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G Protein Activation Stimulates Phospholipase D Signaling in Plants

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We provide direct evidence for phospholipase D (PLD) signaling in plants by showing that this enzyme is stimulated by the G protein activators mastoparan, ethanol, and cholera toxin. An *in vivo* assay for PLD activity in plant cells was developed based on the use of a “reporter alcohol” rather than water as a transphosphatidylation substrate. The product was a phosphatidyl alcohol, which, in contrast to the normal product phosphatidic acid, is a specific measure of PLD activity. When ³²P-labeled cells were treated with 0.1% *n*-butanol, ³²P-phosphatidyl butanol (³²P-PtdBut) was formed in a time-dependent manner. In cells treated with any of the three G protein activators, the production of ³²P-PtdBut was increased in a dose-dependent manner. The G protein involved was pertussis toxin insensitive. Ethanol could activate PLD but was itself consumed by PLD as transphosphatidylation substrate. In contrast, secondary alcohols (e.g., *sec*-butyl alcohol) activated PLD but did not function as substrate, whereas tertiary alcohols did neither. Although most of the experiments were performed with the green alga *Chlamydomonas eugametos*, the relevance for higher plants was demonstrated by showing that PLD in carnation petals could also be activated by mastoparan. The results indicate that PLD activation must be considered as a potential signal transduction mechanism in plants, just as in animals.

INTRODUCTION

Molecular mechanisms by which extracellular information is received, transduced, and converted into specific intracellular responses are of vital importance to the cell, yet in plants these mechanisms are still largely unknown. From animal systems, we know that extracellular information received by cell surface receptors often activates heterotrimeric G proteins, which in turn activate intracellular effector enzymes that catalyze the formation of second messengers (Gilman, 1987; Birnbaumer et al., 1990; Berridge, 1993; Neer, 1995; Nishizuka, 1995). In recent years, biochemical and molecular analyses have demonstrated that heterotrimeric G proteins are present in plants (reviewed in Ma, 1994), and because their activation has been shown to stimulate effector enzymes and induce physiological responses (Bolwell et al., 1991; Warpeha et al., 1991; Legendre et al., 1992, 1993; Musgrave et al., 1992; Quarmby et al., 1992; Neuhaus et al., 1993; Romero and Lam, 1993; White et al., 1993; Yueh and Crain, 1993; Bowler et al., 1994; Coté and Crain, 1994; Elich and Chory, 1994; Ma, 1994; Quarmby and Hartzell, 1994), we assume they play a role in plant cell signaling similar to that described for animals.

In such a scheme, the activated receptor catalyzes the exchange of GTP for GDP on the α subunit and consequently its dissociation from the $\beta\gamma$ complex. Both α and $\beta\gamma$ subunits

have the potential to stimulate effector enzymes (Birnbaumer, 1992; Hepler and Gilman, 1992; Inglese et al., 1995; Neer, 1995). G proteins can be artificially activated by mastoparan, alcohols, and cholera toxin or inhibited by pertussis toxin (Moss et al., 1983, 1994; Gilman, 1987; Birnbaumer et al., 1990; Hoek et al., 1992; Carty, 1994; Law and Northrop, 1994; Ross and Higashijima, 1994), making these compounds powerful tools for studying G protein-coupled signaling pathways. Alcohols and mastoparan exert their effect by influencing the GTP/GDP state of the G protein, pushing it into its activated GTP form. Pertussis toxin can block this activation by maintaining the α subunit in a GDP-inactive state by preventing its reassociation with $\beta\gamma$. Cholera toxin, in contrast, activates by catalyzing the ADP ribosylation of some $G_{\alpha s}$ such that they can no longer revert to the inactive, GDP-bound form. As a consequence, effector enzymes are activated and second messengers produced in the absence of receptor stimulation.

Extensive studies in animal systems have revealed that many G protein-activated pathways operate via phospholipase C (PLC) and phospholipase D (PLD) (for reviews, see Billah and Anthes, 1990; Exton, 1990, 1994; Cockcroft, 1992; Irvine, 1992; Liscovitch, 1992; Berridge, 1993; Billah, 1993; Liscovitch and Cantley, 1994; Roberts, 1994; van Blitterswijk et al., 1994; Divecha and Irvine, 1995; Nishizuka, 1995). Often the two are activated together, with PLC producing a short pulse (seconds) and PLD a more prolonged pulse (minutes) of lipid-derived

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second messengers (Liscovitch, 1992; Nishizuka, 1995). PLC hydrolyzes phosphatidyl 4,5-bisphosphate (PtdInsP₂) into inositol 1,4,5-trisphosphate (InsP₃) and diacylglycerol (DAG). InsP₃ then releases Ca²⁺ from intracellular stores, which activates Ca²⁺-dependent protein kinases and, together with DAG, activates protein kinase C (PKC). DAG is then usually phosphorylated by DAG kinase to produce phosphatidic acid (PtdOH). This lipid has recently emerged as an important novel intracellular signal in animal cells; consequently, PLD has received much attention because it generates PtdOH directly from structural lipids, such as phosphatidylcholine and phosphatidylethanolamine. Because PtdOH can also be dephosphorylated to DAG, the dual activation of PLC and PLD is seen as rapidly generating these two lipid signals in the cell, whose concentration is then maintained for several minutes.

PLD activity was first demonstrated in plants (Hanahan and Chaikoff, 1947), but although it has been associated with a number of plant processes, no one has shown that it is involved in cell signaling (for reviews, see Heller, 1978; Wang, 1993). A good plant system for studying its possible involvement is the biflagellate green alga *Chlamydomonas* spp, because there is already convincing evidence for the presence of PLC signaling activity (Irvine et al., 1989, 1992; Schuring et al., 1990; Brederoo et al., 1991; Drøbak, 1992, 1993; Musgrave et al., 1992, 1993; Quarmbly et al., 1992; Yueh and Crain, 1993; Côté and Crain, 1994; Munnik et al., 1994a, 1994b; Quarmbly and Hartzell, 1994). What is more, this pathway is activated by G protein activators with dramatic effects for gametes; for example, they deflagellate and activate their mating structures, which are two well-established Ca²⁺ responses (Schuring et al., 1990; Musgrave et al., 1992; Quarmbly et al., 1992; Quarmbly, 1994; Quarmbly and Hartzell, 1994; Sanders and Salisbury, 1994). Using phosphorus-32 to monitor phospholipid turnover, these effects were shown to be correlated with the increased turnover of PtdInsP₂ and a dramatic rise in PtdOH, thought to reflect the phosphorylation of DAG by DAG kinase (Musgrave et al., 1992; Quarmbly et al., 1992); in retrospect, this rise in PtdOH could be due partially to PLD activity.

