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Abscisic acid signal transduction in the barley aleurone is mediated by phospholipase D activity

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ABSTRACT **The plant hormones abscisic acid (ABA) and gibberellic acid (GA) are important regulators of the dormancy and germination of seeds. In cereals, GA enhances the synthesis and secretion of enzymes (principally α-amylases) in the aleurone cells of the endosperm, which then mobilize the storage reserves that fuel germination. ABA inhibits this enhanced secretory activity and delays germination. Despite the central role of ABA in regulating germination, the signal transduction events leading to altered gene expression and cellular activity are essentially unknown. We report that the application of ABA to aleurone protoplasts increased the activity of the enzyme phospholipase D (PLD) 10 min after treatment. The product of PLD activity, phosphatidic acid (PPA), also increased transiently at this time. The application of PPA to aleurone protoplasts led to an ABA-like inhibition of α-amylase production, and induction of the ABA upregulated proteins ASI (*a*mylase *s*ubtilisin *i*nhibitor) and RAB (*r*esponsive to *AB*A). Inhibition of PLD activity by 0.1% 1-butanol during the initial 20 min of ABA treatment resulted in inhibition of ABA-regulated processes. This inhibition coincided with the timing of PLD activation by ABA and was overcome by simultaneous addition of PPA. These results suggest that ABA activates the enzyme PLD to produce PPA that is involved in triggering the subsequent ABA responses of the aleurone cell.**

The cereal aleurone exhibits a well defined suite of responses to the phytohormones gibberellic acid (GA) and abscisic acid (ABA). GA promotes the expression and secretion of hydrolytic enzymes (principally α-amylases), which break down the starchy endosperm providing resources for seed germination. ABA inhibits these responses to GA, and causes the upregulation of ABA responsive genes such as HVA1 and RAB (*r*esponsive to *AB*A) (1–5).

Perception of GA and ABA in the aleurone occurs at the plasma membrane (6, 7), although an internal site for ABA action may also be present (8). Candidate signal transduction events that lead from perception to gene induction include changes in cytoplasmic pH (3, 4), membrane potential (5), and protein phosphorylation, possibly through a mitogen-activating protein kinase-like activity (9). Elevated cytoplasmic [Ca^{2+}] has also been shown to be essential for secretory activity in the aleurone (8, 10–13); ABA causes decreases in this ion that are a necessary component of its inhibition of the GA response (8, 11). Despite identification of these candidates for ABA signal transduction elements, the rapid, initial events from receptor to downstream effectors remain obscure.

One of the most well-characterized signal transduction systems in mammalian cells is the phosphoinositide turnover pathway (14). Phosphatidyl-inositol-4,5-bisphosphate in the plasma membrane is hydrolyzed by phospholipase C (PLC) to diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). DAG activates protein kinase C, and IP_3 induces the release of calcium from intracellular stores. The activated PKC and elevated cytoplasmic [Ca^{2+}] then initiate a range of cellular responses. Although PKC has not been identified in plants, DAG (15, 16) and IP_3 (17–22) have all been implicated in plant cell signal transduction events. More recently phospholipase D (PLD) has been proposed to be involved in signaling in animals, algae, and plants (23–25). PLD hydrolyzes phospholipids producing phosphatidic acid (PPA). Several types of PLDs have been identified with differences in their biochemical characteristics and developmental expression (26–29). We therefore investigated the potential role of PLC and PLD in the GA and ABA responses of the aleurone. We provide evidence that ABA rapidly activates PLD in the aleurone and that the product of PLD action, PPA may then trigger the early signal transduction events that lead to the ABA responses of this cell.

MATERIALS AND METHODS

Chemicals and Plant Material. Barley aleurone protoplasts and layers were prepared as described (30, 31) and were incubated in 10 mM $CaCl_2$ with or without 5 μM GA_3 or 10 μM ABA. α-Amylase secretion was assayed as in Bush and Jones (11). Cytoplasmic [Ca^{2+}] levels were determined by using indo-1 as described (8).

Phosphatidic acid and (7-nitro-2-1,3-benzoxadiazol-4-yl)amino-phosphatidylethanolamine (NBD-PE, Avanti Polar Lipids) were stored at −80°C in chloroform. Prior to use they were dried under a stream of N_2, and emulsified by sonication in H_2O, or protoplast medium (30).

