Identification of Diacylglycerol Pyrophosphate as a Novel Metabolic Product of Phosphatidic Acid during G-protein Activation in Plants

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We provide evidence that phosphatidic acid (PtdOH) formed during signaling in plants is metabolized by a novel pathway. In much of this study, 32P-labeled Chlamydomonas cells were used, and signaling was activated by adding the G-protein activator mastoparan. Within seconds of activation, large amounts of [32P]PtdOH were formed, with peak production at about 4 min, when the level was 5-25-fold higher than the control. As the level of [32P]PtdOH subsequently decreased, an unknown phospholipid (PLX) increased in radiolabeling; before activation it was barely detectable. The chromatographic properties of PLX resembled those of lysophos- tidoH and CMP-PtdOH but on close inspection were found to be different. PLX was shown to be diacylglycerol pyrophosphate (DGPP), the product of a newly discovered enzyme, phosphatidate kinase, whose in vitro activity was described recently (Wissing, J. B., and Be- niger, H. (1993) Plant Physiol. 102, 1243-1249). The identity of DGPP was established by co-chromatography with a standard and by degradation analysis as follows: [32P]DGPP was deacylated, and the product (glycerolpyrophosphate, GroPP) was hydrolyzed by mild acid treatment or pyrophosphatase to produce GroP and P, as the only radioactive products. Since DGPP is the pyrophosphate derivative of PtdOH and is formed as the concentration of PtdOH decreases, we assumed that PtdOH was converted in vivo to DGPP. This was confirmed by showing that during a short labeling protocol while the specific radioactivity of DGPP was increasing, the specific radioactivity of the 32P, derived from DGPP as above was higher than that of [32P]GroP. DGPP was also formed in suspension cultures of tomato and potato cells, and its synthesis was activated by mastoparan. Moreover, it was also found in intact tissues of a number of higher plants, for example, carnation flower petals, vetch roots, leaves of fig-leaved goosefoot, and common persicaria and microspores of goosefoot. More- over, it was also found to increase in concentration when signaling is activated. Possible functions of DGPP in phospholipase C and D signaling cascades are discussed.

Phosphatidic acid (PtdOH) is not only an important intermediate in the synthesis of all phospho- and glycolipids but is also suggested to be an intracellular signal in mammalian cells. It is rapidly produced in receptor-stimulated cells by phospholipase D (PLD) and/or the combined action of PLC and diacylglycerol (DAG) kinase (Exton, 1990; 1994a; 1994b; Billah and Anthes, 1990; Liscovitch, 1992; Billah, 1993; Van Blitterswijk et al., 1994; Divena and Irvine, 1995; Nishizuka, 1995). The formation of PtdOH has been linked to a variety of physiological responses such as actin polymerization (Ha and Exton, 1993; Exton, 1994a; 1994b; Ha et al., 1994; Zhou et al., 1995), membrane trafficking (Boman and Kahn, 1995; Liscovitch and Cantley, 1995; Martin, 1995), the respiratory burst in neutro- phils (Baldry et al., 1992; Cockcroft, 1992; Qualliotine-Mann et al., 1993), mitogenesis (for review, see Boarder (1994)), and hormone release (Liscovitch and Amsterdam, 1989; Metz and Dunlop, 1990). How PtdOH operates is still unknown, but there are a number of recent reports describing protein kinases that are specifically activated by the lipid (Lee and Bell, 1989; Bockino et al., 1991; Epand et al., 1992; Nakanishi and Exton, 1992; Stasek et al., 1993; Kahn et al., 1994; Limatola et al., 1994). PtdOH has also been shown to specifically activate Pt- dpnS-P kinase and PLC (J. Ackowski and Rock, 1989; Kroll et al., 1989; Hashizume et al., 1992; Moritz et al., 1992; J Jacob et al., 1993; Jones and Carpenter, 1993; Horstman et al., 1995) and therefore could also function by amplifying the phospho- nitide-PLC signaling cascade (Liscovitch et al., 1994).

