B, circles; control 0.1 μM ABA, downward triangles; 0.1 μM ABA/Lat B, squares.

activation, had the same effect as PD98059, reducing the rate of rise to 59% of the control. Thus blocking the activation of MAP kinase produces a partial inhibition of the ABA-induced efflux transient.

Discussion

The aim of the paper was to test whether actin played a role in stomatal closure, and in particular in the ABA-induced stimulation of K^+/Rb^+ release from vacuole to cytoplasm, and the results show that the actin-modifying agents, jasplakinolide and latrunculin B, did affect this key process, critical for stomatal closure.

Confocal microscopy of GFP-labelled Talin plants was used to confirm that the effects of jasplakinolide and latrunculin B in
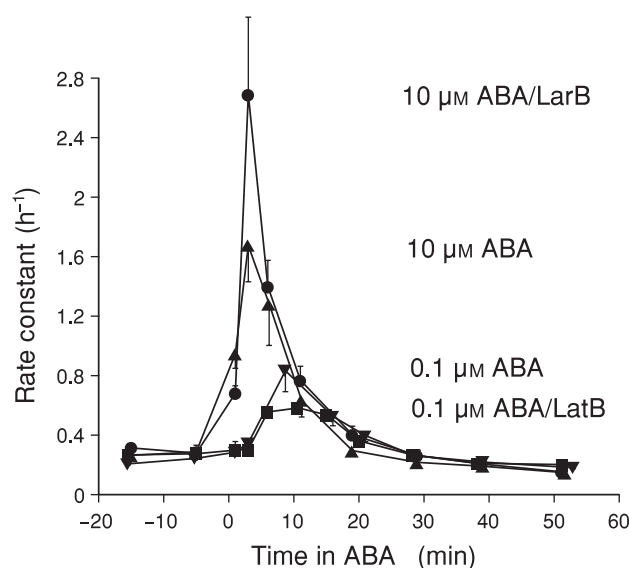
guard cells were as expected. Some caution is needed in the interpretation of GFP-mTalin images, since two problems with its use have been highlighted in recent work (Ketelaar *et al.*, 2004). The first question is the extent to which GFP-mTalin expression interferes with normal growth and function of the cell. In our material, as in most previous studies in which GFP-mTalin was constitutively expressed, rather than inducibly expressed to high concentrations, growth of seedlings and stomatal function appeared to be normal. The second problem is that GFP-mTalin may cause artificial aggregation of actin filaments, and may therefore not give an accurate representation of the true state. Sheahan *et al.* (2004) introduced an alternative tag without these problems, using a fusion protein of GFP tagged to the actin binding domain 2 of *Arabidopsis* fimbrin (GFP-fABD2)), which was effective in visualizing intricate networks not seen with GFP-mTalin, and may give images closer to the true organization. However, for the purposes of this paper, the question is whether treatment with jasplakinolide or latrunculin B produces the expected changes in actin organization in the guard cell. A reorganization of actin can be identified in response to a given treatment, even if neither image is a completely accurate representation of the true network organization, and the results confirm the effects of jasplakinolide in stabilizing actin filaments, and latrunculin B in depolymerizing actin filaments.

