Abscisic acid signal transduction in guard cells is mediated by phospholipase D activity

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In guard cells, the plant hormone abscisic acid (ABA) inhibits stomatal opening and induces stomatal closure through the coordinated regulation of ion transport. Despite this central role of ABA in regulating stomatal function, the signal transduction events leading to altered ion fluxes remain incompletely understood. We report that the activity of the enzyme phospholipase D (PLD) transiently increased in guard cell protoplasts at 2.5 and 25 min after ABA application. Treatment of guard cell protoplasts with phosphatidic acid (PtdOH), one of the products of PLD activity, led to an inhibition of the activity of the inward K^+ channel. PtdOH also induced stomatal closure and inhibited stomatal opening when added to epidermal peels. Application of 1-butanol (1-buOH), a selective inhibitor of PtdOH production by PLD, inhibited the increase in PtdOH production elicited by ABA. 1-BuOH treatment also partially prevented ABA-induced stomatal closure and ABA-induced inhibition of stomatal opening. This inhibitory effect of buOH was enhanced by simultaneous application of nicotinamide, an inhibitor of cADP ribose action. These results suggest that in the guard cell, ABA activates the enzyme PLD, which leads to the production of PtdOH. This PtdOH is then involved in triggering subsequent ABA responses of the cell via a pathway operating in parallel to cADP ribose-mediated events.

The plant hormone abscisic acid (ABA) is a regulator of stomatal apertures via its effects on osmotically driven changes in guard cell turgor. Abscisic acid inhibits the H^+ ATPase (1) and activates both a nonselective Ca^{2+}-permeable channel (2) and a "slow" (S type) anion channel (3, 4). All these effects depolarize the guard cell membrane potential, promoting K^+ efflux and causing stomatal closure. In addition, ABA deactivates K^+ entry channels and activates K^+ efflux channels (5–7), further promoting stomatal closure and inhibiting stomatal opening. How ABA triggers these changes in ion channel activity remains poorly understood. ABA signal transduction in the guard cell is thought to be mediated by changes in cytosolic-free Ca^{2+} concentration, as well as by Ca^{2+}-independent pathways (8–12). Other signaling elements may include activation of phosphoinositide-specific phospholipase C (PI-PLC) (5, 9, 13, 14), protein kinases and phosphatases (15–19), cADP ribose (20), G proteins (21), farnesylation reactions (22), and changes in cytosolic pH (4, 7, 23). Furthermore, many of these proposed regulators have been shown to be involved in regulation of stomatal aperture, including PI-PLC (5, 9, 14), a protein phosphatase (16), cADP ribose (20), and farnesylation (22).

Phospholipase D (PLD) is involved in signaling in animals, and recent studies suggest a similar role in algae and plants (24–31). PLD hydrolyzes phospholipids, producing phosphatidic acid (PtdOH) and the head group. Previously, PLD activation has been implicated in the initial steps of ABA signal transduction in barley aleurone cells (29), where, e.g., inhibition of PtdOH production from PLD resulted in an inhibition of ABA responses. In addition, ethylene- and ABA-induced senescence of detached leaves is impaired in *Arabidopsis* plants transformed with antisense PLD-α (32). We therefore investigated the potential role of PLD in the ABA responses of the stomatal guard cell, one of the most fully characterized ABA-responsive cell types. We provide evidence that ABA activates PLD in these cells and that PtdOH triggers signal transduction events leading to inhibition of the inward K^+ channel and reduction in stomatal aperture. These results suggest that PLD may be a conserved feature of plant responses to ABA.

Materials and Methods

Chemicals and Plant Material. Unless stated otherwise, all chemicals were purchased from Sigma. Guard cell protoplasts of *Vicia faba* were isolated as described previously (23) and stored in the dark on ice for 2 hr before use in patch-clamp experiments. Phosphatidic acid and (7-nitro-2–1,3-bezoxadiazol-4-yl)amino-phosphatidylcholine (NBD-PtdCho; Avanti Polar Lipids) were stored at −80°C in chloroform. Before use they were dried under a stream of N_2(g) and emulsified by sonication in H_2O or bath solution (see below).

Measuring Stomatal Aperture and Electrophysiology. Detached leaves were incubated in H_2O for 1 hr in the dark to initiate stomatal closure or under illumination at 0.18 mmol $m^{-2}\cdot s^{-1}$ (×4 BF 58722 lamps; General Electric) to induce stomatal opening. Epidermal peels were prepared and incubated in 100 mM KCl/1 mM $CaCl_2$/1 mM $MgCl_2$/5 mM Mes, pH 5.6, for a further 1 hr in the dark or light, as required, and treated as indicated, and stomatal aperture was measured (33). Experiments were performed in triplicate and results were confirmed by double-blind assays. U73122 and U73433 (1-[6-([(17β)-3-methoxyoestra-1,3,5(10)-trien-17-yl]amino)hexyl]-1H-pyrrole-2,5-dione and 1-[6-([(17β)-3-methoxyoestra-1,3,5(10)-trien-17yl]amino)hexyl]-2,5-pyrrolidinedione) were used from a stock in DMSO, with a final [DMSO] of 0.01% (vol/vol). Diacylglycerols were used from stocks in ethanol, with a final [ethanol] of 0.01% (vol/vol).