One of the difficulties of measuring PLD activity is that its product, PtdOH, is generated from PLC activity by DAG kinase and is formed as an intermediate in the biosynthetic pathway of phospholipids by acylation of glycerol 3-phosphate. However, *in vitro* studies of plant PLD established that it has a unique ability to transfer the phosphatidyl moiety of a phospholipid to a primary alcohol rather than water, producing phosphatidyl alcohol rather than PtdOH (Dawson, 1967; Yang et al., 1967; Heller, 1978). Thus, in the presence of a low concentration of butanol, the production of phosphatidylbutanol (PtdBut) is a measure of PLD activity. This property has been used as a measure of PLD activity *in vivo* in mammalian cells (Bocckino et al., 1987; Pai et al., 1988; Liscovitch, 1989; Billah and Anthes, 1990; Moehren et al., 1994). We have now adapted this transphosphatidyl transfer assay for use in living plant cells and used it to demonstrate that G protein activators activate PLD in *C. eugametos* and carnation petals.

RESULTS

Mastoparan-Stimulated Formation of PtdOH

Mastoparan activates heterotrimeric G proteins by a mechanism that mimics receptor activation. This makes it a valuable tool for testing whether effector enzymes, such as PLC or PLD, are activated via G proteins (Law and Northrop, 1994; Ross and Higashijima, 1994).

When *C. eugametos* cells, labeled for 3 hr with phosphorus-32, were treated with mastoparan for 3 min, the radioactive lipid patterns were as depicted in Figure 1. The most obvious effects were a three- to 10-fold increase in the formation of PtdOH and the appearance of a novel unidentified phospholipid PLX (recently identified as diacylglycerol-pyrophosphate, a novel metabolic product of PtdOH that will be published elsewhere; T. Munnik, T. de Vrije, R.F. Irvine, and A. Musgrave, unpublished results). Because mastoparan has been shown to activate PLC in this alga (Quarmbly et al., 1992; Yueh and Crain, 1993; Quarmbly and Hartzell, 1994; T. Munnik and A. Musgrave, unpublished results), soybean (Legendre et al., 1993), and carrot (Drøbak and Watkins, 1994; Cho et al., 1995), increasing the level of DAG, which is rapidly phosphorylated to PtdOH, this result has been interpreted as the G protein activation of PLC. However, this interpretation does not account for the contribution that other G protein-activated effector enzymes can make to PtdOH production. For example, G protein activation of PLD is well established in animal cells (for reviews, see Billah and Anthes, 1990; Exton, 1990, 1994; Exton et al., 1991; Liscovitch, 1992; Billah, 1993), and PLD hydrolysis of any of the major radioactive phospholipids present in *C. eugametos* would result in an increase in radioactive PtdOH. Consequently, we tested whether PLD in plants can also be activated through G proteins.

In Vivo Measurement of Phospholipase D and Activation by Mastoparan

To measure specifically PLD activity *in vivo*, we used its ability to transfer the phosphatidyl moiety of a structural lipid to an alcohol (Figure 2), because no other enzyme has this ability (Heller, 1978; Kanfer, 1980; Bocckino et al., 1987; Gustavson and Alling, 1987; Kobayashi and Kanfer, 1987; Pai et al., 1988; Liscovitch, 1989; Moehren et al., 1994). Thus, cells were incubated in *n*-butanol, and the formation of PtdBut was used as a reporter for PLD activity. Cells were prelabeled with phosphorus-32 for 3 hr to label the structural phospholipids (Brederoo et al., 1991; Munnik et al., 1994a) and subsequently stimulated 3 min with mastoparan in the presence or absence of 0.1 or 0.25% *n*-butyl alcohol (*n*-ButOH). Lipids were then extracted and separated using a TLC system separating PtdBut and PtdOH from other phospholipids (Liscovitch and Amsterdam, 1989). Figure 3A shows the autoradiograph of the

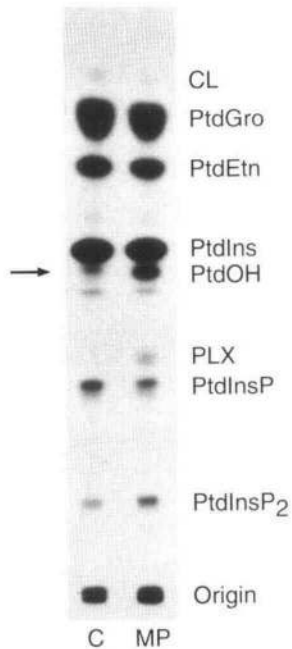


Figure 1. Mastoparan Stimulates the Formation of PtdOH in *C. eugametos*.

Cells were prelabeled with phosphorus-32 for 3 hr and treated for 3 min with H₂O (C, control) or 2 μM mastoparan (MP). Lipids were extracted and separated by alkaline TLC (CHCl₃/MeOH/NH₄OH/H₂O [45:35:2:8 v/v]) as described in Methods. Positions of identified phospholipids are indicated: CL, cardiolipin; PtdGro, phosphatidylglycerol; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdOH, phosphatidic acid; PtdInsP, phosphatidylinositol phosphate; PtdInsP₂, phosphatidylinositol bisphosphate. PLX is an unidentified lipid. Arrow indicates PtdOH.

TLC plate. Mastoparan increased the level of PtdOH (compare lanes 1 and 2), and stimulation in the presence of ButOH resulted in the formation of a new ³²P-labeled lipid that comigrated with a PtdBut standard (arrow). This lipid increased in abundance when the concentration of alcohol was raised (lanes 3 and 4), whereas that of PtdOH did not. This reflects the ability of ButOH to compete with water for transphosphatidylation.

The *in vivo* transphosphatidylation of alcohols and the stimulation by mastoparan were more clearly established by repeating the experiment in the presence of other alcohols, that is, methanol (MeOH), ethanol (EtOH), and *n*-propanol (*n*-PropOH). As shown in Figure 3B, all were used as acceptors for the transphosphatidylation reaction of PLD, each giving rise to a specific product (phosphatidylmethanol, phosphatidylethanol [PtdEt], phosphatidylpropanol, and PtdBut) whose *R_f* value was dependent on the chain length of the alcohol.

Using 2 μM mastoparan, the formation of PtdBut was detectable within 15 sec and increased for at least 60 min (Figure

4A). The stimulation of PtdBut formation was dose dependent and already apparent at a mastoparan concentration of 0.1 μM (Figure 4B).