Measurement of PPA and DAG Levels *in Vivo*. 1.7 ml of freshly isolated protoplasts (0.5×10^6 protoplasts) were incubated with 42.5 μg NBD-PE for 2 hr. GA or ABA was added for the indicated time, and the protoplasts were settled under $1 \times g$ for the final 5 min. Settled protoplasts (500 μl) were added to 1.9 ml chloroform/ethanol (1:2 vol/vol) and vortexed. Chloroform (500 μl) and 500 μl 2M KCl were added, vortexed, and centrifuged for 5 min at $15{,}000 \times g$ (5415 C microcentrifuge, Eppendorf, Westbury, NY), and the lower lipid phase dried under vacuum (Speed Vac SVC 100; Savant). The dried phase was dissolved in 20 μl chloroform:methanol (95:5 vol/vol) and analyzed by TLC as described below.

DAG levels were also assayed by using an Amersham DAG assay kit, according to the manufacturer's instructions. This kit was also used to assay for aleurone DAG kinase activity and its inhibition by the DAG kinase inhibitor R59949 (3-{2-[4-

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Abbreviations: ABA, abscisic acid; DAG, diacylglycerol; GA, gibberellic acid; PPA, phosphatidic acid; PE, phosphatidylethanolamine; PLC, phospholipase C; PLD, phospholipase D; IP_3, inositol-1,4,5-trisphosphate; NBD, (7-nitro-2-1,3-benzoxadiazol-4-yl)amino.
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(*bis*-(4-fluorophenyl)methylene]-1-piperidinyl)ethyl}-2,3-dihydro-2-thioxo-4(1H)quinazolinone) (32). Modifications to the assay were the addition of crude aleurone extract (10 μg protein, prepared as for PLD assays) instead of the purified DAG kinase, use of nonradioactive ATP, and addition of 10 μg 1,2-dioctanoyl-sn-glycerol (a synthetic DAG analog). The assay products were processed and separated by TLC as described below, lipids were visualized by using iodine vapor and quantified by scanning the plate (OneScanner, Apple Computer, Cuppertino, CA) and comparing relative intensities of the spots by using the IPLAB SPECTRUM image analysis software (Signal Analytics, Vienna, VA).

PLD Extraction and Assays. Freshly isolated protoplasts were treated with 10 mM $CaCl_2$, 5 μM GA or 10 μM ABA for a range of times and settled for 5 min at 1 $\times$ *g*. All further procedures were carried out at 4°C. Settled protoplasts (400 μl) were homogenized in a glass/Teflon homogenizer with 800 μl of extraction buffer (50 mM Tris·acetate/5 mM EDTA/5 mM EGTA, pH 8.8/1 mM DTT/10 μg/ml leupeptin, pepstatin, aprotinin) and centrifuged at 2,000 $\times$ *g* for 10 min. Protein concentration was determined by the method of Bradford (33) by using BSA as a standard and the Bio-Rad protein assay kit.

PLD activity was assayed according to Wang *et al.* (26) with the following modifications. The standard assay mixture contained 20 mM Mes/NaOH (pH 6.5), 50 mM $CaCl_2$, 0.25 mM SDS, 5 μl fluorescent substrate (NBD-PE, 1.2 nmol), 1% (vol/vol) 1-butanol, 15 μl extract, total volume 40 μl. The reaction was initiated by the addition of the substrate and incubated at 30°C for 30 min with shaking (100 rpm). The reaction was stopped by the addition of 150 μl chloroform:methanol (1:2, vol/vol). Chloroform (40 μl) and 40 μl 2M KCl were added, the mixture was vortexed and centrifuged at 15,000 $\times$ *g* for 2 min. The phases were separated and 100 μl chloroform added to the aqueous phase, vortexed, and centrifuged at 15,000 $\times$ *g* for 2 min, and the lower chloroform phases from each step pooled. Each sample was dried under a stream of N_2 and 20 μl chloroform:methanol (95:5, vol/vol) added. PLD activity was measured as the production of phosphatidylbutanol in each sample, determined by TLC, as described below.

For preparation of the phosphatidylbutanol used to treat protoplasts, the PLD assay was run as described, the phosphatidylbutanol separated by TLC, the spot corresponding to phosphatidylbutanol scraped and eluted as outlined below.

TLC and Quantification of Fluorescent Lipids. Samples were spotted onto TLC plates (silica gel G, Fisher Scientific) and developed with 2,2,4-trimethylpentane:acetic acid:H_2O:ethyl acetate (2:3:10:13, vol/vol/vol/vol). When DAG was assayed the solvent was methanol:chloroform:toluene (5:15:85, vol/vol/vol). Fluorescently labeled lipids were visualized by using a UV light box (FBTIV-88, Fisher Scientific), and the regions corresponding to phosphatidylbutanol and PE, or PPA, DAG and PE marked. The spots marked were scraped from the plates and placed in 600 μl chloroform:methanol:H_2O (5:5:1, vol/vol/vol), vortexed, and centrifuged for 5 min at 15,000 $\times$ *g*, and the fluorescence (excitation 460 nm, emission 534 nm) from the eluted lipids measured in a fluorescence spectrophotometer (F-2000; Hitachi). Lipid spots were identified by comparison to standards (Avanti Polar Lipids) visualized by using iodine vapor. The difference in relative mobilities between NBD-labeled and unlabeled standards was less than 5%. A standard curve was constructed for the *in vitro* PLD assays by using a range of PE concentrations treated as samples (no aleurone extract was added and the lipid was not incubated for 30 min). The relationship between amount of PE standard and fluorescence measured was linear throughout the range 10 ng–10 μg. The limit of detection was between 5–20 ng.