While the speed and magnitude of second messenger forma- tion are important, cells must also down-regulate their effective concentrations to ensure that the response level is tightly coupled to the stimulation level. In general, PtdOH is thought to be converted by PtdOH phosphatase to DAG or by PLA2, to lyso-PtdOH or to be converted to CMP-PtdOH for the resynthesis of certain phospholipids (Kent, 1995). Recently, a new mecha- nism of PtdOH attenuation was discovered (Van Blitterswijk and Hilkmann, 1993) in which PLD transfers the phosphatidyl moeity of phosphatidylinositol to endogenous DAG, resulting in the formation of the novel phospholipid bisphosphatidic acid (BisPtdOH). This uncommon lipid is therefore the condensa- tion product of PLC and PLD signaling pathways and attenuates the formation of both DAG and PtdOH.

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The abbreviations used are: PtdOH, phosphatidic acid; PLC, phospholipase C; PLD, phosphatidase D; DAG, diacylglycerol; BisPtdOH, bisphosphatidic acid; DGPP, diacylglycerol pyrophosphate; PtdInsP2, phosphatidylinositol bisphosphate; PtdInsP3, phosphatidylinositol monophosphate; TLC, thin layer chromatography; PtdIns, phosphati- dylinositol; GroP, glycerol 3-phosphate; GroPP, glycerol 3-phosphate pyrophosphate; PEI, polyethyleneimine; PLAP, phospholipase A2; PtdEtn, phosphatidylethanolamine; PtdGro, phosphatidylglycerol.
In plants, evidence for lipid-derived second messenger pathways is increasing, as reflected in the number of recent reviews (Drøbak, 1992, 1993; Coté and Crain, 1993, 1994). One of the best studied systems is the green alga Chlamydomonas that was first used to show that G-protein activators such as mastoparan (Law and Northrop, 1994; Ross and Higashijima, 1994) and ET(OH) (Hoeck et al., 1992) rapidly activate the formation of PtdOH (Musgrave et al., 1992; Quarmby et al., 1992). The increase was originally explained as the combined activities of PLC and DAG kinase. Similar effects of mastoparan on PLC increase was first used to show that G-protein activators such as mastoparan rapidly activate the formation of PtdOH (Wissing and Behrbohm, 1993a, 1993b; Wissing et al., 1994) and DGPP was further shown to be phosphorylated in plants is unknown. How the PtdOH levels are down-regulated in plants is unknown.

Recently, a new enzyme activity was extracted from many different higher plants that was able to phosphorylate PtdOH to diacylglycerol pyrophosphate (DGPP) and was called phosphatidate kinase (Wissing and Behrbohm, 1993a, 1993b; Wissing et al., 1994). As yet, all demonstrations of activity were in vitro. Here we show that when Chlamydomonas cells were treated with mastoparan, the initial rise in PtdOH formation was counteracted by its conversion to DGPP, illustrating that this novel lipid kinase is highly active in vivo when intracellular signaling is stimulated. DGPP was further shown to be present in several higher plants and their different tissues, and its synthesis was shown to be stimulated when cells were activated by mastoparan.

EXPERIMENTAL PROCEDURES

Materials—Silica 60 TLC plates and reagents for lipid extraction and analyses were from Merck. Mastoparan (Vespuca livisii), glycerophosphate (GroP), CMP-PtdOH, PLA2 (Apis mellifera), and inorganic pyrophosphatase (Escherichia coli and Saccharomyces cerevisiae) were purchased from Sigma. PEI cellulose TLC polygram sheets were from Machery-Nagel (Düren, Germany). [32P]Orthophosphate (carrier-free) (Dupont NEN) and tritiated phosphate (GroPP) was dried by vacuum centrifugation, dissolved in 20 mM, pH 7.4 (KOH), 1 mM MgCl2, 1 mM CaCl2, 1 mM KCl (HMCK). After 2–16 h, cells were harvested, washed twice with HMCK by centrifugation for 5 min at 500 × g, and resuspended at a final concentration of 1–2 × 106 cells/ml in HMCK. Phospholipids were metabolically labeled by incubating cells (1–2 × 106 cells/ml) with 100 μCi of carrier-free 32P-O4 in HMCK for the times indicated in the figure legends. When necessary, excess label was removed by washing the cells twice with HMCK. For the identification of CMP-PtdOH, cells were labeled for 5 h with 10 μCi of [3H]cytidine (100 μl). After extraction and TLC, the plate was sprayed with En3Hance (DuPont NEN) and exposed for 3 weeks to visualize 3H-labeled CMP-PtdOH by autoradiography.