These results establish that phosphatidylalcohols are formed as transphosphatidylation products of PLD activity and that the enzyme is stimulated in cells treated with mastoparan. This means that part of the mastoparan-stimulated production of PtdOH registered in the first section must result from PLD rather than PLC activity. The quantitative relation between these two pathways is currently being studied and will be presented in a later paper. However, PtdOH can be seen as a biologically active second messenger, because the two known responses to G protein activation, namely, deflagellation and mating structure activation (Schuring et al., 1990; Musgrave et al., 1992; Quarby et al., 1992; Quarby and Hartzell, 1994), are also induced by PtdOH treatment. Figure 5 shows the dose-dependent deflagellation induced by PtdOH. Because the lipid was administered as a suspension in water, we assume that the concentration entering the cell was much lower than indicated

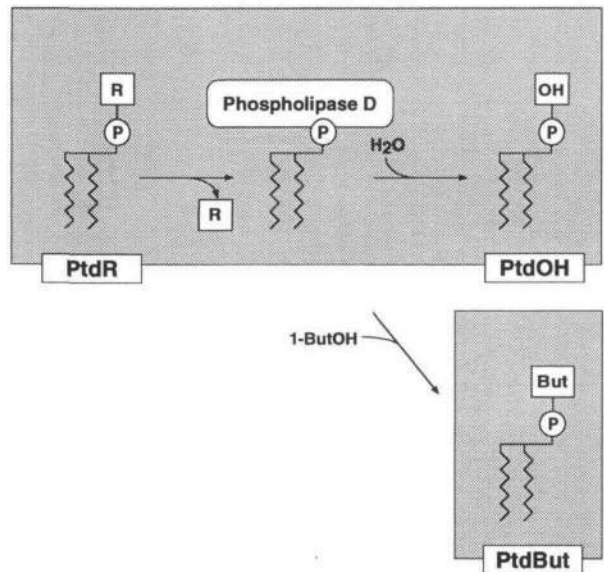


Figure 2. Schematic Representation of PtdOH and PtdBut Formation by PLD-Mediated Transphosphatidylation Activity.

Removal of the head group R from a structural phospholipid (PtdR) by PLD results in the formation of a short-lived "phosphatidyl-PLD" intermediate. Under normal conditions, PLD then transfers the phosphatidyl moiety to H₂O, resulting in the formation of PtdOH, representing the natural hydrolysis of a PtdR. However, when incubated in the presence of an alcohol such as ButOH, the alcohol can be used as an alternative transphosphatidylation substrate, resulting in the formation of PtdBut. The formation of PtdBut can be used as a reporter for PLD activity *in vivo*.

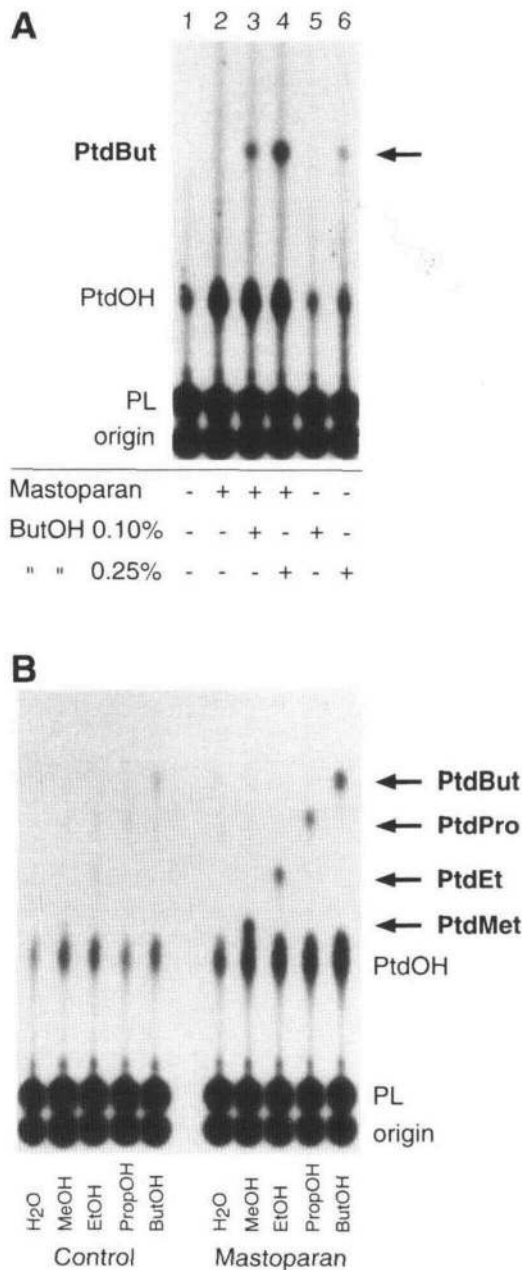


Figure 3. Mastoparan Stimulates the in Vivo Formation of Phosphatidylalcohol in *C. eugametos*.

C. eugametos cells prelabeled with phosphorus-32 for 4 hr were treated for 5 min with H₂O (control) or 2 μM mastoparan in the presence or absence of an alcohol. Lipids were then extracted, separated by ethyl acetate TLC (ethyl acetate/iso-octane/HAc/H₂O [13:2:3:10 v/v]), and autoradiographed. The locations of PtdBut, phosphatidylpropanol (PtdPro), PtdEt, and phosphatidylmethanol (PtdMet) are indicated by arrows. Most of the phospholipids remained at or near the origin and are labeled PL.

(A) Cells were treated with (+) or without (-) mastoparan in the pres-

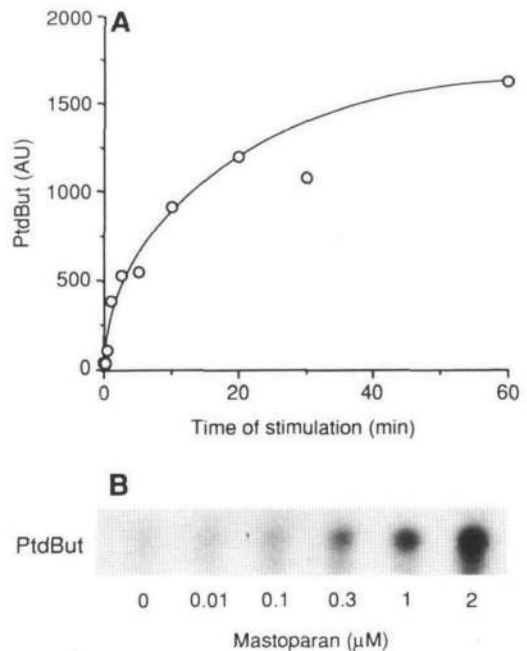


Figure 4. Mastoparan-Stimulated PtdBut Formation Is Time and Dose Dependent.

³²P-labeled *C. eugametos* cells were stimulated by mastoparan for the times and in the concentrations indicated, and their lipids were extracted, separated by TLC, and visualized by autoradiography.

(A) Time course of PtdBut formation in cells stimulated with 2 μM mastoparan in the presence of 0.1% *n*-ButOH. PtdBut formation was quantified by scanning the autoradiograph and is expressed in arbitrary units (AU) representing the density of each spot.

(B) Autoradiograph showing PtdBut formation after 3 min of stimulation with different concentrations of mastoparan in the presence of 0.1% *n*-ButOH. Only the relevant part of the autoradiograph is shown. Densitometric measurements of PtdBut were as follows (from left to right): 132, 267, 375, 676, 721, and 1299 AU. Similar results were obtained in two independent experiments.

in Figure 5. Using 800 μM PtdOH, 12% of the cells had also activated their mating structures (data not shown).