SDS/PAGE and Western Blot Analysis. Protein was extracted from protoplasts as described above for PLD assays. 12% SDS/PAGE mini-gels (mini protean II; Bio-Rad) and semi-dry blotting (Transblot SD; Bio-Rad) to polyvinylidine difluoride membranes were performed according to the manufacturer's instructions. Protein blots were then incubated for 1 hr in 3% (wt/vol) BSA and for 3 hr in anti-α-amylase (1:2,000), anti-RAB, anti amylase subtilisin inhibitor (1:1,000) (34), or anti-tubulin (1:500). Cross-reacting proteins were visualized with goat, anti-rabbit alkaline phosphatase conjugate (35).

RESULTS

PPA Inhibits the GA Response of Aleurone in an ABA-like Manner. To test the possible role of phospholipases in ABA signaling in the aleurone, we added the products of PLC and PLD action (DAG and PPA, respectively) to aleurone protoplasts and monitored the effect on ABA-regulated processes. PPA caused an ABA-like inhibition of the GA-induced α-amylase when applied to protoplasts or layers (Fig. 1*A*). Fig. 1*A* also shows that PPAs with different acyl chains varied in their capacity to inhibit the GA activated α-amylase activity. We do not know if this is due to differing biological activities, stability, or uptake. In subsequent experiments we used the most effective PPA forms determined from this assay. Fig. 1*B* shows that the application of PPA also resulted in the reduction of the amount of α-amylase protein that could be extracted from protoplasts as well as enhancement of the levels of the ABA up-regulated proteins RAB and ASI (*a*mylase *s*ubtilisin *i*nhibitor) in an ABA-like manner. In contrast, the levels of the constitutively expressed protein tubulin were unaffected. ABA is also known to reduce cytoplasmic [Ca^{2+}] in GA-treated protoplasts (8, 12). PPA mimicked ABA action by causing such a decrease in cytoplasmic [Ca^{2+}] when applied to GA-treated protoplasts, or prevented any increase in cytoplasmic [Ca^{2+}] when applied simultaneously with GA (Fig. 1*C*). Taken together, these results suggested that PPA was mimicking the action of ABA both in repressing the GA response and triggering ABA-induced events.

We also noted that DAG led to an identical induction of ABA-like events in the aleurone (Fig. 2*A*). PPA is the phosphorylated form of DAG. We therefore sought to determine whether PPA or DAG was the active component mimicking ABA action by preventing the interconversion of DAG to PPA by inhibiting DAG kinase(s). The DAG kinase inhibitor R59949 (32) was found to inhibit aleurone DAG kinase activity (IC_{50}, $\approx$5 μM, Fig. 2*B*). Treating aleurone protoplasts with this compound prevented DAG inhibition of α-amylase production (Fig. 2*A*), but did not reduce the inhibitory action of PPA or ABA.

ABA Induces an Increase in PPA but Not DAG. The ABA-like effect of applying PPA to aleurone protoplasts led us to investigate whether changes in the endogenous levels of PPA occurred in response to GA or ABA. Levels of PPA were transiently elevated for 10–15 min in protoplasts treated with ABA, but only slowly increased with GA (Fig. 3). Measurements made for a further 2 hr showed no additional changes in PPA level for any treatment tested (Fig. 3*C* and data not shown). The levels of newly synthesized DAG (TLC analysis, Fig. 3*D*) or total DAG (Amersham DAG assay kit, Fig. 3*E*) failed to reveal an equivalent ABA-induced transient increase in DAG, or other obvious hormone-stimulated pattern of change in DAG level for up to 2 hr after treatment with ABA or GA.