For whole-cell patch clamping, performed as described previously (34), the pipette solution contained 80 mM K-glutamate, 20 mM KCl, 2 mM $MgCl_2$, 2 mM ATP, 10 mM Hepes (pH 7.8), and sorbitol to a final osmolality of 500 mmol kg^{-1}. The bath solution contained 100 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM Hepes, 5 mM Mes (pH 5.6), and sorbitol to a final osmolality of 460 mmol kg^{-1}. (+/−)-ABA was added to the bath solution at a final concentration of 15 μM from a stock in 10 mM TAPS (*N*-tris[hydroxymethyl]methyl-3-amino propane sulfonic acid; ([hydroxy-1,1-bis(hydroxymethyl)ethyl]amino)-1-propane sulfonic acid).

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Abbreviations: ABA, abscisic acid; buOH, butanol; DAG, diacylglycerol; NBD, (7-nitro-2–1,3-bezoxadiazol-4-yl)amino; PtdOH, phosphatidic acid; PtdBut, phosphatidylbutanol; PtdCho, phosphatidylcholine; PI-PLC, phosphoinositide specific-phospholipase C; PLD, phospholipase D; U73122, 1-[6-([(17β)-3-methoxyoestra-1,3,5(10)-trien-17-yl]amino)hexyl]-1H-pyrrole-2,5-dione; U73433, 1-[6-([(17β)-3-methoxyoestra-1,3,5(10)-trien-17yl]amino)hexyl]-2,5-pyrrolidinedione.

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Measurement of PtdOH, PtdBut, DAG Levels, and PLD Activity. Guard cell protoplasts were prepared as described previously (35). For *in vitro* assays, 100-μl aliquots (approximately 2.5×10^5 protoplasts) were treated with 10 μM (+/−)-ABA. After various times each sample was processed as described in ref. 29, with the following modifications to the PLD assay: a 40-μl sample was assayed in a total volume of 80 μl, and the fluorescent substrate was NBD-PtdCho.

For *in vivo* measurement of PtdOH, phosphatidylbutanol (PtdBut), and diacylglycerol (DAG), protoplasts were incubated in 0.5 mg/ml NBD-PtdCho for 80 min on ice, and then 100-μl aliquots were transferred to 22°C for 10 min. In experiments for Fig. 5, 0.1% (vol/vol) 1-buOH also was added at the start of the 22°C incubation. (+/−)-ABA (10 μM) was added from a stock of 50 mM in 95% ethanol (final [ethanol], 0.02% vol/vol). After the required time, the samples were processed and NBD-labeled PtdOH, PtdBut, and DAG were quantified as described previously (29).

Microinjection and Calcium Measurement. Epidermal peels were mounted cuticle-side down in an open perfusion chamber (total volume, 500 μl) (10) and incubated under the conditions described for aperture measurements. Guard cells surrounding open stomata were impaled with borosilicate glass micropipettes and pressure-microinjected with Indo-1 conjugated to a 10-kDa dextran (Molecular Probes) (36). Microinjected cells were allowed to recover for 30 min. Only cells that showed a successful cytoplasmic injection (10) and maintained viability (assessed by a stable resting [Ca^{2+}], maintained turgor and cytoplasmic structure, and cytoplasmic staining with fluorescein diacetate at the end of the experiment) were included in the analysis (10). Calcium ratio imaging and Indo-1 calibration were performed by using an LSM 410 confocal microscope (Zeiss) (36). Autofluorescence and dark current represented <10% of the Indo-1 signal. PtdOH (50 μM) did not affect the responsiveness of Indo-1 to *in vitro* calibration (data not shown). Neither ABA nor PtdOH affected the cytoplasmic localization of the Indo-1 (Fig 3 *A–D*).

Results

Phosphatidic Acid Induces Stomatal Closure. ABA causes closure of open stomata and inhibits the light-induced opening of closed stomata. To test the possible role of PLD in ABA signaling in the guard cell, we added PtdOH to epidermal peels and monitored the effect on stomatal aperture. Addition of 50 μM PtdOH to open stomates induced stomatal closure (Fig. 1 *A* and *B*). PtdOH also inhibited opening of stomata that first had been closed (to 3.5 ± 0.3 μm) by preincubation in the dark and then transferred to the light (Fig. 1*C*). PtdOH was approximately 50% as effective as ABA in causing stomatal closure or inhibiting stomatal opening. Fig. 1*D* shows that increasing [PtdOH] to above 50 μM did not have any further effect. Simultaneous addition of 15 μM ABA and 50 μM PtdOH caused a stomatal closure response similar to ABA alone ($P > 0.05$, *t* test; Fig. 1*A*).