Activation of PLD by Alcohols

Although alcohols can be used as substrate for transphosphatidylation by PLD in *C. eugametos*, they are also known to activate heterotrimeric G proteins in animals (Hoek et al.,

ence (+) or absence (-) of 0.1 or 0.25% ButOH. Arrow indicates the position of a PtdBut standard.

(B) Cells were treated with H₂O (control) or mastoparan in the presence of 0.2% ButOH or 0.4% *n*-PropOH, or 1% EtOH, or 1.5% MeOH.

1992) and *C. eugametos* (Musgrave et al., 1992). This could mean that alcohols can stimulate their own phosphatidylation (Kiss and Anderson, 1989), which was tested by incubating ^{32}P -prelabeled cells separately with ethanol and *n*-butanol at different concentrations and for different time periods. Subsequently, lipids were extracted and separated by thin layer chromatography (TLC), and their radioactivity was quantified by liquid scintillation counting. Figure 6A shows that the production of PtdOH and PtdBut increased when cells were treated with higher concentrations of ButOH. Maximum PtdBut formation was reached with $\sim 0.75\%$ ButOH in the medium. Using that concentration, the formation of PtdBut was linear for ~ 1 min and continued for ~ 6 min (Figure 6B). EtOH also stimulated the formation of PtdOH (data not shown; Musgrave et al., 1992), and with increasing concentrations, more PtdEt was formed (Figure 6C). Note that higher concentrations of EtOH than ButOH were needed to obtain a particular effect. This difference in potency was found to be dependent on the chain length of the alcohol (MeOH < EtOH < *n*-PropOH < ButOH < hexanol; results not shown) and is consistent with the known higher efficacy of longer chain alcohols to activate G proteins (Kiss and Anderson, 1989; Hoek et al., 1992). Note also that the PtdBut spot is composed of two overlapping spots. This is probably due to PLD using two different phospholipids with different fatty acid compositions to phosphatidylate ButOH. The two PtdBut species are then partially separated based on their fatty acid content.

The fact that the production of phosphatidylalcohol increases with higher concentrations of alcohol does not necessarily mean that the alcohol stimulates PLD activity; it may simply

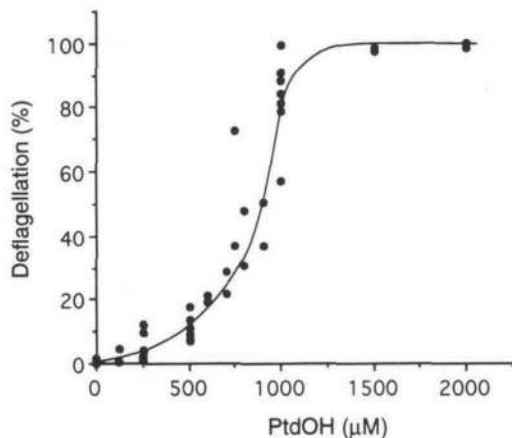


Figure 5. PtdOH Induces Deflagellation in *C. eugametos*.

Aliquots of cells were incubated with different concentrations of PtdOH for 30 sec. Samples were fixed, and the percentage of cells that had lost both flagella was quantified. Each point in the figure represents the score of at least 125 cells. Data from three independent experiments are included (SE < 5%).

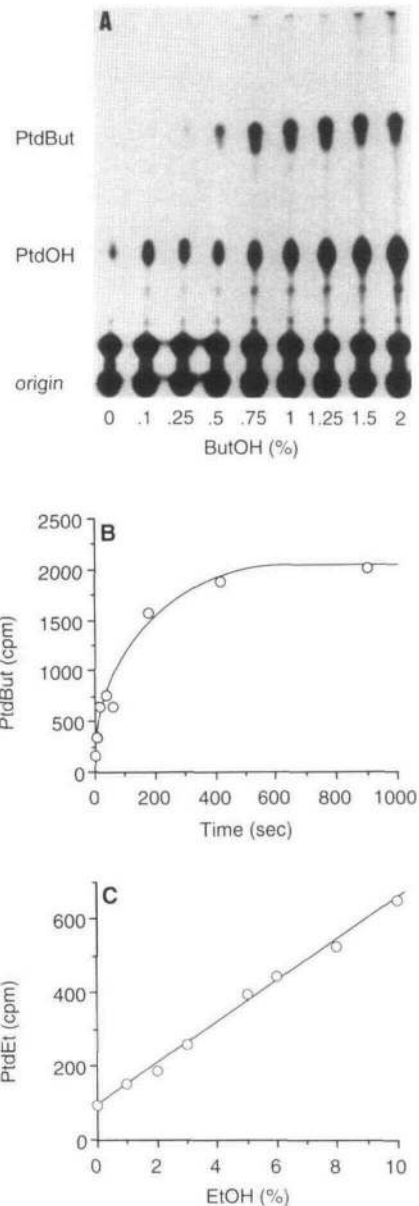


Figure 6. Alcohol-Dependent Phosphatidylalcohol Formation in *C. eugametos*.

Cells were prelabeled with phosphorus-32 and incubated in concentrations of ButOH or EtOH for the times indicated. Lipids were then extracted, separated by TLC, and visualized by autoradiography. Individual phospholipid spots were scraped from the TLC plate and quantified by liquid scintillation counting.

(A) Autoradiograph of a TLC plate showing PtdBut formation in cells stimulated for 5 min with different concentrations of *n*-ButOH. The positions of PtdBut and PtdOH are indicated.

(B) Time course of PtdBut formation in cells incubated in 0.75% *n*-ButOH.

(C) PtdEt formation after a 5-min incubation with different concentrations of EtOH. The radioactivity at 0% EtOH represents background.

reflect an increase in substrate concentration. We therefore performed an experiment in which two alcohols were used: one as a reporter and a second as an activator. The reporter concentration was kept constant to monitor PLD activity, whereas that of the activator alcohol was varied to study its ability to activate PLD. The results are summarized in Figure 7. Using *n*-ButOH as the reporter and increasing amounts of EtOH as the activator, it was clear that low concentrations of EtOH activated PLD and thus stimulated the formation of PtdBut (Figure 7A). However, EtOH concentrations above 3% were less effective. This was to be expected because EtOH competes with *n*-ButOH as a transphosphatidyl substrate, in agreement with the linear increase in PtdEt with increasing EtOH concentration, as shown in Figure 7A.

To exclude any artifacts due to differences in the diffusion rates of the two alcohols or PLD's preference for one of the alcohols as substrate or activator, the reporter and the activator alcohols were reversed. As evident from Figure 7B, ButOH also activated PLD, with a resulting increase in PtdEt formation. Together, these results indicate that alcohols can also activate PLD.