PLD Activity Is Increased in Response to ABA Application. The enzyme responsible for PPA production is PLD. The activity of PLD in protoplasts increased $\approx$2-fold 5–10 min after treatment with ABA, but was unaffected by GA over this time period (Fig. 4*A*). To test if the ABA-induced increase in PLD activity was due to increased expression levels, rather than increased activity of existing enzyme, we pretreated protoplasts with the protein synthesis inhibitor cycloheximide at 5

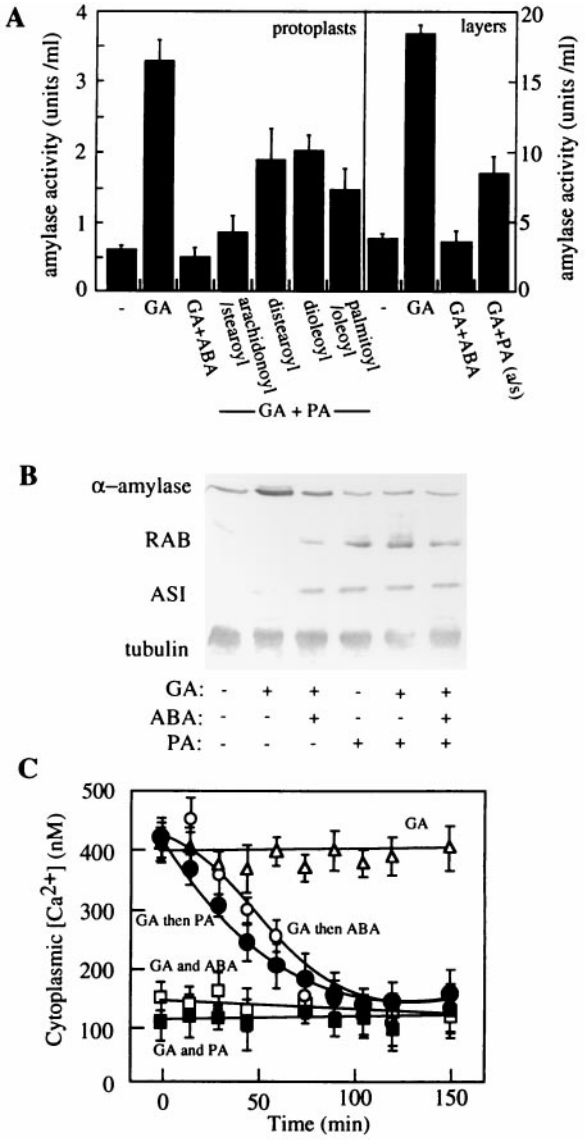

FIG. 1. The effects of PPA on aleurone protoplasts and layers. (*A*) Freshly isolated protoplasts and layers were treated with GA (5 μM), ABA (5 μM), or PPA (50 μM) and secreted α-amylase activity assayed after 48 hr. For protoplasts four types of PPA were used, differing in their acyl chains as denoted, for layers PPA with the acyl chain arachidonoyl/stearoyl was used. The data shows mean ± SEM, $n = 3$. (*B*) Western blot analyses show the effect of arachidonoyl/stearoyl PPA on levels of α-amylase, tubulin, and the ABA-induced proteins RAB and ASI. These blots reflect the levels of the proteins in the protoplasts rather than secreted activity assayed in *A*. Protoplasts were treated for 48 hr, protein extracted, and SDS/PAGE and immunoblotting carried out as described in *Materials and Methods*. (*C*) Effect of arachidonoyl/stearoyl PPA on cytoplasmic [Ca^{2+}] levels. Protoplasts were treated with either GA for 24 hr and then with PPA (GA then PPA) or ABA (GA then ABA), or freshly isolated protoplasts were treated simultaneously with GA and PPA or GA and ABA.

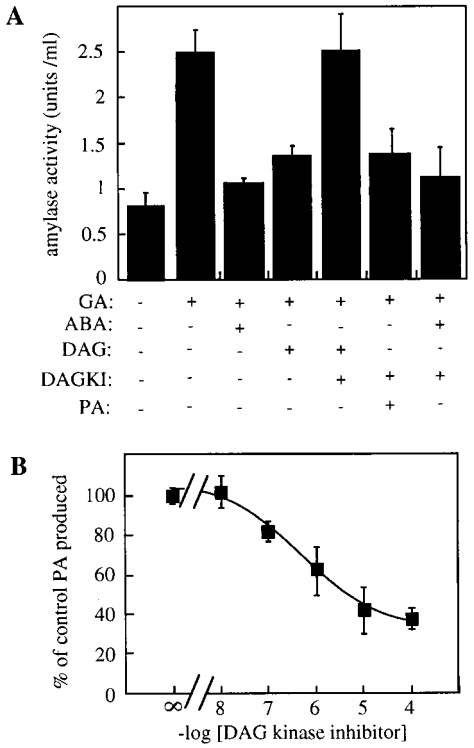

FIG. 2. The effect of DAG and DAG kinase inhibitor (DAGKI) on aleurone protoplasts. (*A*) Freshly isolated protoplasts were treated with GA (5 μM), ABA (5 μM), DAG (1,2-dioctanoyl-sn-glycerol; 50 μM), and DAG kinase inhibitor (R59949; 10 μM). After 48 hr, secreted α-amylase activity was assayed. (*B*) The effect of DAG kinase inhibitor on aleurone DAG kinase. Protein was extracted and DAG kinase activity assayed in the presence a range of DAGKI concentrations as described in *Materials and Methods*. Data represent mean ± SEM, $n = 3$.