We tested a range of other phospholipids to determine the specificity of the effect of PtdOH. Treating guard cells with up to 50 μM phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, and phosphatidylinositol had no detectable effect on stomatal aperture (data not shown). The PtdOH-breakdown products DAG and lysophosphatidic acid (LysoPtdOH) also could have been responsible for the observed effects of PtdOH treatment. However, at levels up to 200 μM, neither dioleoylglycerol, the more stable DAG analog dioctanoylglycerol, nor LysoPtdOH had any significant effect on stomatal closure (Fig. 1*A*; $P > 0.05$, *t* test, and data not shown). These results suggested that the effects of PtdOH were not a general feature of lipid or lipid-turnover products on guard cell function.

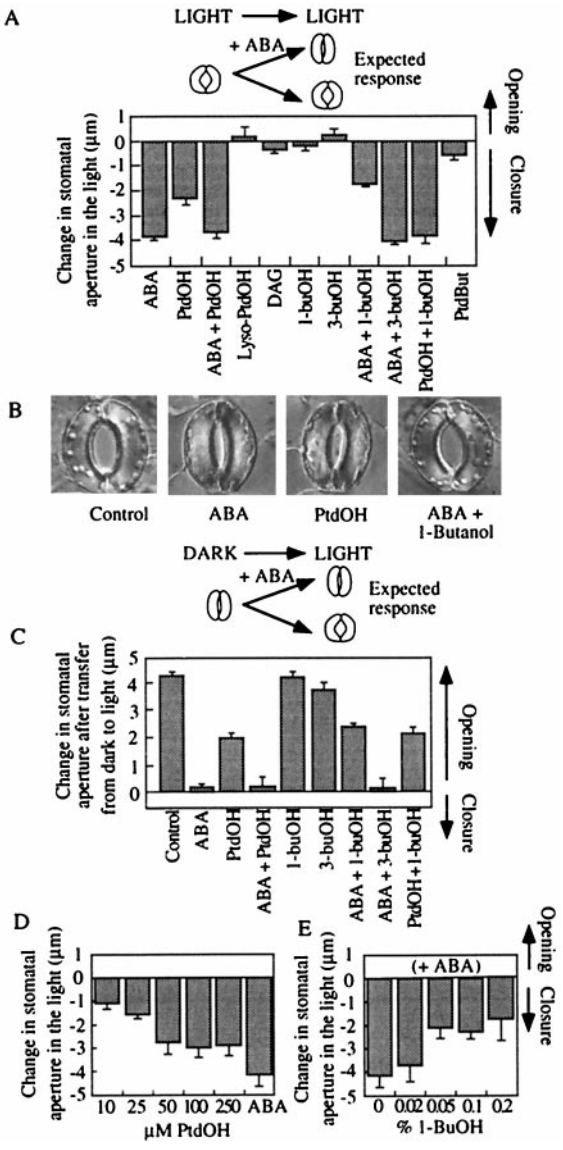

Fig. 1. The effects of PtdOH and buOH on stomatal aperture. (*A*) Epidermal peels were kept in the light for 1 hr to induce stomatal opening and then treated (still in the light) as indicated. (*B*) Bright-field images of stomata treated as described. (Bar = 10 μm.) (*C*) Epidermal peels were kept in the dark for 1 hr to induce stomatal closure and then transferred to the light and treated as shown. Concentrations used: (+/−)-ABA, 15 μM; PtdOH, LysoPtdOH, DAG, and PtdBut, 50 μM; 1- and 3-buOH, 0.1% (vol/vol). (*D* and *E*) Epidermal peels were prepared as for *A* and treated with a range of PtdOH concentrations (*D*) or with ABA plus a range of 1-buOH concentrations (*E*). Stomatal apertures were measured after 1 hr of treatment and compared with the aperture of untreated controls [light, 7.9 ± 0.2 μm (*A*); dark, 3.5 ± 0.3 μm (*C*)]. The results show the mean ± SEM of at least three independent experiments measuring at least 60 stomata on 3 separate peels.

1-BuOH Inhibits ABA Action. The effects of PtdOH on stomatal aperture suggested to us that the ABA signal might be transduced through PLD activation in the guard cell. We therefore applied 1-buOH, an inhibitor of PtdOH production by PLD (24, 29), to see whether this would block the response of guard cells to ABA. 1-BuOH inhibits PtdOH production because PLD can catalyze the transfer of a phosphatidyl group onto an alcohol molecule, producing a phosphatidylalcohol (in this case, PtdBut) instead of PtdOH. This transphosphatidylation activity is unique to PLD and competitively inhibits PtdOH production (24, 37).

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PLD is known to transphosphatidylate primary alcohols such as 1-buOH but not other alcohols such as 3-buOH (24). Hence, 1-buOH, but not 3-buOH, can inhibit PtdOH production.