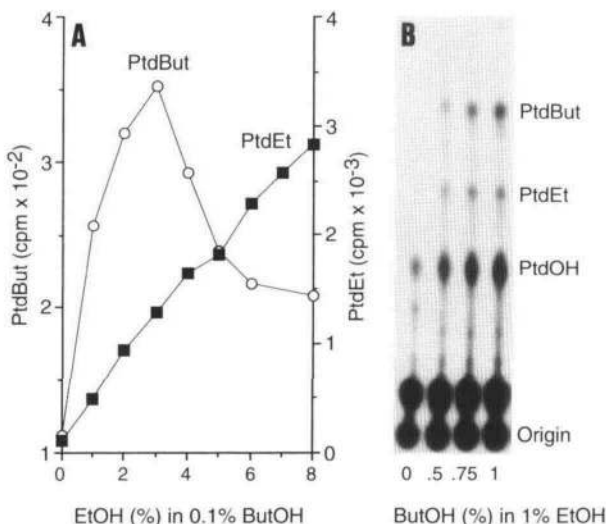


Figure 7. Phosphatidylalcohol Formation in *C. eugametos* Is Activated by Alcohols.

Cells were prelabeled with phosphorus-32 and stimulated with different concentrations of EtOH in the presence of ButOH or vice versa. Reactions were stopped after 5 min, and lipids were extracted, separated by TLC, and visualized by autoradiography. Regions corresponding to the radioactive spots were scraped from the TLC plate and quantified by liquid scintillation counting.

(A) Radioactivity present as PtdBut and PtdEt in cells treated with different concentrations of EtOH in the presence of 0.1% *n*-ButOH.

(B) Autoradiograph of a TLC plate showing lipids extracted from cells treated with different concentrations of *n*-ButOH in the presence of 1% EtOH.

Alcohols as Activators and Substrates of PLD

Alcohols are substrates for measuring the transphosphatidyl activity of PLD, and they can also activate PLDs via G proteins. With the idea of separating these two properties, we tested the structural isomers of ButOH as activators and transphosphatidyl substrates of PLD in living *C. eugametos* cells. The activation was monitored by incubating ³²P-prelabeled cells in a low concentration of EtOH as a constant source of transphosphatidyl substrate. Thus, if a ButOH isomer activates PLD, the result is an increased level of PtdEt; if it is also a substrate, PtdBut is formed as well. As shown in Figure 8A, the primary alcohols *n*-ButOH and isobutyl alcohol were both able to activate PtdEt formation and function as a substrate, giving rise to PtdBut. Note the slight difference in migration on TLC due to the different ButOH head groups on the phosphatidyl moiety. The secondary alcohol, *sec*-butyl alcohol, was also able to activate PLD, as judged by the elevated PtdEt formation compared with the control level, but clearly was itself unable to act as a transphosphatidyl substrate. Incubations in higher concentrations still did not give rise to PtdBut formation, whereas PtdEt formation was stimulated in a dose-dependent manner (Figure 8B). Thus, *sec*-butyl alcohol has the attractive property of being able to activate PLD without being a potential substrate. *Tert*-butyl alcohol neither activated PLD nor gave rise to a PtdBut species, and even in the presence of activating concentrations of EtOH, no PtdBut was formed (data not shown). Table 1 summarizes the properties of primary, secondary, and tertiary alcohols with regard to G protein-regulated PLD in *C. eugametos* and mammalian cell lines (Heller, 1978; Kiss and Anderson, 1989; Hoek et al., 1992).

PLD Activation by Other G Protein-Modulating Compounds

Activation of PLD by mastoparan and alcohols supported the idea that heterotrimeric G proteins are naturally involved and that it was therefore of interest to study the influence of other G protein modulators in vivo. Cholera toxin is known to activate G proteins by ADP ribosylation of the G_α subunit at its intrinsic GTPase site. This prevents hydrolysis of GTP and maintains the G protein in its active GTP-bound form. Pertussis toxin ADP ribosylates at a conserved cysteine at the C terminus of G_α, thereby preventing reassociation with the βγ subunit. As a result, the G protein remains in its inactive GDP-bound form. Whereas the toxins can enter the cell, they are only active on those classes of G proteins that contain these particular ADP-ribosylating sequences (Moss et al., 1983; Gilman, 1987; Birnbaumer et al., 1990; Hepler and Gilman, 1992; Carty, 1994; Moss et al., 1994).

As shown in Figure 9, treatment of cells with cholera toxin for 10 min activated PLD in a dose-dependent manner (top). In contrast, pretreatment of cells with pertussis toxin (up to

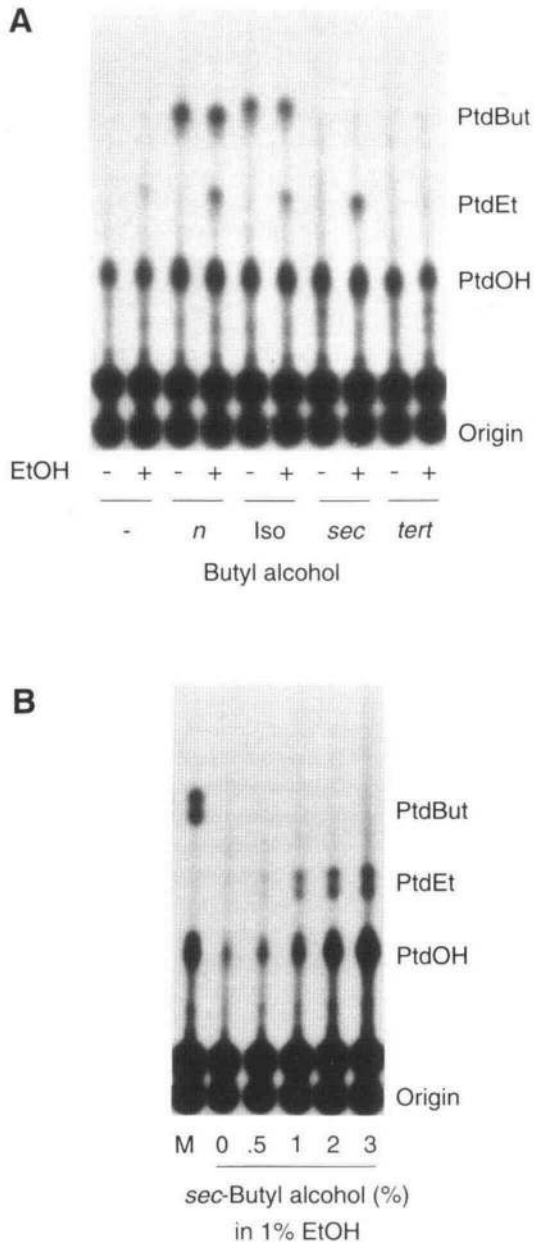


Figure 8. Ability of Different ButOH Isomers To Stimulate PLD and Function as a Transphosphatidylation Substrate.