$\mu g/ml^{-1}$ for 2 hr. We then treated the protoplasts with ABA or GA and measured PLD activity over 1 hr. Fig. 4*B* shows that in the presence of cycloheximide the increase in PLD in response to ABA was unaffected ($P > 0.05$, Student's t test), though the activity after 1 hr of GA treatment declined slightly. Fig. 4*B* (*Inset*) shows that 5 $\mu g\ ml^{-1}$ cycloheximide effectively inhibited protein synthesis, such as GA-stimulated synthesis of α-amylase in protoplasts.

The Application of 1-Butanol to Aleurone Cells Inhibits the ABA Response. We next applied an inhibitor of PLD activity, 1-butanol, to see if inhibiting this enzyme would block the response of aleurone cells to ABA. PLD can catalyze the transfer of a phosphatidyl group onto an alcohol molecule, producing a phosphatidylalcohol instead of PPA. This transphosphatidylation activity is unique to PLD, competitively inhibits PPA production, and has been used to distinguish PLD from PLC activity (23, 36). In addition PLD is known to transphosphatidylate 1-butanol but not 2- or 3-butanol (23), hence 1-butanol, but not the other forms, can inhibit the activity of PLD. We treated aleurone layers with 0.1% (vol/vol) 1-, 2-, or 3-butanol and assessed their effects on ABA action. Fig. 5*A* shows that in the absence of added GA or ABA, 1-butanol-stimulated α-amylase production, but the levels obtained in repeated experiments ($n = 8$) were significantly lower than those of GA-treated controls. When aleurone layers were treated simultaneously with GA and ABA, the ABA inhibited GA-induced amylase production. However 1-butanol prevented this inhibitory action of ABA. 2- and 3-Butanol had no significant effect on ABA inhibition of GA-induced α-amylase production ($P > 0.05$, Student's t test). 1-butanol also inhibited ABA induction of the ABA upregulated protein RAB but had no effect on constitutive protein levels (tubulin, Fig. 5*B*).

At higher concentrations of alcohols (>0.5%, vol/vol) the production of α-amylase was inhibited even in the absence of ABA (data not shown); we attribute this to a nonspecific toxic effect on the cells. Other alcohols (methanol, ethanol) at 0.1% had no effect on amylase production (data not shown); these other primary alcohols are known to be poor PLD substrates at this concentration (23).

To determine the timing of the events inhibited by 1-butanol, aleurone layers were treated with GA for 4 hr to induce