The two actin-modifying agents had opposite effects on ABA-induced changes in stomatal aperture. Stomatal closing induced by 10 μM ABA was inhibited by jasplakinolide but enhanced by latrunculin B. Jasplakinolide also inhibited
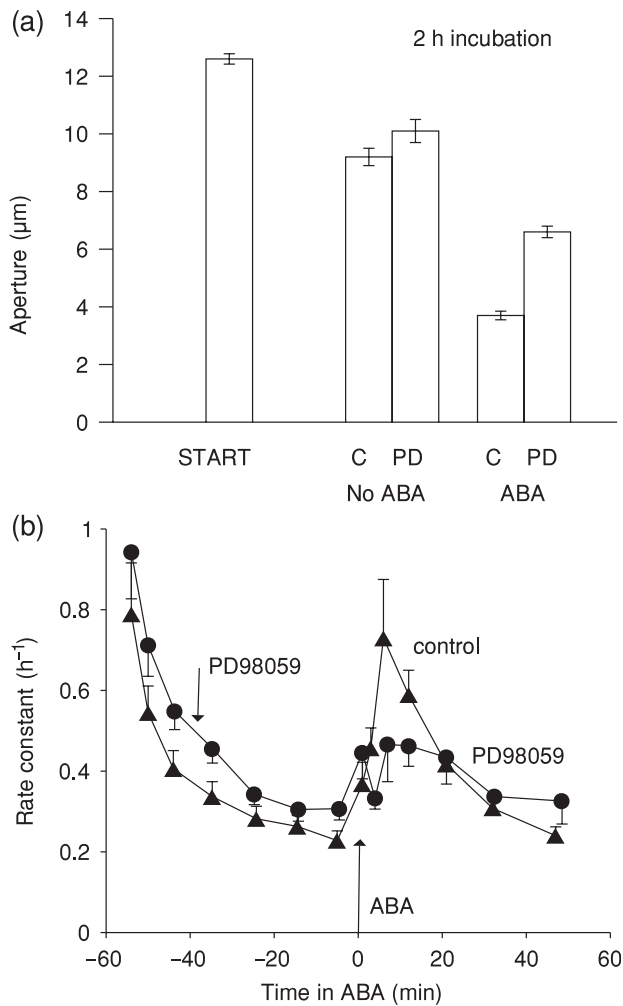
Fig. 6 PD98059 inhibits abscisic acid (ABA)-induced stomatal closing and ABA-induced vacuolar efflux transient. (a) Stomatal aperture (mean ± SEM), at the start and after 2 h incubation in light, with (PD) or without (C) 100 µM PD98059, and with or without 50 µM ABA. Starting aperture, mean of 12 strips; ABA treatments, mean of four strips; no ABA treatments, mean of two strips each; 17–21 pores measured on each strip. (b) Rate constant for ^{86}Rb$^+$ efflux plotted against time. Each point shows mean and SEM of four strips. Pretreatment with 50 µM PD98059 for 40 min before adding 10 µM ABA at zero time on the graph, at 60 min of washout. Solutions contained 20 mM RbCl. Triangles, control; circles, PD98059.

concentration of ABA, the ABA-induced transient stimulation of tonoplast efflux was reduced by jasplakinolide, but was enhanced by latrunculin B. The flux results suggest that actin is involved in the control of the activation of tonoplast ion fluxes by ABA, and provide an explanation of the aperture effects. In the response to 10 µM ABA, Ca^{2+} influx makes the major contribution to the increase in cytoplasmic Ca^{2+} which triggers the vacuolar efflux transient, and the results could be explained if the Ca^{2+} influx channel was maintained in the closed configuration by interaction with a component of filamentous actin. Evidence for this explanation may be provided by the recent identification in guard cells of an osmosensitive, stretch-activated, Ca^{2+}-permeable channel, which is regulated by actin dynamics, activated by actin depolymerization (Zhang *et al.*, 2007). Such a channel could also explain the turgor-sensitivity of the vacuolar K$^+$/Rb$^+$ channel, stimulated at high turgor and reduced at low turgor (MacRobbie, 2006). However, in the present results latrunculin B did not affect the resting efflux of Rb$^+$ from the vacuole, but did enhance the ABA-induced stimulation of this flux. If the actin-regulated, stretch-sensitive, Ca^{2+}-permeable channel is involved, there may be additional control mechanisms in the resting state.