Routinely, cells were treated with mastoparan in a total volume of 1 ml for the time and concentrations indicated within the figure legends. Occasionally, for the preparation of labeled standards, larger volumes were used. Incubations were stopped by the addition of 3.75 volumes of CHCl3/MeOH/HCl (50:100:1 (v/v/v)), and lipids were extracted, separated, and quantified as described below.

Extraction and Analysis of Chlamydomonas Lipids—The extraction method was completed, and a two-phase system was induced by the addition of 3.75 volumes of CHCl3, and 1 volume of 2 M HCl. Tubes were vortexed for 15 s and centrifuged for 2 min in a microcentrifuge. The organic lower phase was washed once with 3.75 volumes of CHCl3, MeOH, 1 M HCl (3:48:47 (v/v/v)) and dried by vacuum centrifugation. Lipids were dissolved in CHCl3, and stored under nitrogen at −20 °C.

Routinely, lipids were separated by TLC using an alkaline solvent system (CHCl3, MeOH, 25% NH4OH, H2O; 90:70:4:16 (v/v/v)) as described previously (Munnik et al., 1994a). For the purification and quantification of 32P-labeled DGPP, two-dimensional TLC was used. After the alkaline solvent system in the first dimension, plates were dried and chromatographed in a second dimension using CHCl3/pyridine/formic acid (35:30:7 (v/v/v)).

Phospholipids were detected by autoradiography. Individual spots were scraped from the TLC plate for further processing or quantification by liquid scintillation counting. Alternatively, radioactivity was determined directly on the TLC plate using a PhosphorImager (Molecular Dynamics). Unlabeled phospholipid standards (10 μg) were visualized by exposure to iodine vapor.

Controlled Breakdown of DGPP—32P-Labeled DGPP was purified by two-dimensional TLC as described above. The lipid was scraped from the TLC plate and deacylated with monomethylamine at 53 °C for 30 min as described in Munnik et al. (1994a). The resulting glycerophosphate (GroPP) was dried by vacuum centrifugation, dissolved in H2O, and subsequently desalted on a cation-exchange column (Bio-Rad AG 1X8, 88–200–400, H+ form). Samples were concentrated by vacuum centrifugation and stored at −20 °C in H2O.

Mild acid hydrolysis of the pyrophosphate bond of GroPP into P and GroP was achieved by treating the sample with 1 M trichloroacetic acid at 100 °C for 5 min. Trichloroacetic acid was removed by extracting the sample three times with water-saturated diethyl ether. Alternatively, GroPP was hydrolyzed using 4 M formic acid for 30 min at 100 °C. Both treatments hydrolyzed [32P]JATP but left [32P]GroP intact (data not shown); however, the formic acid method had the advantage that samples could be dried directly without leaving salt traces that interfered with subsequent analyses.

Enzymatic breakdown of GroPP was performed by treatment with inorganic pyrophosphatase. Two different sources were used, each applied to the supporting TLC plate gel (BioRad, Ultrolite, 0.25 mm; Sigma, 0.75 mm). One source of GroPP isolated from S. cerevisiae (112 units, 0.8 mg, 85% buffer salts) was dissolved in 122 μl of 10 mM Hepes at pH 7.2, while that from E. coli (1 mg, 100 units, 15% buffer salts) was dissolved in 100 μl of 100 mM Tris-HCl, pH 8.9. Samples were incubated with 5 units of enzyme in 50 μl of buffer for 2 h at room temperature. Protein was removed by ETOH precipitation. Samples were dried by vacuum centrifugation and stored in H2O at −20 °C.