We treated epidermal peels with 0.1% (vol/vol) 1- or 3-buOH and ABA to assess the effect of buOH action on the ABA response. 1-BuOH [0.1% (vol/vol)] both inhibited ABA-induced stomatal closure (Fig. 1 *A* and *B*; $P < 0.05$, *t* test) and antagonized ABA's inhibitory effect on stomatal opening (Fig. 1*C*; $P < 0.05$, *t* test). 3-buOH [0.1% (vol/vol)] did not affect ABA responses in either of these assays (Fig. 1 *A* and *C*), consistent with 3-buOH being a poor substrate for PLD. Treatment of peels with 0.1% (vol/vol) 1- or 3-buOH in the absence of ABA had no detectable effect on the aperture of open stomata maintained in the light (Fig. 1 *A* and *C*) or closed stomata maintained in the dark (data not shown).

Fig. 1*E* indicates that 0.1% (vol/vol) 1-buOH elicited a maximal inhibition of the ABA response of approximately 50%. Similarly, addition of 50 μM PtdOH together with 0.1% (vol/vol) 1-buOH led to a reduction in stomatal aperture (Fig. 1*A*) or inhibition of stomatal opening (Fig. 1*C*) to approximately 50% of the levels elicited by ABA. However, application of PtdOH to 1-buOH + ABA-treated cells resulted in stomatal closure identical to a normal ABA response (3.5 ± 0.3-μm reduction in aperture, $n = 60$; $P > 0.05$ *t* test). These results are consistent with 1-buOH inhibiting ABA-stimulated PtdOH production from a PLD that is required for the full extent of ABA action. This inhibition then is overcome by the application of the product of PLD activity, PtdOH.

To ensure that the effects of PtdOH and 1-buOH were not due to nonspecific cytotoxic effects, we assessed the viability of treated guard cells. Bright-field images of untreated guard cells and those treated with ABA, PtdOH, or ABA plus 1-buOH (Fig. 1*B*) showed no obvious morphological indication of cytotoxicity. Staining with the vital dye fluorescein diacetate revealed no loss of viability in PtdOH- and 1-buOH-treated cells (data not shown). In addition, stomates treated with ABA, PtdOH, or 1-buOH all showed subsequent increases in aperture upon treatment with 1 μM fusicoccin for 2 hr (data not shown). Fusicoccin promotes stomatal opening by functional guard cells, indicating that the response systems of the treated cells remained intact.

Although the product of PLD activity in the presence of 1-buOH (PtdBut) is thought to be biologically inactive, we also tested whether PtdBut might be affecting the stomatal response. PtdBut [0.1% (vol/vol)] had no effect on stomatal aperture (Fig. 1*A*). This result suggests that the effect of 1-buOH on the guard cells was attributable to its action of preventing PLD from making normal levels of PtdOH, rather than to an effect of the PtdBut produced during this inhibition.

PtdOH Inhibits the Inward K^+ Channel. Another well characterized measure of the ABA response in guard cells is the inhibition of inward K^+-channel activities. We performed whole-cell patch clamping of guard cell protoplasts to assess the effect of PtdOH on the inwardly rectifying K^+ channel in *V. faba* (5–7, 34). Fig. 2 shows that ABA and PtdOH each inhibit the inward K^+ channel, with PtdOH inhibition reaching approximately 50% that of the maximal ABA response at -160 mV. These results are consistent with ABA activating PLD to produce PtdOH that then mediates part of the inhibition of the inwardly rectifying K^+ channel. Fifty micromolar of the PtdOH-breakdown product LysoPtdOH had no effect on channel activity ($n = 7$, data not shown), consistent with its inactivity in the epidermal peel experiments (Fig. 1). At +60 mV, the mean outward K^+ current in protoplasts treated with PtdOH was also lower than those treated with ABA or controls. However, although stable within an individual cell, under our experimental conditions this outward current was variable between cells and the apparent

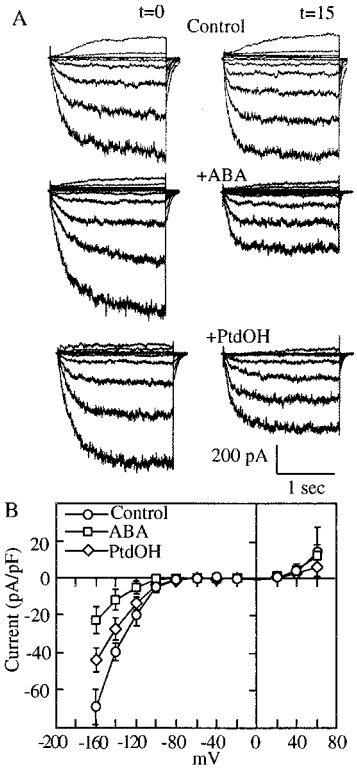

Fig. 2. The effects of ABA and PtdOH on whole-cell K^+-channel currents. (*A*) Families of current recordings from guard cells before ($t = 0$) and 15 min after ($t = 15$) treatment with 15 μM (+/−)-ABA or 50 μM PtdOH. Stable recordings were obtained for at least 20 min before each experimental treatment. Voltage was stepped in 20-mV increments from −160 mV to +60 mV. Representative traces are shown. (*B*) Current–voltage plots constructed from leak-subtracted K^+ currents. Currents were measured 10–20 min after treatment with 15 μM (+/−)-ABA or 50 μM PtdOH (applied to the bath solution). Each plot shows the mean ± SEM for: control, $n = 10$; ABA, $n = 9$; PtdOH, $n = 9$.