The effects of actin-modifying agents on the guard cell in response to 10 µM ABA suggest signalling chains similar to those established for cold acclimation in plant cells, in which cold induces Ca^{2+} influx, leading to expression of cold-induced genes (Örvar *et al.*, 2000; Sangwan *et al.*, 2001). Cold-induced gene expression (of *cas30* in alfalfa, or *BN115* in *Brassica napus*) requires, sequentially, membrane rigidification (which can be produced by DMSO at room temperature), remodelling of the actin cytoskeleton and Ca^{2+} influx. At 4°C, Ca^{2+} influx and consequent gene expression are inhibited by jasplakinolide, whereas depolymerization of actin microfilaments (by cytochalasin D in alfalfa or latrunculin B in *Brassica*), activates Ca^{2+} influx and associated gene expression at 25°C. These results suggest that a component of F-actin acts as a negative regulator on Ca^{2+}-channel opening. There is also evidence that in pollen tubes of *Arabidopsis*, Ca^{2+}-permeable channels in the plasmalemma are activated by disassembly of F-actin by cytochalasins, increasing cytoplasmic Ca^{2+} (Wang *et al.*, 2004). The effects of jasplakinolide and latrunculin B on the ABA-induced efflux transient in guard cells, in conditions where Ca^{2+} influx provides the trigger, suggests that this mechanism, in which a component of F-actin acts to maintain a Ca^{2+}-permeable channel in its closed configuration, also features in guard cell signalling. This form of regulation may therefore be common in plant cells, featuring in a number of different signalling chains.

Further work on cold acclimation suggested that this signalling chain, with membrane rigidification, remodelling of the actin cytoskeleton, Ca^{2+} influx and CDPK activation, then terminates in activation of a specific MAP kinase, SAMK (Sangwan *et al.*, 2002). Involvement of a MAP kinase cascade in ABA signalling in guard cells is an attractive hypothesis,

dark-induced stomatal closure, with no effect on light-induced opening. The aperture results are not identical to the effects observed earlier, using phalloidin and cytochalasins ((Kim *et al.*, 1995); the inhibition of ABA-induced closing by jasplakinolide is similar to that observed with phalloidin, but the earlier work found no effect of cytochalasin on ABA-induced closure, whereas latrunculin B clearly enhances the ABA response.

Neither jasplakinolide nor latrunculin B affected the resting tonoplast efflux, but the two agents had opposite effects on the response of the tonoplast efflux to 10 µM ABA. At this

and is proposed by Burnett *et al.* (2000); they observed activation by ABA of a MAP kinase in guard cells of *Pisum sativum*, and partial inhibition of ABA responses by PD 98059, which blocks such activation. Jiang *et al.* (2003) found that PD98059 prevented ABA-induced H_2O_2 production in guard cells of *Vicia faba* (Pei *et al.*, 2000). Compromised guard-cell responses to ABA and H_2O_2 were also observed in transgenic *Arabidopsis* plants with guard cell-specific antisense *MPK3* (Gudesblat *et al.*, 2007). ABA-induced *rab16* gene expression in barley aleurone also involves activation of a MAP kinase, and both processes are blocked by PAO (Knetsch *et al.*, 1996). The present results show that in *Commelina* guard cells responding to 10 μM ABA, PD98059 produces partial inhibition of both ABA-induced stomatal closure (as found in *Pisum* by Burnett *et al.*, 2000), and the ABA-induced vacuolar efflux transient. The effect on the vacuolar efflux transient provides an explanation for the effect on aperture, and gives further support to the suggestion that the vacuolar efflux response shares signalling chains with the response to cold.