Samples were spotted on PEI cellulose anion-exchange TLC polygram sheets, and GroP, P, and GroPP were separated using a mixture of 0.5 M ammonium formate and 0.2 M formic acid as a solvent. 32P-labeled P standard was from Amersham Corp. A [32P]GroP standard was prepared by deacylation of metabolically labeled Chlamydomonas PtdOH, which had been purified by two-dimensional TLC as described above. The identities of P, and GroP were confirmed by ionophoresis on Whatman No. 1 paper (56 cm) using 0.1 M sodium oxalate buffer, pH 1.5, at 2000 V-h as described by Seiffert and Agranoff (1965). Using this system the radioactivities relative to that of P, for GroP, 2,3-diphosphoglycerate (P), and GroPP were 1.44, 1.92, 2.11, and 2.0, respectively (results not shown).

Radioactive spots were detected by autoradiography and quantified by liquid scintillation counting of the corresponding regions of the chromatogram. Unlabeled phosphate standards (−5 μg) were visualized by spraying with a P reagent that was specifically designed for cellulose-based chromatograms and was prepared as follows. 8 ml of stock solution A (4% ammonium molybdate (w/v) in 9% HClO4 (w/v)) was mixed immediately before use with 24 ml of stock solution B (100 ml of H2O, 10 ml of HClO4 and 1 ml of concentrated HCl). The chro...
PLX—When 32Pi-prelabeled a strong UV source to develop the blue colors. autoradiographs were then dried at room temperature and irradiated with a strong UV source to develop the blue colors.

Phospholipid Analysis of Other Plants—Carnation petal-discs (Diathuis caryophyllus L. cv. White Sim) were labeled with 32P, for 1 h, and their lipids were extracted as described previously (Munnik et al., 1994b).

A potato (Solanum tuberosum L.) suspension culture, prepared from callus of an inbred line, was cultivated at 26 °C in a Murashige-Skoog type liquid medium (Murashige and Skoog, 1962) supplemented with 30 glt sucrose, 5 mg/l 1-naphthylacetic acid, and 0.1 mg/l 6-benzyladenine. Cells were subcultured in intervals of 1 week and were used 4 days after transfer. Prior to labeling, cells were washed 3 times with medium without Pi by decanting the supernatant after the culture had settled due to gravity after 2 min. Cells were labeled at room temperature in the same medium using 100 μCi of 32P/ml for the times indicated. Samples were treated with mastoparan by withdrawing aliquots of 90 μl and adding them to 10 μl of mastoparan or H2O (control) as specified within the figure. Lipids were extracted as described above for Chlamydomonas.

Tomato suspension cells (Lycopersicum esculentum cv. Money Maker) were grown in Murashige-Skoog medium (Murashige and Skoog, 1962), which was supplemented with B vitamins (Gamborg et al., 1968), 30 glt sucrose, 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.1 mg/l kinetin. Cells were grown on a rotary shaker (125 rpm) at 25 °C in the dark. Radiolabeling, mastoparan treatment, and lipid extractions were performed essentially as described above for potato suspension cells.

Leaves from common persicaria (Polygonum persicaria) and fig-leaved goosefoot (Chenopodium ficifolium) were harvested from mature plants growing in the university grounds. Leaf-discs were 32P-labeled for 3 h, and their lipids were extracted as described earlier for carnation petal discs (Munnik et al., 1994b).

RESULTS AND DISCUSSION

Mastoparan-induced Formation of a Novel Phospholipid PLX—When 32P-prelabeled Chlamydomonas cells were stimulated with the G-protein activator mastoparan, the subsequent analysis of their lipids revealed two clear responses (Fig. 1): a dramatic increase in the level of PtdOH and the appearance of an unknown 32P-containing lipid, hereafter called phospholipid X (PLX). The PtdOH response is the result of G-protein-activated PLD and PLC activities (Cramby et al., 1992; Munnik et al., 1995). The product of PLC activity is actually DAG, but much of it is rapidly converted to PtdOH by DAG kinase. The origin and nature of PLX, however, were unknown and therefore studied further.

