The effects of manipulating phospholipase C on guard cell ABA-signalling

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Abstract

Studies using stably transformed tobacco plants containing very low levels of PI-PLC in their guard cells show that this enzyme plays a role in the events associated with the inhibition of stomatal opening by ABA, but not in the cellular reactions that are responsible for ABA-induced stomatal closure. However, Commelina communis guard cells microinjected with the InsP3 antagonist, heparin, fail to close on addition of ABA. There are three possible explanations for this apparent data mismatch. The differences may be indicative of species-specific signalling pathways, the presence of a PI-PLC isoform(s) that is not down-regulated in these transgenic lines and/or they may reflect differences between short-term (acute) administration of an inhibitor and long-term (chronic) effects of gene manipulation. It is possible that the guard cell is a robust signalling system that is able to adapt or compensate for the chronic loss of PI-PLC, but which is unable to adjust quickly to acute loss of this component. It would be interesting to investigate this possibility further using either transient manipulation of gene expression or through the use of an inducible promoter.

Key words: Abscisic acid, calcium ions, guard cell, phospholipase C, signalling, stomata.

Introduction

Stomata have proved to be an excellent model for investigating signal transduction in plants (Assmann and Wang, 2001; Hetherington and Woodward, 2003). Not only do they respond to many different signals, but changes in stomatal aperture also provide the robust readout essential for investigating the subtle phenotypic effects produced by pharmacological intervention or gene manipulation. One of the best-understood guard cell responses to an external signal are the cellular events that follow perception of the plant hormone abscisic acid (ABA) (Blatt, 2000; Hetherington, 2001; Schroeder et al., 2001). Application of ABA to open stomata results in a decrease in the stomatal pore that is achieved by inhibiting the processes associated with stomatal opening and promoting the cellular events that occur during stomatal closure.

It has been suggested that guard cell signalling exhibits properties similar to scale-free networks and that an increase in cytosolic free calcium concentration ([Ca2+]cys) is an important node or hub involved in the response of stomata to numerous extracellular signals (Hetherington and Woodward, 2003). In guard cell ABA signalling, an increase in [Ca2+]cys can be generated through the involvement of plasma membrane calcium-permeable channels, phosphatidyl inositol specific phospholipase C (PI-PLC), cyclic ADP ribose (cADPR), sphingosine-1-phosphate (S1P), and, possibly, inositol hexakisphosphate (InsP6) (reviewed in Hetherington, 2001; Schroeder et al.,...
Materials and methods

Chemicals
All chemicals used were obtained from the Sigma–Aldrich Company Ltd. (Dorset, UK) unless otherwise stated.

Plant material and growth conditions
Commelina communis was grown in Levington M3 compost under controlled conditions, 16 h photoperiod 26/20 °C day/night, relative humidity 34% with illumination provided by 400 W metal halide lamps (Osram, UK) with a minimum photon flux density of 100 μm m⁻² s⁻¹ (400–700 nm). Experiments were conducted on 5–6-week-old plants. The production of Nicotiana tabacum cv. Wisconsin 38, in which the levels of the NrPLC2 gene (Staxen et al., 1999) had been manipulated such that transgenic lines exhibited low levels of PI-PLC protein in guard cells, was described in Hunt et al. (2003). The plants were grown in Levington M3 compost in a growth room at 24 °C, relative humidity 30–45%, a 16/8 h light/dark cycle, and a light intensity of 130 μmol m⁻² s⁻¹.

Isolated epidermal peel stomatal bioassays
The lower epidermis was removed from the sixth leaf from the shoot apex of 7–8-week-old plants and stored in 10 mM MES-2-[N]-morpholinoethane sulphonic acid/KOH, pH 6.15 at 20 °C until required. Inhibition of stomatal opening by ABA was investigated as described in Hunt et al. (2003). Promotion of stomatal closure by ABA was investigated by first incubating epidermal strips for 3 h in conditions designed to promote stomatal opening (Hunt et al., 2003) and then transferring the strips to 10 mM MES/KOH, 100 mM KCl, pH 6.15, containing various concentrations of 0–10 μM ABA for a further 2 h and then measuring stomatal apertures as described by McInish et al. (1995). Data were analysed using SigmaStat 2.03 software (SPSS Inc., USA). A Kruskal–Wallace One-way analysis of Variance on Ranks was used to analyse the aperture measurements for each treatment. Dunn’s method was used to identify treatments that were significantly different from the control. Significance was assigned at the 5% level.