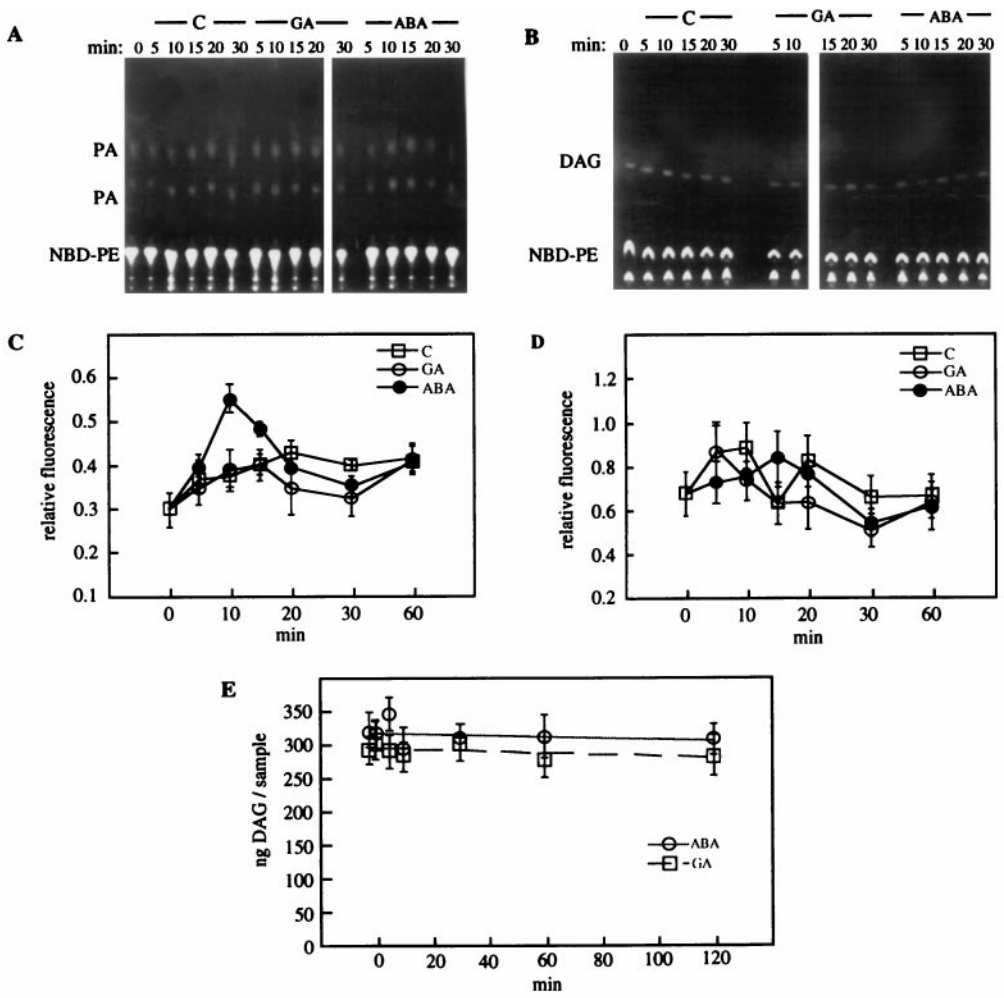

FIG. 3. Changes in levels of PPA and DAG in response to GA and ABA. (*A* and *B*) Fluorescence image of TLC plates separating PPA (*A*), or DAG (*B*), extracted from protoplasts treated with or without GA (5 μM) or ABA (5 μM) for the indicated times (C, control, no hormone). The protoplasts were loaded with NBD-PE as described in *Materials and Methods*. (*C* and *D*) Quantification of PPA and DAG levels from TLC plates shown in *A* and *B*, the data shows the mean ± SEM, $n = 3$ separate experiments. (*E*) Total protoplast DAG assayed by using the Amersham DAG assay kit. Samples were taken at the indicated times after GA or ABA treatment. Data represent mean ± SEM, $n = 3$.

α-amylase production. ABA was then added and the tissue treated with 0.1% 1-butanol at various times after this ABA treatment. Fig. 5*C* shows that 1-butanol prevented the ABA inhibition of α-amylase production only if it was added within 20 min of ABA application. The application of PPA and 1-butanol together at 0 min after ABA addition resulted in low levels of α-amylase activity (Fig. 5*D*) and induction of RAB with or without ABA (data not shown), suggesting that the PPA "overrides" the action of the 1-butanol.

Though the product of PLD transphosphatidylation, phosphatidylalcohol is not readily metabolized further *in vivo*, it has been reported to affect an enzyme activity in mammalian cells (37). Phosphatidylbutanol applied at 0.1% had no effect on either the GA or ABA response (data not shown), suggesting the biological action of 1-butanol was most likely attributable to PLD inhibition rather than the effect of the phosphatidylalcohol.

DISCUSSION

The application of PPA to aleurone protoplasts led to the induction of ABA-like events in the cell. PPA led to the inhibition of the GA response, as measured by the reduction of secreted α-amylase activity. This inhibition was not due to a nonspecific toxic effect of PPA; because the protein levels of the ABA enhanced genes ASI and RAB (3–5, 32) increased in protoplasts treated with PPA, while levels of the constitutively expressed protein tubulin were unaffected (Fig. 1*B*). Additionally, PPA mimicked ABA action in causing a reduction in cytoplasmic $[Ca^{2+}]$ (Fig. 1*C*), which requires the functioning of an intact, energy dependent Ca^{2+} homeostatic system in these cells (38). These data suggest that the ABA signal may be transduced in the aleurone cell through activation of PPA production, as depicted in Fig. 6. Although the available data suggests a plasma membrane perception site for ABA (as implied in Fig. 6), multiple ABA receptors, both internal and external may exist (39).