difference between ABA- and PtdOH-treated cells was not significant ($P > 0.05$, *t* test).

Attempts to inhibit ABA action in these patch clamp experiments by addition of 0.1% (vol/vol) 1-buOH were unsuccessful. Both pretreatment of protoplasts with this alcohol and addition of 1-buOH to protoplasts already in the whole-cell configuration led to rapid loss of the GΩ seal between the patch pipette and the plasma membrane. The reason for this reduction in seal resistance is unknown. It did not reflect a general cytotoxic effect of buOH on the protoplasts because they showed no obvious morphological signs of cytotoxicity and maintained >95% viability as assessed by FDA staining after 1 hr of treatment with 0.1% buOH.

PtdOH Action Is Not Associated with Changes in Cytoplasmic Ca^{2+}. The 50% efficiency with which PtdOH induced stomatal closure compared with ABA led us to investigate whether PLD/PtdOH might comprise just part (50%) of an ABA-signaling network. Ca^{2+}-dependent and -independent signal transduction pathways have been proposed to operate in parallel in guard cells in response to ABA. We therefore asked whether PtdOH was acting in a Ca^{2+}-dependent or -independent manner. We first monitored the effect of PtdOH and ABA on cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) levels by using ratio imaging of guard cells microinjected with the Ca^{2+}-sensitive fluorescent dye Indo-1. ABA can elicit $[Ca^{2+}]_{cyt}$ increases in guard cells of several species (8, 10–12), and these changes can trigger stomatal closure (9). *V. faba* guard cells showed a stable, resting Ca^{2+} level of 100–200

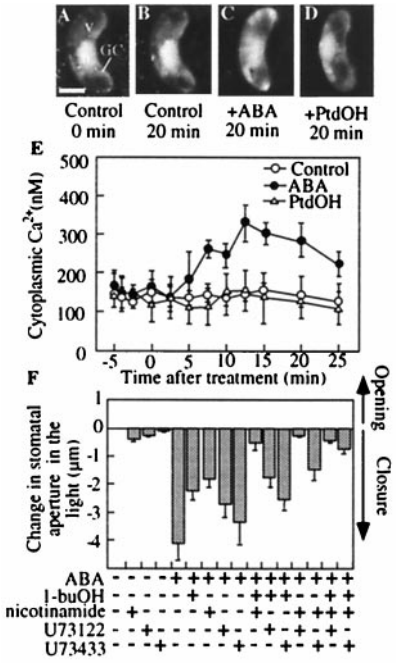

Fig. 3. The effect of PtdOH on guard cell cytoplasmic [Ca^{2+}] and the effect of inhibitors of the ABA response on stomatal aperture. (*A–D*) Midplane confocal sections of guard cells microinjected with Indo-1 dextran and imaged before (*A*) or after 20-min incubation (*B*) or after 20-min treatment with 10 μM (+/−)-ABA (*C*) or 50 μM PtdOH (*D*). (*E*) Average cytoplasmic [Ca^{2+}] determined by confocal ratio analysis of guard cells microinjected with Indo-1-dextran and treated with 10 μM (+/−)-ABA or 50 μM PtdOH as indicated. Each plot shows the mean ± SEM: control, $n = 10$; ABA, $n = 12$; PtdOH, $n = 10$. (*F*) Epidermal peels were kept in the light for 1 hr to induce stomatal opening and then treated (still in the light) as indicated. Concentrations used: (+/−)-ABA, 10 μM; 1-buOH, 0.1%; nicotinamide, 50 mM; U73122 and U73433, 1 μM. The results show the mean ± SEM of at least three independent experiments measuring at least 60 stomata on 3 separate peels.

nM (Fig. 3), and, upon ABA treatment, Ca^{2+} was seen to rise by 200–500 nM in 67% of guard cells. Such variability in the Ca^{2+} response to ABA is well characterized in stomata from several species (10–12) including *V. faba* (39). Fig. 3*E* shows the average response of the population of guard cells analyzed, but the trend of ABA-induced Ca^{2+} increase is clearly visible. In no case ($n = 10$) was an increase in $[Ca^{2+}]_{cyt}$ observed to follow PtdOH treatment (Fig. 3*E*).