Stomatal conductance measurements
Porometry: Tobacco plants were grown to 6 weeks as described above with the exception that John Innes loam-based No. 2 compost (J Arthur Bowers, Lincoln, UK) and Cem Pak Horticultural Grit (Cem Pak, Dewsbury, UK) in an 8:1 ratio was used to allow more uniform soil drying. Plants were watered until saturation on day 0 and then water was withheld and then rewatered after 2 d. Abaxial surface gas exchange was measured with a Delta-T AP4 Porometer (Delta-T devices, Cambridge, UK). Two measurements were taken from each plant from the fifth and sixth leaf from the shoot apex.

Infrared gas analysis: Leaf stomatal conductance was determined using an IRGA (Ciras-one, PP systems). The leaf chamber was set to CO2 concentration 350 ppm (ambient), a photon flux density of 550 μmol photons m⁻² s⁻¹ (white light), relative humidity 55%, and a gas flow rate 200 ml min⁻¹. Plants were dark adapted for 1 h prior to the start of the experiment. Measurements were carried out on mature leaves from approximately 10-week-old plants. Control readings were taken from plants that had been watered daily. Stomatal conductances of well-watered control and low-PLC plants did not differ significantly (data not shown). Plants were then subjected to a 3 d drought by withholding water, and IRGA readings repeated. The droughted plants were then rewatered before taking the IRGA readings. In each case measurements were taken from two leaves from each plant and at least three plants from each line.

Heparin microinjection
Freshly prepared epidermis of C. communis was perfused with CO₂-free 10 mM MES/15 mM KCl, pH 6.2 at 25 °C and guard cells were cytosolically loaded with either low molecular weight heparin (6000 Da) or dextran (19 500 Da), at a pipette concentration of 0.5 mM, in an injection buffer containing 1 mM lucifer yellow CH and 50 mM KCl using pressure microinjection (Leckie et al., 1998). Visualization of loaded cells, the subsequent promotion of stomatal opening, and half aperture measurements following perfusion with 1 μM ABA were as described previously (Leckie et al., 1998).

Results
To investigate the contribution of PI-PLC to guard cell ABA signalling, use was made of transgenic tobacco plants in which the level of PI-PLC protein in the guard cell had been reduced to very low levels (Hunt et al., 2003). It was demonstrated previously that reducing the level of PI-PLC in guard cells partially interferes with the ability of ABA to inhibit stomatal opening. Figure 1A shows that stomata from the reduced guard cell PI-PLC plants were significantly less sensitive to the application of ABA than the (transformed) control plants. By contrast, Fig. 1B shows that when the ability of ABA to promote stomatal closure was investigated using the same plants, there was no significant difference between the response of the reduced PI-PLC line and its control. Similar results were seen in a second reduced PI-PLC line (data not shown).

To investigate whether the results obtained using isolated epidermal strips reflected the situation in the intact plant, leaf gas exchange techniques used. Figure 2
shows the effects of soil drying on the tobacco reduced PI-PLC plants relative to a (transformed) control. It is clear that withdrawing water from the reduced PI-PLC line and the control plants, a treatment that would be expected to increase leaf ABA levels, leads to progressive reductions in stomatal conductance. These data suggest that the ability to reduce stomatal conductance in response to soil drying, and therefore ABA, was not compromised in the reduced PI-PLC plants. By contrast, when IRGA was used to investigate the ability of stomata of droughted plants, both reduced PI-PLC and control plants subjected to a 3 d drought treatment, which had been dark-adapted for 1 h to reopen in response to light, it was apparent that the resulting increase in stomatal conductance was much greater in the reduced PI-PLC plants than in the control plants (Fig. 3). The same results were observed with another reduced PI-PLC line (data not shown). On the assumption that the soil-drying treatment used in this experiment results in an increase in leaf ABA levels, these data suggest that the ability of ABA to inhibit stomatal opening is compromised in the reduced PI-PLC plants. Therefore, the data in Figs 2 and 3 fully support the conclusion arrived at using the isolated epidermal strips (Fig. 1) that PI-PLC appears to play a role in the inhibition of stomatal opening by ABA but not in the ABA-induced promotion of stomatal closure. However, it should be
noted that ABA-induced promotion of stomatal closure still requires calcium as the calcium chelator, EGTA, partially interferes with ABA-induced closure in both the reduced PI-PLC and control lines (Fig. 4).