Mastoparan-induced Formation of PtdOH and PLX Is Time- and Dose-dependent—Fig. 2A shows the kinetics of formation of PtdOH and PLX after mastoparan stimulation. The PtdOH response was very fast and transient, increased within seconds, peaked at about 4 min, and decreased quickly thereafter. The increase in 32P-labeled PLX was slower but, significantly perhaps, correlated with the disappearance of [32P]PtdOH. When different concentrations of mastoparan were used to stimulate cells for 7 min (Fig. 2B), the amount of PLX formed again seemed related to the amount of PtdOH formed. Based on these observations, we hypothesized that PLX was a metabolic product of PtdOH.

PLX Is Not Lyso-PtdOH or CMP-PtdOH—The immediate biological response to treating Chlamydomonas cells with mastoparan is deflagellation. Since this is followed by the complete regeneration of new flagella within 90 min, lipid synthesis could be rapidly activated on adding mastoparan, and so we considered whether PLX was a key intermediate in the biosynthesis of lipids. Both lyso-PtdOH and CMP-PtdOH were good
candidates since they are more polar derivatives of PtdOH. Accordingly, we isolated [32P]PtdOH from 32P-labeled cells that had been stimulated with mastoparan, treated it with PLA2, to form lyso-PtdOH, and compared its chromatographic properties with those of PLX. As is obvious from Fig. 3, their Rf values were different.

In order to identify CMP-PtdOH on chromatograms, Chlamydomonas cells were labeled with [3H]cytidine, and the lipids were isolated and separated. While the only radioactive spot co-chromatographed with a CMP-PtdOH standard, it did not co-migrate with PLX (Fig. 4). These results established that PLX was not one of the well known metabolic products of PtdOH involved in lipid synthesis (Kent, 1995).

PLX Is Not BisPtdOH but Co-migrates with the Novel Phospholipid DGPP—PtdOH is the biologically active product of PLD activity in Chlamydomonas cells (Munnik et al., 1995) and so must be considered a potential signal molecule. Since increases in levels of other signaling molecules are invariably attenuated by metabolism to less active derivatives, the same can be expected of PtdOH. For example, BisPtdOH is a newly discovered condensation product of PtdOH and DAG, whose formation in bradykinin-stimulated human fibroblasts attenuates both signals (Van Blitterswijk and Hilkmann, 1993). Since both PtdOH and DAG are formed in Chlamydomonas cells treated with mastoparan, we considered whether PLX is BisPtdOH. However, we immediately found that the former is so much more polar than the latter (Rf values in the alkaline TLC system were 0.39 and 0.92, respectively) that the two cannot be confused (results not shown). Another derivative of PtdOH has recently been reported by Wissing and Behrbohm (1993a). They discovered an in vitro enzyme activity in plants that utilized ATP and PtdOH to produce a new phospholipid (Wissing and Behrbohm, 1993a, 1993b). NMR analysis revealed it to be DGPP, and therefore they called the enzyme phosphatidate kinase. To test whether PLX could be DGPP, we compared their TLC properties using a standard generously provided by Dr. Wissing (GBF, Braunschweig, Germany). On a two-dimen-

![Image](http://www.jbc.org/)

**Fig. 3.** Comparison of the chromatographic properties of lyso-PtdOH and PLX. 32P-labeled lyso-PtdOH was prepared by treating [32P]PtdOH, isolated from mastoparan-stimulated Chlamydomonas cells, with (−) or without (−) PLA2, as described under "Experimental Procedures." After 1 h, the lipids were extracted and separated by TLC together with some starting material to mark the position of PLX (M). The lyso-PtdOH present in the untreated PtdOH (middle lane) represents the breakdown that occurred during PtdOH isolation from the silica.

**Fig. 4.** Comparison of the chromatographic properties of CMP-PtdOH and PLX. Chlamydomonas cells were labeled with [3H]cytidine, and their lipids were extracted, separated by TLC, and visualized by autoradiography. [3H]Labeled CMP-PtdOH was identified by co-migration with a standard that was visualized by exposure to iodine vapor (not shown, indicated by an arrow). Lipids from 32P- prelabeled Chlamydomonas cells treated with mastoparan were co-chromatographed to compare their chromatographic behavior.