We next assessed whether PLD and Ca^{2+}-mobilizing signal transduction elements were acting in parallel. U73122 is an inhibitor of PI-PLC and has been shown to inhibit both ABA-dependent Ca^{2+} changes in guard cells and ABA-induced stomatal closure in *Commelina communis* (14). Similar effects have been reported for nicotinamide, an antagonist of the Ca^{2+}-mobilizing intracellular messenger cADP ribose (20). Fig. 3*F* shows that the responses of *V. faba* guard cells to these inhibitors follow those reported for *C. communis.* Thus, 1 μM U73122 and 50 mM nicotinamide both partially inhibit stomatal closure elicited by ABA. The U73122 analog U73433 showed only a small inhibitory activity toward ABA, similar to that seen on *C. communis* (14) and consistent with its poor inhibitory effect on PLC (40). We reasoned that if U73122 or nicotinamide were acting on signaling elements in the same pathway as PLD, then inhibiting PLD (with 1-buOH) in guard cells already treated with these inhibitors would have no further effect on reducing the ABA response. However, if U73122 or nicotinamide were acting on a signaling pathway in parallel to that mediated by PLD, the effect of PLD inhibition would be additive with the other inhibitor. Similar experiments already have suggested that PI-PLC and cADP ribose act in parallel pathways in guard cells (E. MacRobbie, personal communication). We therefore treated guard cells with ABA and 1-buOH and determined the effect of the further addition of U73122 or nicotinamide on stomatal closure. In these experiments, U73122 led to no additional inhibition of ABA-induced closure whereas nicotinamide increased the inhibition of ABA-induced closure caused by 1-buOH by 80% (Fig. 3*F*). This suggests that 1-buOH and U73122 are acting to inhibit elements in the same pathway whereas nicotinamide may be acting on a parallel ABA-related signaling pathway. Consistent with this model, ABA-induced stomatal closure could be fully inhibited by either U73122 + nicotinamide or 1-buOH + nicotinamide (Fig. 3*F*).

PtdOH Production Is Increased in Response to ABA. The experiments described above suggesting a role for PLD in the ABA response of *V. faba* guard cells led us to investigate whether changes in the endogenous levels of PtdOH and PLD activity occur in response to ABA. In protoplasts loaded with the fluorescent phospholipid NBD-PtdCho, NBD-labeled PtdOH and DAG are produced *in vivo* by PLD and PLC activity, respectively. Fig. 4 *A* and *C* shows a 2-fold increase in NBD-PtdOH production after 20 min of ABA treatment as compared with controls, consistent with PLD activation. The levels of newly synthesized DAG failed to reveal an equivalent ABA-induced increase (Fig. 4 *C* and *D*).

Because PtdOH can be formed and metabolized by a range of different enzymes, changes in levels of PtdOH are only a partial reflection of PLD activity. However, it is possible to assess the activity of this enzyme *in vivo* by using its transphosphatidylation activity. Because 1-buOH is a selective substrate for PLD (22) and the PtdBut produced is not readily metabolized further, the rate at which PtdBut accumulates in cells treated with 1-buOH is a measure of PLD activity *in vivo*. Fig. 5*A* shows that guard cells treated with 0.1% 1-buOH did not exhibit the ABA-induced increase in PtdOH normally seen in response to ABA (compare with Fig. 4*C*). This result indicated that 0.1% 1-buOH was acting to efficiently divert PLD activity from PtdOH production, and, thus, the resulting PtdBut accumulation would most likely reflect PLD activity *in vivo*. Fig. 5*B* shows that levels of NBD-PtdBut in protoplasts treated with ABA are higher than in untreated controls, indicating higher levels of PLD activity in these cells. PLD activity is also reflected by the rate of NBD-PtdBut production. The rate of such accumulation was highest at 2.5–5 and 15–25 min after ABA addition, suggesting a transient stimulation of PLD at these times.

PLD Activity Increases in Response to ABA. We also assayed PLD activity *in vitro* from protein extracts of guard cell protoplasts that had been treated with ABA for various times. This analysis revealed transient peaks in PLD activity: one at 2.5–7.5 min and one at 20–25 min after ABA treatment (Fig. 5*C*). There were no further changes in PLD activity in samples assayed for an additional 1.5 hr (data not shown). Thus, the timing of ABA activation of PLD monitored by *in vitro* assay (Fig. 5*C*) corresponds to that assayed *in vivo* by using NBD-PtdBut production (Fig. 5*B*). The timing of changes in PLD activity assayed *in vivo* (Fig. 5*B*) and *in vitro* (Fig. 5*C*) was consistent in three independent experiments.