Although these data from stably transformed tobacco plants do not support a role for PI-PLC in ABA-induced stomatal closure, there are other data in the literature, obtained by manipulating PLC activity acutely, that strongly support a role for this enzyme in ABA-induced loss of guard cell turgor. For example, administration of the aminosteroid PLC inhibitor U73122 to isolated epidermal strips partially interferes with the ability of ABA to induce stomatal closure (Staxen et al., 1999; Jacob et al., 1999; MacRobbie, 2000). These results suggest that guard cells respond differently to short-term (acute) or long-term (chronic) manipulation of PLC activity. As there are reports in the animal literature that U73122 can have non-specific effects (Pulcinelli et al., 1998; Walker et al., 1998; Cenni and Picard, 1999) it was decided to shift the focus to inositol (1,4,5) trisphosphate (InsP$_3$), the calcium mobilizing intracellular messenger produced through the action of PI-PLC on phosphatidyl inositol (4,5) bisphosphate. In order to investigate the effect of interfering with InsP$_3$ activity Commelina communis was used, a species that has both been extensively used in guard cell signalling research and is also amenable to microinjection. To interfere with InsP$_3$ signalling, low molecular mass heparin ($M_r$ 6000) was used, which is a potent inhibitor of InsP$_3$-mediated Ca$^{2+}$ release from vacuolar stores in plants (Brosnan and Sanders, 1990, 1993). Guard cells of C. communis were pressure-microinjected with low molecular weight ($M_r$ 6000) heparin or dextran ($M_r$ 19 500), to investigate whether this interfered with ABA-induced stomatal closure. Guard cells loaded with low molecular weight ($M_r$ 6000) heparin failed to reduce turgor to the same extent as the uninjected control cell from the same stoma (Fig. 5C, D, arrowed); the half stomatal apertures of the heparin-loaded and unloaded guard cells 20 min after perfusion in 1 $\mu$M ABA were 3.5±0.3 $\mu$m and 1.8±0.3 $\mu$m, respectively ($n=6$). To rule out the possibility that heparin was exerting its effects through alterations to guard cell osmotic conditions, guard cells were microinjected with a relatively high molecular weight dextran ($M_r$ 19 500). The introduction of dextran into the guard cell cytosol had no effect on the subsequent response to ABA; the dextran-injected and uninjected control cell from the same stoma both reduced turgor to the same extent (Fig. 5A, B, arrowed); the half stomatal apertures of the dextran-loaded and unloaded guard cells 20 min after perfusion in 1 $\mu$M ABA were 2.2±0.5 $\mu$m and 1.9±0.5 $\mu$m, respectively ($n=3$). The results from these experiments using the acute application of an inhibitor of InsP$_3$ action fully confirm the previous investigations in which PLC has been shown to be associated with the ABA-induced promotion of stomatal closure (Staxen et al., 1999; Jacob et al., 1999; MacRobbie, 2000). Further support for this hypothesis comes from recent results (Jung et al., 2002) demonstrating that Vicia guard cells transiently expressing GFP-PLC8IPH (believed to interfere with phosphoinositol signalling by binding to the substrate and product of the

**Fig. 4.** Calcium is required for ABA-induced stomatal closure in Nicotiana tabacum guard cell reduced PI-PLC plants. Epidermal strips of Nicotiana tabacum reduced guard cell PI-PLC and control (vector only transformed) plants were incubated under conditions designed to promote stomatal opening and then challenged with ABA in the presence or absence of 2 mM EGTA. Reduced PI-PLC, black bars; control, open bars; reduced PI-PLC+EGTA, hatched bars; control+EGTA, cross-hatched bars. Standard errors from the mean are shown.

**Fig. 5.** Microinjection of heparin into the guard cell cytosol alters the response to ABA. (A) Dextran-loaded cell (tip concentration 500 $\mu$M, arrowed) 60 min after perfusion in MES/KCl buffer under constant illumination. (B) The same cell, 20 min after perfusion in MES/KCl and 1 $\mu$M ABA. (C) Heparin-loaded cell (tip concentration 500 $\mu$M, arrowed) 60 min after perfusion in MES/KCl. (D) The same cell, 20 min after perfusion in MES/KCl and 1 $\mu$M ABA. Bar=10 $\mu$m. Data are representative of measurements on three cells (A, B) and six cells (C, D).
reaction catalysed by PI-PLC, PI(4,5)P2 and InsP3) show decreased ABA-induced reductions in stomatal aperture.