To further establish the identity of PLX as DGPP, we devised the scheme shown in Fig. 6. It predicts that removal of the fatty acids will result in the formation of GroPP. However, because no standard was available, the molecule was further degraded. Since a pyrophosphate bond is much more acid-sensitive than a monoester phosphate, mild acid hydrolysis was used to degrade GroPP to GroP and Pi, for which standards were available. In addition, the sensitivity to inorganic pyrophosphatase was tested.

32P-Labeled PLX was purified from mastoparan-stimulated Chlamydomonas cells by two-dimensional TLC and deacylated, and the water-soluble headgroup (i.e., PX or GroPP) was analyzed by anion-exchange TLC. Fig. 7 shows the deacylation product of PLX before and after mild acid hydrolysis. Before the hydrolysis, PX contained all of the radioactivity of PLX and migrated as a single spot whose Rf was different from PX or GroPP. Since increases in levels of other signaling molecules are invariably attenuated by metabolism to less active derivatives, the same can be expected of PtdOH. For example, BisPtdOH is a newly discovered condensation product of PtdOH and DAG, whose formation in bradykinin-stimulated human fibroblasts attenuates both signals (Van Blitterswijk and Hilkmann, 1993). Since both PtdOH and DAG are formed in Chlamydomonas cells treated with mastoparan, we considered whether PLX is BisPtdOH. However, we immediately found that the former is so much more polar than the latter (Rf values in the alkaline TLC system were 0.39 and 0.92, respectively) that the two cannot be confused (results not shown). Another derivative of PtdOH has recently been reported by Wissing and Behrbohm (1993a). They discovered an in vitro enzyme activity in plants that utilized ATP and PtdOH to produce a new phospholipid (Wissing and Behrbohm, 1993a, 1993b). NMR analysis revealed it to be DGPP, and therefore they called the enzyme phosphatidate kinase. To test whether PLX could be DGPP, we compared their TLC properties using a standard generously provided by Dr. Wissing (GBF, Braunschweig, Germany). On a two-dimen-

![Image](http://www.jbc.org/)
Together, these results indicate that PX is GroPP, and therefore we deduce that PLX is DGPP.

PtdOH Is Phosphorylated to DGPP in Vivo—The kinetics of formation of PtdOH and DGPP (Fig. 2) suggested that DGPP is formed by phosphorylation of PtdOH. To test this we used the strategy of Stephens and Downes (1990) and determined the relative specific radioactivities of the individual phosphate groups in DGPP after a short labeling period. Assuming that both phosphorylations use the γ-phosphate of the same ATP pool, then while the specific radioactivity of ATP is still increasing, the phosphate with the highest specific activity is the one added last during synthesis. Accordingly, cells were labeled with $^{32}$P$_i$ for 5 min and stimulated with mastoparan for 4 min, a period during which $^{32}$P$_i$ is still being taken up into the cells and incorporated into the ATP pool. DGPP was isolated by two-dimensional TLC and hydrolyzed in the presence of excess nonradioactive GroP and P$_i$ to prevent a specific loss. The radioactivity in GroP and P$_i$ was then determined after separation on PEI cellulose. DGPP samples taken after longer periods of mastoparan stimulations (up to 25 min), the radioactivity in P$_i$ was always higher than in GroP, also see the data in Fig. 8. These results are consistent with DGPP being formed by phosphorylation of PtdOH.

Occurrence in Other Plants—To verify the presence of DGPP in other living plant tissues, we labeled leaves of Polygonum persicaria and Chenopodium ficifolium, microspores of Brassica napus, Vicia sativa roots, and flower petals of Dianthus caryophyllus with $^{32}$Pi and analyzed their lipids. All extracts contained a radioactive lipid that co-chromatographed with DGPP on both one- and two-dimensional TLC. Its headgroup comigrated with Chlamydomonas GroPP and was degraded into GroP and P$_i$ after acid hydrolysis (not shown). It is a minor lipid; for example after 1–3 h of incubation in $^{32}$Pi, the radioactivity in DGPP accounted for 2.5, 1.5, 1.0, 5.3, and 1.0% of the total present in the lipid fractions extracted from P. persicaria, C. ficifolium, B. napus, V. sativa, and D. caryophyllus, respectively. The molecular percentages are expected to be lower because DGPP contains two phosphates that are rapidly labeled via ATP and because no attempt was made to reach isotopic equilibrium. These results are consistent with DGPP being formed by phosphorylation of PtdOH.