Elevation of PPA levels by ABA could occur through several routes. PPA is the phosphorylated from of DAG and the two molecules are readily interconvertable by a DAG kinase and PPA phosphatase. Thus PPA could be produced directly from the action of PLD, or via phosphorylation of DAG produced from, for example, PLC activity. DAG is a central molecule in one of the most well-characterized signal transduction pathways of animal cells, the DAG and IP_3 producing phosphoinositide signaling pathway (14). The role of DAG and IP_3 in plants is less well defined. Both DAG and IP_3 have been shown to elicit (opposite) responses in guard cells (16, 19) and to accelerate and retard mitotic progression in Tradescantia stamen hairs (15). IP_3 has been proposed as a regulator of pollen tube growth (40) and shown to increase transiently in response to GA in the aleurone (22). Although these results

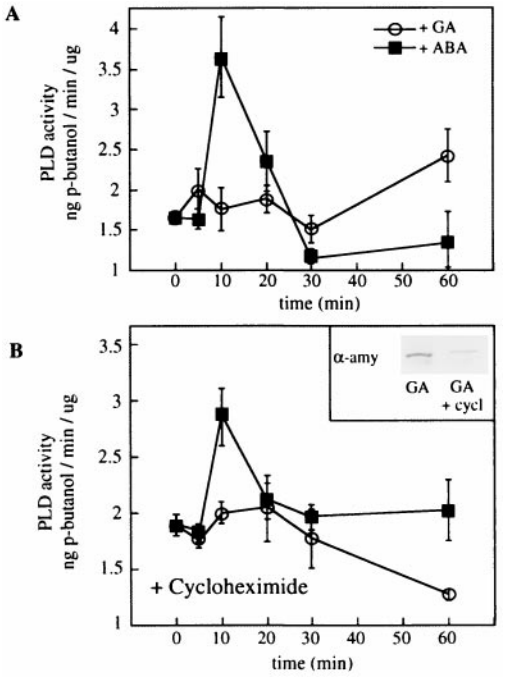

FIG. 4. PLD activity in aleurone protoplasts treated with GA or ABA. (*A*) GA (5 μM) or ABA (5 μM) was applied to protoplasts for various times and PLD activity assayed *in vitro* as described in *Materials and Methods*. (*B*) Protoplasts were incubated in 5 μg/ml cycloheximide for 2 hr and then treated with ABA or GA, and PLD assayed as for *A*. Mean ± SEM, $n = 3$ separate experiments. (*B Inset*) Protoplasts were treated with 5 μM GA, with (+cycl) or without cycloheximide (5 μg/ml) for 48 hr. Protein was extracted, and SDS/PAGE and immunoblotting carried out as described in *Materials and Methods*.

suggest hormone-regulated changes in the activity of PLC could produce DAG and IP_3 in the aleurone, and that these second messengers could underlie hormone signal transduction, we think this unlikely for ABA signaling on several counts. We have found that microinjection of IP_3 in the aleurone cytoplasm does not alter cytoplasmic Ca^{2+}, nor can an IP_3 mobilizable pool of Ca^{2+} be identified in isolated membranes *in vitro* (S.G., unpublished data). Similarly, inhibitors of mammalian PLC (neomycin and D609) and inositol phosphate metabolism (wortmannin and $LiCl_3$) do not alter GA or ABA responses in aleurone (unpublished data). Fig. 3 indicates that large elevations in DAG levels do not occur in response to GA or ABA. Furthermore, the ABA-like activity of DAG is blocked by a DAG kinase inhibitor, whereas ABA action is unaffected. This result suggests that any ABA-like activity of DAG is due to its conversion to PPA by DAG kinase, but that this conversion is not a normally required part of ABA signal transduction. Thus it seems unlikely that a classical DAG/IP_3 signaling pathway is acting in the ABA response of the aleurone cell.

Production of DAG does not appear to provide the mechanism for an ABA-induced increase in PPA. However, we have observed a rapid (within 10 min), transient, ABA-dependent activation of PLD in aleurone. Inhibiting this activation with 1-butanol prevented ABA action, whereas adding the product of PLD activity, PPA, overcame this inhibition. These results are consistent with an ABA activation of PLD to produce PPA that then propagates the ABA signal. Inhibition of PLD activity was only effective in preventing ABA action during the 10–20 minutes of PLD activation induced by ABA, suggesting the ABA signal had been passed on from the PLD activation to PPA, and PPA-regulated activities, over this time.

The downstream events affected by PPA are unknown. One possibility is that a PPA breakdown product propagates the