Discussion

The results presented in this paper provide further evidence that PI-PLC is involved in guard cell ABA-signalling. Using tobacco in which the level of guard cell PI-PLC protein had been reduced, the results of a previous paper (Hunt et al., 2003) showing that PI-PLC is involved in the events associated with the inhibition of stomatal opening by ABA (Fig. 1A) were extended. Since these experiments were carried out on isolated epidermal strips, it was also investigated whether the same situation applied at the level of the whole plant. The IRGA results presented in Fig. 3 show that this is the case. However, when it was investigated whether PI-PLC was also involved in the promotion of stomatal closure by ABA no evidence was found, either in isolated epidermal strips (Fig. 1B) or in whole plants (Fig. 2), for the involvement of this enzyme in guard cell turgor loss. On the basis of these results it is possible to conclude that the involvement of PI-PLC in guard cell ABA-signalling is restricted to the processes associated with the inhibition of stomatal opening, not with the promotion of closure and inhibition of stomatal opening.

There are other data in the literature indicating that the promotion of closure and inhibition of stomatal opening are controlled by different processes and that these involve, at least some, separate components. For example, Assmann and colleagues (Wang et al., 2001) showed that the stomata of the Arabidopsis gpa1 mutant (GPA1 encodes a putative subunit of a heterotrimeric G protein) failed to exhibit the inhibition of opening response, but displayed wild-type behaviour in the ABA promotion of stomatal closure bioassay. Additional evidence that the control of stomatal aperture by ABA is mediated by separable components responsible for inhibiting opening and promoting closure is to be found in work from the Neill laboratory. Desikan and co-workers (Desikan et al., 2002) found that stomata of the Arabidopsis nitrate reductase double mutant nia1,nai2 failed to close in response to ABA, but exhibited wild-type behaviour in the inhibition of stomatal opening bioassay. These results suggest that ABA-mediated nitric oxide synthesis is required for the promotion of closure by ABA, but not in the inhibition of stomatal opening.

The data obtained with stably transformed tobacco plants clearly show that although the promotion of stomatal closure by ABA requires an increase in \( [Ca^{2+}]_{cyt} \), this does not appear to require the participation of PI-PLC. However, these data contrast with other investigations carried out using pharmaceutical intervention to investigate the same phenomenon. Administration of the aminosteroid PLC inhibitor U73122 to guard cells on isolated epidermal strips of Commelina communis (Staxen et al., 1999; MacRobbie, 2000) or Vicia faba (Jacob et al., 1999) partially interferes with the ability of ABA to induce stomatal closure.

The results from the inhibitor-based experiments place PI-PLC in the series of cellular events responsible for the promoting stomatal closure after the application of ABA. In order to ensure that these results did not reflect a non-specific effect of U73122, (even though the experiments were controlled through the use of the inactive analogue U73143), it was decided to interfere with PI-PLC signalling at the level of InsP3 activity. In order to do this heparin was used, which has been used as an InsP3 antagonist in plants (Brosnan and Sanders, 1990, 1993). When guard cells of C. communis were microinjected with heparin, they failed to reduce turgor fully (i.e. close) in response to the application of ABA (Fig. 5). This experiment confirms that interfering with PI-PLC signalling, in this case by antagonizing InsP3 action, partially blocks ABA-induced stomatal closure.

There are at least three explanations to account for the apparent disparity in results obtained from the gene manipulation and pharmaceutical intervention experiments. The gene manipulation studies were carried out in tobacco while the inhibitor studies were carried out in Commelina or Vicia. Therefore, it is possible that PI-PLC plays a role in ABA-induced stomatal closure in the latter two species, but not in the former. It is also possible that ABA-induced stomatal closure in tobacco is supported by a PI-PLC isoform(s) that is neither down-regulated in the transgenic lines nor recognized by the antibody raised against NrPLC1 (Hunt et al. 2003). Alternatively, or additionally, it is possible that the differences observed might reflect the results of short-term (acute) inhibitor treatments compared with long-term (chronic) effects of gene manipulation. This raises the possibility that the plant is able to adapt to, or compensate for, the chronic loss of PI-PLC in ABA-induced stomatal closure whereas in the short-term the system does not display this level of robustness or tolerance. If this is part of the explanation for the disparity between the two sets of results, it will be of great interest to investigate it further. Experiments in this area might be especially illuminating as robustness is a recognized emergent property of scale-free networks (Hetherington and Woodward, 2003). Manipulating guard cell PI-PLC expression through the use of an inducible promoter or through transient gene manipulation might provide further information on how robust the network is to a fairly rapid loss of a component. This, in turn, might provide useful insights into the mechanism by which robustness is maintained. Indeed experiments using Arabidopsis plants stably transformed with an inducible AtPLC1 antisense construct have shown that (following...
induction), seed germination is insensitive to inhibition by ABA (Sanchez and Chua, 2001). However, disruptions to the guard cell ABA signalling pathway have not been reported for these plants.

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