Discussion

In this paper we provide evidence that transduction of the ABA signal in guard cells is mediated by PLD activation. Addition of PtdOH both inhibited stomatal opening and promoted stomatal closing in epidermal peel experiments, activities also associated with ABA action. The mechanisms of stomatal opening and closing are thought to operate through distinct signal/response elements (40), and our results indicate that PLD activation may

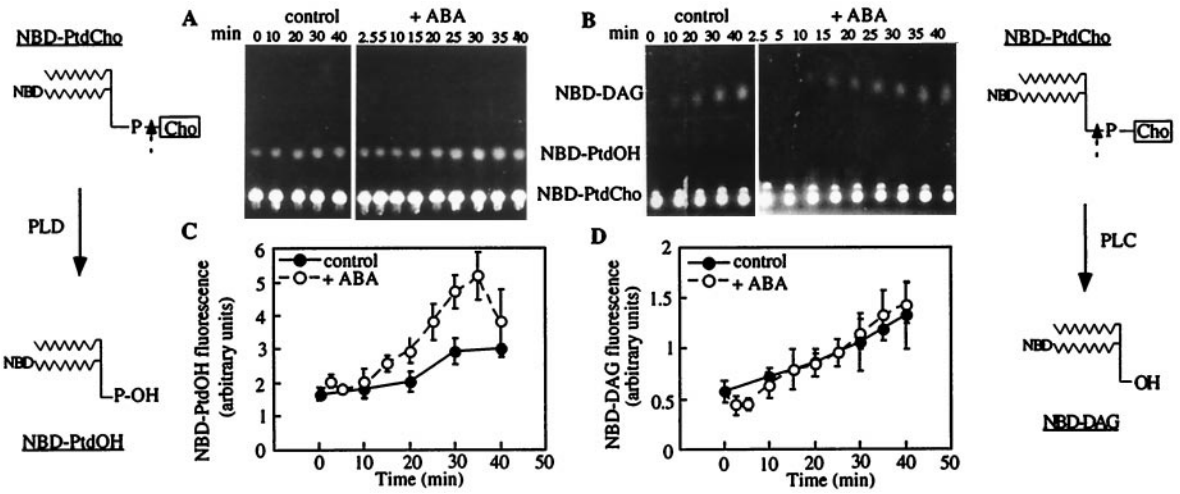

Fig. 4. The effect of ABA on *in vivo* levels of PtdOH and DAG. (*A* and *B*) Fluorescence images of TLC plates separating PtdOH (*A*) or DAG (*B*), extracted from protoplasts prelabeled with the fluorescent PLD substrate NBD-PtdCho, and treated with 10 μM (+/−)-ABA. (*C* and *D*) Quantification of PtdOH and DAG levels from TLC plates as shown in *A* and *B*. The data shown in *C* and *D* are the mean ± SEM of at least three separate experiments for each time point.

act in both of these aspects of the ABA response. The effect of PtdOH was dose-dependent, being maximal at 50 μM, a concentration similar to that which elicits ABA responses in the barley aleurone (29) but 10-fold lower than that which triggers PLD-dependent deflagellation in *Chlamydomonas* (24). The largest effects of PtdOH on inducing stomatal closure, inhibiting stomatal opening, or inhibiting the K^+-uptake channel activity were approximately 50% of the maximal ABA response (Figs. 1 and 2). Similarly, inhibiting PLD with 1-buOH inhibited approximately half of the ABA response. This effect suggests a model with parallel pathways for ABA signal transduction, with PLD carrying 50% of the signal.

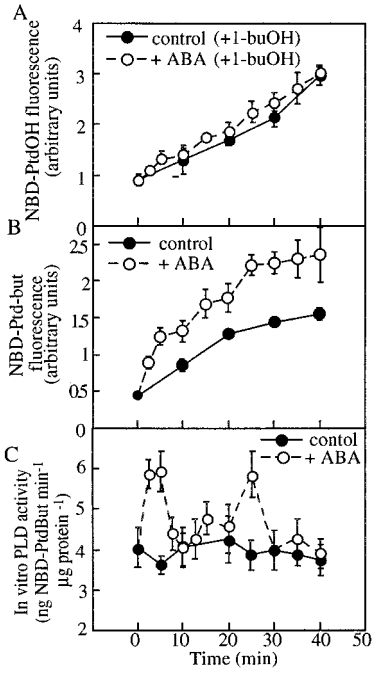

Fig. 5. The effect of ABA on *in vivo* levels of PtdOH, PtdBut, and PLD activity. Protoplasts and NBD-labeled phospholipids were prepared as for Fig. 4. (*A* and *B*) Quantification of NBD-PtdOH (*A*) and NBD-PtdBut (*B*) levels from protoplasts treated with 10 μM (+/−)-ABA and 0.1% (vol/vol) 1-buOH. (*C*) PLD activity in extracts of protoplasts. The data show the mean ± SEM of at least three separate experiments for each time point.