C. eugametos cells labeled with phosphorus-32 were incubated for 5 min with different ButOH isomers in the absence or presence of 1% EtOH. Lipids were then extracted, separated by ethyl acetate TLC, and visualized by autoradiography.

(A) TLC autoradiograph showing lipids from cells treated with different ButOH isomers (0.75%) in the presence (+) or absence (-) of 1% EtOH for 5 min.

(B) TLC autoradiograph showing lipids from cells treated with different concentrations of sec-butyl alcohol in the presence of 1% EtOH. M is a lipid extract of cells incubated in 0.75% *n*-ButOH for 15 min to mark the position of PtdBut.

Table 1. Structural Relationship of Different ButOH Isomers and Their Ability To Stimulate PLD and Function as a Transphosphatidylation Substrate

Alcohol		PLD	
Name	Type	Transphosphatidylation Substrate	Activation
<i>n</i> -Butyl alcohol	Primary	+	+
Isobutyl alcohol	Primary	+	+
sec-Butyl alcohol	Secondary	-	+
tert-Butyl alcohol	Tertiary	-	-

2 hr) was not able to block the mastoparan-activated PtdBut formation (bottom).

Effect of Mastoparan on PLD Activity in Carnation Flower Petals

To test whether the results from a single-celled alga were of general relevance to higher plants, we tested whether PLD activity could be measured in carnation petals and whether it was elevated by mastoparan. The use of a reporter alcohol as a transphosphatidylation substrate to measure PLD activity was just as successful as in *C. eugametos*, and, as shown in Table 2, the formation of PtdOH and PtdBut was stimulated by mastoparan in a dose-dependent manner. Similar results have recently been found for oilseed rape microspores (T. Munnik and M.S.A. Parzer, unpublished results), indicating that the PLD assay can be applied generally and that many if not all the higher plants contain a G protein-regulated form of PLD.

DISCUSSION

PLD Activity Assay

The activation of PLD and the subsequent increase in concentration of the second messenger PtdOH have emerged as an important new signal transduction mechanism in animals (Billah and Anthes, 1990; Exton, 1990, 1994; Exton et al., 1991; Liscovitch, 1992; Billah, 1993; Boarder, 1994; Liscovitch and Cantley, 1994; Roberts, 1994; van Blitterswijk et al., 1994; Divecha and Irvine, 1995; Nishizuka, 1995). Animal PLD activity has been measured both in vivo and in vitro, based on its ability to use primary alcohols, such as *n*-ButOH, as transphosphatidylation substrate, resulting in the formation of PtdBut. Such activity has been shown to be a unique property of PLD (Kanfer, 1980; Bocckino et al., 1987; Gustavson and Alling, 1987; Kobayashi and Kanfer, 1987; Pai et al., 1988; Liscovitch, 1989; Moehren et al., 1994), so the formation of PtdBut can be taken as unequivocal evidence for its presence.

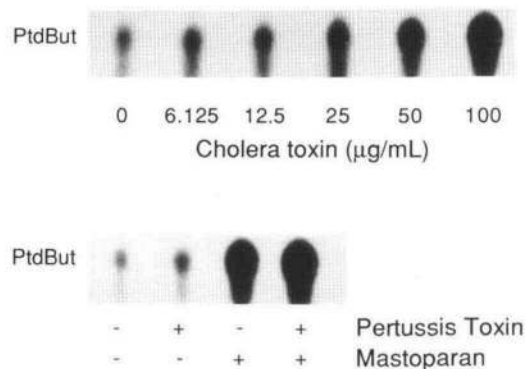


Figure 9. Effect of Cholera Toxin and Pertussis Toxin on PLD Activity in *C. eugametos*.

Cells were prelabeled with phosphorus-32 and treated with cholera toxin or first pretreated with pertussis toxin and then stimulated with mastoparan or H₂O (control). Lipids were extracted, separated by TLC, and visualized by autoradiography.

(**Top**) Autoradiograph showing PtdBut formation after 10-min stimulation with different concentrations of cholera toxin in the presence of 0.2% *n*-ButOH.

(**Bottom**) Labeled cells were pretreated with pertussis toxin (10 µg/mL) for 2 hr and then stimulated with 1 µM mastoparan or H₂O (control) for 10 min in the presence of 0.2% *n*-ButOH. Each (+) and (–) represents treatment with or without mastoparan or pertussis toxin.

Transphosphatidyl activity was first demonstrated for a plant PLD (Dawson, 1967; Yang et al., 1967), and this property was reconfirmed with the recent cloning and characterization of a PLD gene from castor bean (Wang et al., 1994). After expression in *Escherichia coli*, extracts were shown to hydrolyze phosphatidylcholine and to transphosphatidylate ethanol in vitro, whereas control extracts were devoid of such activity.

In this article, we have illustrated how the same assay can be used to measure in vivo PLD activity in plants and demonstrated that, as in animals, transphosphatidyl activity is specific for primary as opposed to secondary and tertiary alcohols (Table 1; Heller, 1978). Although most of our experiments were performed with the unicellular alga *C. eugametos*, the assay worked just as well with higher plant tissue. A successful assay is dependent on the initial incorporation of phosphorus-32 (or other tracer) into lipids, such as phosphatidylcholine and phosphatidylethanolamine, which are preferred substrates for PLD (Heller, 1978; Wang, 1993), and the rapid penetration of ButOH (0.1 to 0.2%) into the cells in question. Under these conditions, the phospholipid PtdBut is formed; it can be easily extracted and separated from all common native phospholipids by TLC and thereafter quantitated. Because ButOH competes with water, the physiological substrate for phosphatidyl, PtdBut formation only provides a relative rather than an absolute measure of PLD activity.

Activation of PLD

PLD activity was first characterized in plants (Hanahan and Chaikoff, 1947); however, its role in intracellular signaling has only recently been established in animal cells, where it is involved in the regulation of membrane trafficking (Brown et al., 1993; Kahn et al., 1993; Cockcroft et al., 1994; Liscovitch et al., 1994; Boman and Kahn, 1995; Liscovitch and Cantly, 1995), the respiratory burst in neutrophils (Bauldry et al., 1992; Cockcroft, 1992; Qualliotine-Mann et al., 1993), actin polymerization (Ha and Exton, 1993; Ha et al., 1994), and mitogenesis (reviewed in Boarder, 1994). PLD is frequently activated together with or downstream from PLC (Liscovitch, 1992). Both tyrosine kinase receptors and G protein-coupled receptors have been shown to activate PLC and PLD (Billah and Anthes, 1990; Exton, 1990, 1994; Exton et al., 1991; Xie and Dubyak, 1991; Bourgoin and Grinstein, 1992; Cano et al., 1992; Cockcroft, 1992; Geny and Cockcroft, 1992; Uings et al., 1992; Jiang et al., 1994).