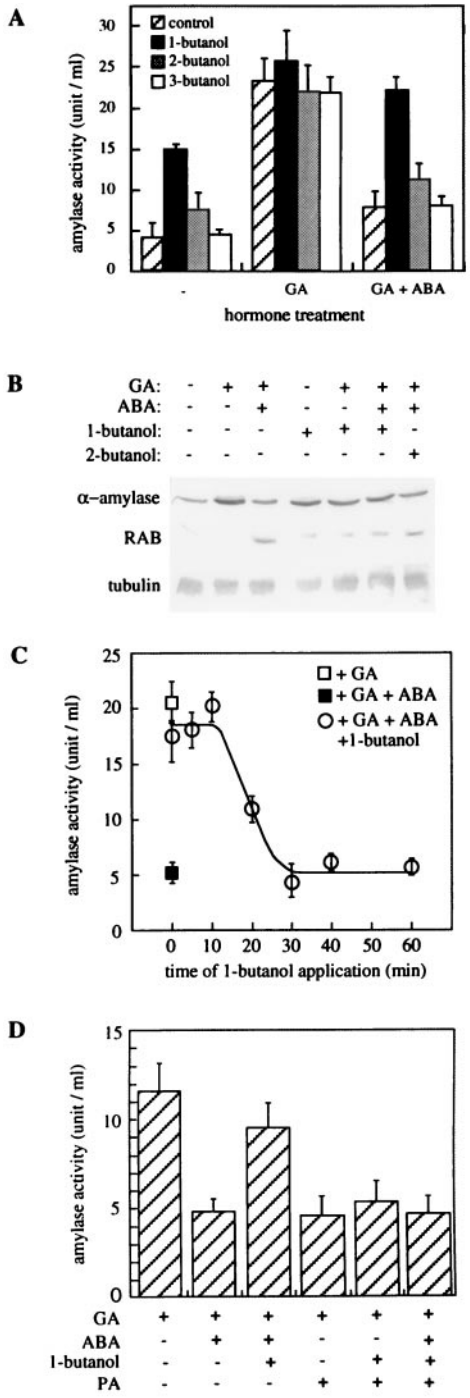

FIG. 5. The effect of butanol on amylase activity and protein levels of aleurone cells. (*A*) Aleurone tissue was treated with GA (5 μM), ABA (5 μM), 0.1% 1,2, or 3 -butanol as denoted. α-Amylase secretion was then assessed after 48 hr. Results represent mean ± SEM, $n = 4$. (*B*) Aleurone tissue was treated as in A, and the levels of α-amylase, tubulin and the ABA-induced protein RAB assessed by western blotting. These immunoblots indicate the levels of proteins extracted from the protoplasts rather than the secreted activity assayed in *A*. (*C*) Aleurone tissue was treated with 5 μM GA for 4 hr, then with 5 μM ABA, then with 0.1% 1-butanol at various times after the ABA. α-Amylase activity was assayed after 48 hr treatment. Results represent mean ± SEM, $n = 4$. (*D*) Aleurone tissue was treated with GA (5 μM), ABA (5 μM), 0.1% 1-butanol, and 50 μM PPA (acyl chains arachidonoyl/stearoyl) as noted and α-amylase secretion assessed after 48 hr. The data represent mean ± SEM, $n = 3$.

ABA signal. Although we have tested the effects of several putative PPA turnover products such as fatty acids (palmytic,

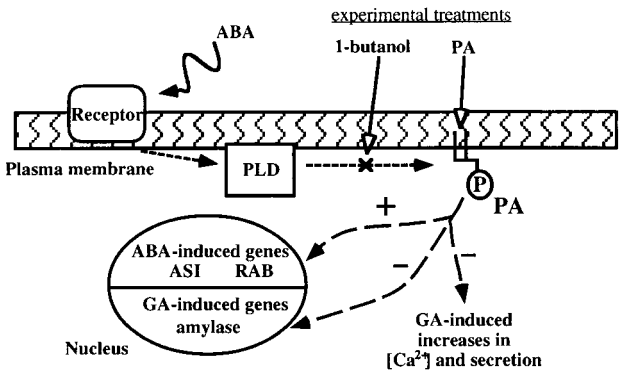

FIG. 6. A schematic diagram of the involvement of PLD and PPA in the ABA signal transduction process in the barley aleurone. Solid-headed arrows represent events proposed to occur during the response of the aleurone to ABA, open-headed arrows represent manipulations to the system carried out in experiments described in this work.

oleic, arachidonic, stearic), lysophosphatidic acid, and lysophosphatydylcholine, none of these elicit the ABA-like responses shown by PPA (data not shown). Other candidates for PPA-dependent signaling activities include the protein kinases and phosphatases that have been implicated in ABA signaling in other plant cells (41–43). Lipid modulation of Ca^{2+}-dependent kinases has been reported (44, 45) and considering the Ca^{2+} dependency of the hormonal regulation of aleurone (38), these enzymes represent attractive candidates for integrating signals in these cells. Messenger RNAs corresponding to calmodulin-like domain protein kinases have been identified in oat aleurone (46) and a calmodulin-like domain protein kinase-like activity associated with the GA response has been identified in barley aleurone (47). Defining these downstream events, as well as the regulators between ABA receptor and PLD should provide insights into the ABA signal transduction system of the aleurone. The PLD-based propagation of the ABA signal in aleurone also highlights the potential role for this enzyme in transduction of the ABA signal in other types of plant cells.

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