We tested whether the apparent PLD-dependent and -independent pathways of ABA action might also reflect the Ca^{2+}-dependent and -independent pathways associated with the ABA response of guard cells (8–12). Using fluorescence ratio imaging to monitor $[Ca^{2+}]_{cyt}$ levels, we observed an increase in Ca^{2+} upon ABA treatment of guard cells in 67% of cells (Fig. 3*E*). No such increase was seen in response to PtdOH, suggesting that PtdOH is either acting through a Ca^{2+}-independent signaling pathway or acting downstream of any Ca^{2+} changes. The results of simultaneous treatment of guard cells with 1-buOH, to inhibit PLD action, and an inhibitor of PI-PLC (U73122) or cADP ribose action (nicotinamide; Fig. 3*F*) suggest that PLD and PI-PLC may operate in the same pathway, which acts in parallel with cADP ribose-dependent events to mediate ABA action.

Consistent with a role for PLD in ABA action, we observed rapid and transient ABA-dependent stimulation of PLD in guard cells. When PLD activity was measured *in vivo* (by PtdBut accumulation; Fig. 5*B*) or *in vitro* (by using extracted enzyme; Fig. 5*C*), distinct peaks of increased activity were noted; however, when we measured the *in vivo* production of PtdOH (Fig. 4*C*), we observed only the peak at 25 min. This implies that much of the PtdOH produced by the initial peak of PLD activation is turned over rapidly, possibly by another phospholipase. This turnover appears to be suppressed later on, leading to the accumulation of PtdOH after 20 min. The transient nature of the PLD activation also implies some kind of feedback control mechanism.

Elevation of PtdOH levels by ABA could occur directly from the action of PLD or via phosphorylation of DAG produced from PLC activity. Indeed, PI-PLC and one of its products, inositol-1,4,5-trisphosphate, have been proposed to mediate stomatal responses (9, 13, 14, 41). However, we think DAG is unlikely to be the source of PtdOH that triggers downstream, ABA-related responses in our experiments. Elevations in the level of DAG were not detected in response to ABA (Fig. 4), although such elevations could have gone undetected if DAG were turned over rapidly. Also, the substrate used to monitor DAG production had the head group choline, and the PLC involved in signaling may be specific for phospholipids with other head groups, such as inositol-2-phosphate, as found for the signaling-related PLC in animal cells. However, treatment of peels with up to 200 μM DAG did not elicit stomatal closure

whereas 50 μM PtdOH was effective under the same assay conditions (Fig. 1*A*). This result is similar to a previous report on DAG action in guard cells (41), where DAG was shown to have little effect on closure but to cause stomatal opening and promote H^+-efflux, effects opposite of those seen with ABA.

The mechanism by which PLD activity is transiently stimulated remains to be investigated. PLDs contain C2-type Ca^{2+}-phospholipid-binding domains that may confer Ca^{2+} dependence to regulation *in vivo* (42). However, the stimulation of PLD by ABA was evident in the *in vitro* assays we conducted and, thus, survived extraction in high concentrations of the Ca^{2+} chelator EGTA and assay at a fixed (mM) $CaCl_2$ concentration. Hence, it is unlikely that increases in $[Ca^{2+}]_{cyt}$ are the direct cause of the elevation in PLD activity, although such increases could function upstream in the PLD activation pathway. A further possibility is that PLD is activated by a G protein, as seen in many mammalian PLD-based signal transduction pathways (30). G protein activation of PLD also has been reported in *Chlamydomonas* (24, 43), and there are extensive data implicating G proteins as intermediates in signal transduction in guard cells (40, 44).

The downstream elements of the PLD/PtdOH-mediated ABA-signaling pathway remain to be determined. PtdOH may directly modulate signaling elements such as protein kinases or act through one of its turnover products. The lack of effect of DAG and LysoPtdOH in our experiments suggests that these molecules, which can be derived from PtdOH, are not likely candidates for such downstream components. Likewise, fatty acids, such as arachidonic and linolenic acid, are reported to elicit stomatal opening in the dark (45) and have an opposite effect on K^+ channels to ABA. These observations suggest that fatty acids produced from PtdOH are also unlikely to propagate the PtdOH signal. However, many other possible products of PtdOH metabolism exist. For example, diacylglycerol pyrophosphate, the phosphorylated form of PtdOH, has been observed to accumulate during signaling events in which PtdOH levels are elevated (46). Defining whether PtdOH acts directly or through a turnover product such as diacylglycerol pyrophosphate represents an exciting area for future guard cell research.

A role for PLD in the ABA response is not confined to guard cells of *V. faba* because we have observed a similar ABA-induced increase in PLD activity in tobacco guard cell protoplasts (unpublished data). Thus, PLD now has been implicated in ABA responses in leaves [guard cells (this study) and ABA-related senescence in *Arabidopsis* (32)] and seed tissue [barley aleurone cells (29)]. The ABA responses of these systems involve alterations in cellular processes ranging from gene expression to ion transporter activity. This involvement of PLD in the ABA responses of disparate cell types and varied physiological processes suggests this enzyme may be a conserved element in the transduction of the ABA signal in many types of plant cells.

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