The G protein-activated signaling cascades can be artificially activated by alcohols and mastoparan (see reviews in Hoek et al., 1992; Law and Northrop, 1994; Ross and Higashijima, 1994). With the recent demonstrations that these artificial stimulators also activate PLC in plants (Musgrave et al., 1992; Quarby et al., 1992; Legendre et al., 1993; Drøbak and Watkins, 1994; Cho et al., 1995), we tested their ability to activate PLD. Both were very effective, and PtdBut formation in *C. eugametos* increased approximately fivefold, whereas a similar increase in carnation petals was found after treatment with mastoparan. Even though we do not yet know what the natural receptors are, these results provide suggestive evidence for PLD signaling in plants and its control directly or indirectly via G protein-coupled receptors. In *C. eugametos*, the G protein is cholera toxin sensitive and pertussis toxin insensitive. We have not tested the effects of these compounds on carnation petals.

In plants, G protein-coupled signaling has been invoked in the activation of PLC and the elicitation of the oxidative burst

Table 2. Mastoparan Stimulates PLD Activity in Carnation Flower Petals^a

Mastoparan (µM)	PtdBut	
	% of Total PL	Stimulation
0	0.28 ± 0.07 (n=3)	1.0
5	0.50 ± 0.04 (n=2)	1.8
25	1.45 ± 0.45 (n=3)	5.2

^a Petal discs prelabeled with ³H-palmitic acid were incubated for 60 min with or without mastoparan in the presence of 0.5% *n*-ButOH. Lipids were extracted and separated on TLC as described in Methods. Lipids were scraped from the TLC and quantified by liquid scintillation counting. The radioactivity as PtdBut and PtdOH is expressed as a percentage of that in the total phospholipid (PL) pool.

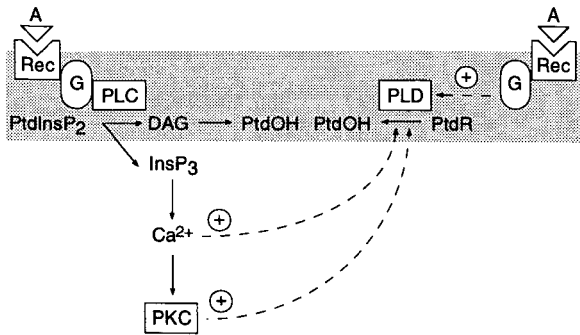
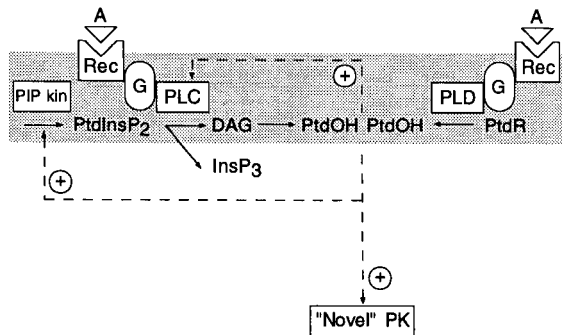
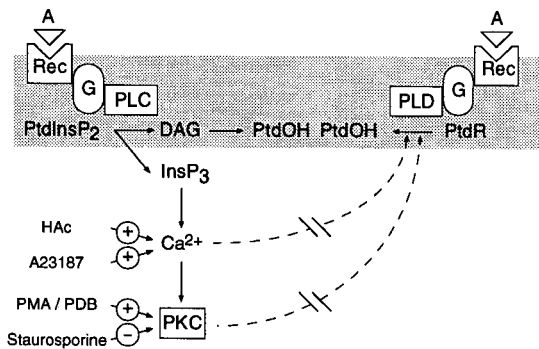
A Mechanisms of PLD activation in mammalian cells**B Targets of PtdOH****C Regulation of *C. eugametos* PLD**

Figure 10. Models of PtdOH Generation by G Protein-Activated PLC and PLD (see Discussion).

(A) Mechanisms by which PLD is activated in mammalian cells: (1) activation directly through a G protein or downstream from PLC, (2) through Ca^{2+} , or (3) through PKC.

(B) Targets of PtdOH. PtdOH has been reported to stimulate a novel type of protein kinase activity and to amplify the PLC signaling cascade by activating PtdInsP kinase and PLC.

(C) Regulation of *C. eugametos* PLD. The stimulation of PLD by G protein activators can occur by the three mechanisms depicted in (A). However, increasing the intracellular Ca^{2+} concentration by treating

in soybean cells (Bolwell et al., 1991; Legendre et al., 1992, 1993), in coupling activated phytochrome to light-induced responses (Romero et al., 1991; Neuhaus et al., 1993; Bowler et al., 1994; Elich and Chory, 1994), as well as in deflagellation and mating structure activation in the green alga *Chlamydomonas* spp that was used in our study (Schuring et al., 1990; Musgrave et al., 1992; Quarmby et al., 1992; Yueh and Crain, 1993). Clearly, it would be interesting to know whether PLD activation plays a role in any of these processes. As yet, it only has the potential to play a role in deflagellation and mating structure activation in *Chlamydomonas* spp because G protein activators stimulated both processes as well as induced the production of PtdOH within seconds. In support, PtdOH alone also induced deflagellation and mating structure activation when added to gametes, suggesting that it could be the downstream effector (Figure 10A). However, we must add that flagellar excision is a Ca^{2+} -controlled mechanism that is complete within 5 sec of adding mastoparan (Musgrave et al., 1992; Quarmby et al., 1992; Yueh and Crain, 1993; Sanders and Salisbury, 1994). It is thought to be induced by G protein-activated PLC activity producing InsP_3 and releasing intracellular Ca^{2+} (Musgrave et al., 1992; Quarmby et al., 1992; Quarmby and Hartzell, 1994), but because PtdOH is known to enhance PLC signaling in animals (see below; Figure 10B), it may operate in a similar manner in *Chlamydomonas* spp.

Activation of PLC and PLD

PLD activity generates PtdOH and indirectly DAG, due to PtdOH-phosphatase activity. Conversely, PLC activity generates DAG and indirectly PtdOH due to DAG kinase activity. This suggests an interrelationship between the two signaling routes and, because they can supplement each other, may explain why many animal hormones and growth factors activate both pathways (Liscovitch, 1992; van Blitterswijk et al., 1994; Nishizuka, 1995). The G protein activation of PLD reported here for *C. eugametos* should also be seen as complementing the G protein-activated PLC in *Chlamydomonas* spp (Musgrave et al., 1992; Quarmby et al., 1992; Yueh and Crain, 1993; Quarmby and Hartzell, 1994).

Dual activation in itself emphasizes the potential importance of the common products in both signaling routes, namely, DAG and PtdOH. Although DAG is established in animals as a second messenger that activates PKC, this is not the case in plants; as yet, no one has cloned a plant PKC or provided a convincing

cells with A23187 (up to 50 μM) or HAc (up to 50 mM) did not activate PLD. In addition, the PKC stimulators phorbol 12-myristate 13-acetate and phorbol 12,13-dibutyrate (0.1 to 100 μM) did not activate PLD, and the activation by mastoparan could not be blocked by pretreatment with the PKC inhibitor staurosporine (up to 1000 nM; 2 hr).