There is evidence that both cADPR and inositol trisphosphate ($InsP_3$), triggers of release of Ca^{2+} from internal stores, are also involved in ABA-induced stomatal closure (Leckie *et al.*, 1999; Staxen *et al.*, 1999). Specifically, it was found that at suboptimal concentrations of ABA, internal Ca^{2+} release, rather than Ca^{2+} influx, was the major contributor to the triggering of the ABA-induced efflux transient, with both cADPR and $InsP_3$ as triggers (MacRobbie, 2000). The effects of latrunculin B on the ABA-induced efflux transient were therefore also tested at suboptimal conditions, at 0.1 μM ABA. In these conditions, latrunculin B inhibited the efflux transient. One explanation could be inhibition of the release of Ca^{2+} from the endoplasmic reticulum by latrunculin B, as has been shown in hippocampal neurons, whether mediated by the ryanodine receptors or by $InsP_3$ receptors (Wang *et al.*, 2002). Hence the results suggest multiple roles for actin in the ABA signalling processes concerned with triggering vacuolar ion release, with components of filamentous actin exerting a negative role on Ca^{2+} influx, but a positive role on internal Ca^{2+} release.

There are two other examples where it is argued that F-actin maintains an ion channel in the closed state: a Na^+ channel in human myeloid leukaemia cells (Negulyaev *et al.*, 1996, 2000), and in control of the inward K^+ channel in the guard cell plasmalemma (Hwang *et al.*, 1997; Liu & Luan, 1998). Here it is argued that a Ca^{2+} influx channel in guard cells, or other plant cells as discussed, appears to show such regulation, with F-actin maintaining the channel in the closed state. However, it should be noted that the effect on inward K^+ channels is likely to be direct, since this channel is Ca^{2+}-inhibited. In guard cells, such control of a Ca^{2+}-permeable channel has now been directly demonstrated for the stretch-activated, osmosensitive, Ca-permeable channel identified by Zhang *et al.*, 2007). Such control is likely to be exerted through association of specific actin filaments with membranes,

actin-binding proteins, channel regulators or channels, in macromolecular complexes, rather than through changes in the gross organization of the bulk of actin filaments. The relation, causal or otherwise, between stomatal opening/closing and gross reorganization of actin fibrils in guard cells remains uncertain. Evidence for actin involvement comes from the effects of actin-modifying agents on aperture and fluxes, rather than from the correlation between the pattern of actin organization in guard cells and stomatal aperture.

However, although modifying actin dynamics does affect ABA-induced vacuolar ion release, there are two reasons for suggesting that actin is unlikely to be the main target for PAO in the inhibition of tonoplast ion efflux by that inhibitor. The first is that the effects of the actin-modifying agents are much less dramatic than those of PAO, which reduced the resting tonoplast efflux to c. 20% of the control and abolished any stimulation by ABA. Neither jasplakinolide nor latrunculin B affected the resting tonoplast efflux, and the effects on the size of the ABA-induced efflux transient were clear, but moderate. Jasplakinolide inhibited but did not abolish the ABA-induced efflux transient. At 10 μM ABA, jasplakinolide inhibited the ABA-induced efflux transient to 43–66% of the control, and 5 μM latrunculin B increased the peak to $157 \pm 7\%$ ($n = 3$), or 1 μM latrunculin B to $141 \pm 10\%$ ($n = 3$). Secondly, the different effects of latrunculin B on efflux transients at different concentrations of ABA suggest modulation of Ca^{2+} fluxes, rather than direct interaction between actin and the tonoplast efflux channel (directly or via a specific channel regulator protein). Taken together, these results suggest that the primary target for PAO in the regulation of tonoplast efflux, located in the signalling chains downstream of Ca^{2+}, is not actin. The target remains to be identified, but it is possible that it is the SV channel, recently molecularly identified as the TPC1 protein by Peiter *et al.* (2005). The SV channel is a strong candidate as the channel responsible for K^+ release from vacuole to cytoplasm (Ward & Schroeder, 1994), and is strongly inhibited by PAO (Scholz-Starke *et al.*, 2004). The effect of PAO on the other strong candidate channel, the VK channel (Ward & Schroeder, 1994), is not known. The action of PAO in blocking vacuolar ion release may reflect a direct interaction with the tonoplast efflux channel (or with a specific channel regulator protein), whereas actin seems to exert its control less directly, upstream of Ca^{2+}.

Acknowledgements

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