In order to test whether higher plant cells synthesize more DGPP when signaling is stimulated, both potato- and tomato-
suspension cultures were prelabeled with $^{32}$P and then treated with mastoparan. Lipid were then extracted and separated as illustrated in Fig. 9. In analogy with Chlamydomonas, the synthesis of PtdOH and DGPP was again stimulated by mastoparan. While this implies that these cells have receptors that are coupled by G-proteins to PLC, it also indicates that plants in general metabolize PtdOH to DGPP, in particular when signaling is stimulated. In a separate study, human platelets and U937 cells were prelabeled with $^{32}$P. The platelets were then stimulated with thrombin or mastoparan, and the U937 cells were stimulated with ATP. While these treatments increased the level of radioactively labeled PtdOH, no radioactive DGPP spot was detected on TLC. Thus the phosphorylation of PtdOH to form DGPP could be confined to plants.

Possible Functions—We have previously shown that G-protein activators such as mastoparan stimulate both PLC and PLD signaling in plant cells (Munnik et al., 1995). The subsequent rapid synthesis of PtdOH and its metabolism to DGPP indicate that both compounds could play an important role in signaling. Since PtdOH is the direct product of PLD activity, it must be considered a signal in its own right, just as in animal cells where it is reported to activate specific protein kinases (Boccino et al., 1991; Epand et al., 1992; Nakanishi and Exton, 1992; Stasek et al. 1993; Khan et al. 1994; Limatola et al., 1994). In support, we have shown that PtdOH added to Chlamydomonas cells induces the same biochemical responses as the G-protein activators themselves (Munnik et al., 1995). Because cells must be able to respond to repeated stimuli, increased signal levels have to be constantly attenuated, and the conversion of PtdOH to DGPP may be an example of signal attenuation. This is represented in our model of signaling in a plant cell (Fig. 10), where the increase in PtdOH concentration is attenuated by PtdOH-kinase, which is known to be located at the plasma membrane (Wissing and Behrbohm, 1993b). While this hypothesis emphasizes the potential importance of PtdOH as a signal, it relegates DGPP to the realm of biologically inactive compounds, and that may not be the case. As a minor polar lipid that dramatically increases and then decreases in concentration when cells are activated, it has itself the potential to be a signal molecule. While there is no evidence for or against this (see question mark in Fig. 10), it remains possible that DGPP formation is not just a means of inactivating PtdOH.

2 T. Munnik, K. A. Hinchcliffe, F. T. Cooke, and L. R. Stephens, unpublished data.
DGPP, a Novel Phosphatidic Acid Metabolite

![Diagram of PLC Signaling and Signal Attenuation]

Another possibility for DGPP stems from the fact that it is, like CMP-PtdOH, an activated phosphatidate molecule. In the presence of a phosphatidylinositol transferase and an appropriate substrate, e.g. inositol, it could be converted to other lipids such as phosphatidylinositol. As such, it could help maintain the PtdIns cycle, whereby polyphosphoinositides hydrolyzed during PLC signaling are refurbished via DAG, PtdOH, DGPP, and PtdIns formation. In animal cells, the possibility of resynthesis of PtdIns from PtdOH in the plasma membrane remains controversial (e.g. Rara and Hokin (1990)) and it may be that PtdOH is transferred from the plasma membrane to the endoplasmic reticulum where it can be converted to PtdInsP2 and PtdIns formation. In animal cells, the possibility of resynthesis of PtdIns from PtdOH in the plasma membrane remains controversial (e.g. Rara and Hokin (1990)) and it may be that PtdOH is transferred from the plasma membrane to the endoplasmic reticulum where it can be converted to PtdInsP2 and PtdIns formation.

Fig. 10. Model of DGPP as the attenuator of a PtdOH signal formed by G-protein activation of PLC and PLD. The possible role of DGPP as a signal molecule is depicted as a dashed arrow to the physiological response. For details and other possible functions see text.

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