Activation is indicated by (+) and inhibition by (-). A, agonist; G, G protein; PDB, phorbol 12,13-dibutyrate; PIP kin, PtdInsP kinase; PK, protein kinase; PMA, phorbol 12-myristate 13-acetate; PtdR, structural phospholipid; Rec, receptor.

biochemical characterization of a classic PKC that is activated by Ca^{2+} and DAG. Although the lack of conviction about DAG signaling in plants is not an argument for the importance of PtdOH, its acceptance as a signal in animal cells, together with our demonstration of its biological activity in *C. eugametos*, means that it should now receive more attention from plant cell biologists. In animal cells, no consensus picture has yet formed about how PtdOH operates, but a number of recent reports describe a novel type of protein kinase that is specifically activated by this lipid (Figure 10B; Bocckino et al., 1991; Epand et al., 1992; Nakanishi and Exton, 1992; Stasek et al., 1993; Khan et al., 1994; Limatola et al., 1994). Another potential site of action appears to be the PLC signaling cascade itself, because PtdOH has been found to activate PtdInsP 5-kinase and PLC specifically (Jackowski and Rock, 1989; Kroll et al., 1989; Moritz et al., 1989; Hashizume et al., 1992; Jacob et al., 1993; Jones and Carpenter, 1993), in this way amplifying the PLC signaling cascade (Figure 10B).

The interrelations between PLC and PLD signaling can be complex, for in animal cells, PLC activation often precedes PLD, and for some systems, PLD has been shown to lie downstream from PLC (Liscovitch, 1992; Gustavson et al., 1994; van Blitterswijk et al., 1994). In those cases, PLD activation is dependent on the increase in intracellular Ca^{2+} via InsP_3 or the activation of PKC through DAG and Ca^{2+} , as illustrated in Figure 10A. Because mastoparan (Quarmby et al., 1992; Yueh and Crain, 1993; Quarmby and Hartzell, 1994) and EtOH (Musgrave et al., 1992) have been shown to activate PLC in *Chlamydomonas* spp, it was possible that the observed PLD activation was due to an upstream activation of PLC. However, all experiments we performed to test the possible involvement of PKC and Ca^{2+} gave negative results. These are summarized in Figure 10C and include treatment with the ionophore A23187 and acetic acid, which is known to increase the influx of Ca^{2+} into *Chlamydomonas* spp and to activate PLC (Schuring et al., 1990; Quarmby et al., 1992; Yueh and Crain, 1993; Quarmby and Hartzell, 1994), the PKC stimulators phorbol 12-myristate 13-acetate and phorbol 12,13-dibutyrate, and the PKC inhibitor staurosporine that was tested in combination with mastoparan. All of these compounds were without effect on PtdBut formation. Therefore, in the absence of clear evidence for the involvement of Ca^{2+} and PKC, we suggest that PLD activation does not lie downstream from PLC in *Chlamydomonas* spp but is directly activated via a G protein, as depicted in Figure 10.

Alcohols as Activators and Substrates for PLD

In this report, we analyzed the capacity of all ButOH isomers to act as transphosphatidylation substrates for PLD and at the same time to activate PLD via G proteins. The results were clear and illuminating. For example, high concentrations of primary alcohols (e.g., 1% *n*-ButOH) are convenient for screening for PLD activity because they act as both substrate and activator. However, *n*-ButOH also has a drawback, namely, that

PLD consumes it as it activates. *sec*-ButOH is then the alcohol of choice because it has only activating activity. The level of activation can then be measured by using a low concentration of reporter alcohol. *tert*-ButOH is without any effect and thus may serve as a control in comparison with *n*-ButOH for determining whether a process is G protein activated or not.

METHODS

Materials

Mastoparan from *Vesputa lewisii* was purchased from Sigma. Reagents for lipid extraction and subsequent analyses, as well as Silica 60 thin layer chromatography (TLC) plates (0.25 × 200 × 200 mm), were from Merck (Darmstadt, Germany). ^{32}P -orthophosphate (carrier free) and ^3H -palmitic acid were obtained from Amersham International (*s*-Hertogenbosch, The Netherlands). Pertussis toxin and cholera toxin were from List Laboratories (Campbell, CA). Standards of phosphatidylethanol (PtdEt) and phosphatidylbutanol (PtdBut) were generously provided by W.J. van Blitterswijk (Dutch Cancer Institute, Amsterdam, The Netherlands).

Chlamydomonas Cell Culture, ^{32}P -Phospholipid Labeling, and Phospholipase D Assay

The unicellular, biflagellate green alga *Chlamydomonas eugametos* (strain 17.17.2) was cultivated on agar-containing M1 medium in Petri dishes, as described by Schuring et al. (1987). Cultures were maintained at 20°C in a 12-hr-light/12-hr-dark regime with an average photon flux of 30 $\mu\text{E m}^{-2} \text{sec}^{-1}$ provided by Philips (Eindhoven, The Netherlands) TL 65W/33 fluorescent tubes.

Swimming gamete suspensions were obtained by flooding 3- to 4-week-old plate cultures with 20 mL of 10 mM Hepes, 1 mM MgCl_2 , 1 mM CaCl_2 , 1 mM KCl, pH 7.4 (HMCK). After 2 to 16 hr, cells were harvested, washed twice with HMCK by centrifugation for 5 min at 500g, and resuspended at a final concentration of 1 to 2 × 10⁷ cells per mL in HMCK.

Cellular phospholipids were metabolically labeled by incubating cells (1 to 2 × 10⁷ cells per mL) with 100 μCi phosphorus-32 (carrier free) per mL in HMCK for 2 to 5 hr. ^{32}P -prelabeled cells were incubated with mastoparan, alcohols, or other compounds in a total volume of 100 μL at the concentrations and for the times indicated. For the assays of phospholipase D (PLD)-stimulated ^{32}P -phosphatidylalcohol accumulation, 80 μL of ^{32}P -labeled cells in HMCK were added to 20 μL of a solution containing the test compound and the reporter alcohol.

Reactions were quenched by adding 900 μL of ice-cold $\text{CHCl}_3/\text{MeOH}/\text{HCl}$ (100:100:1 [v/v]), and lipids were extracted, separated, and quantified as described below.

Extraction and Analysis of *C. eugametos* Lipids

Lipids were extracted as described previously (Munnik et al., 1994a), dried, dissolved in 20 μL of CHCl_3 , and separated on TLC plates that had been heat activated for at least 1 hr at 115°C. Two different TLC solvent systems were used. For the separation of the different phospholipid classes and the polyphosphoinositides, lipids were chromatographed

on impregnated (1% K-oxalate, 2 mM EDTA in MeOH/ H₂O [2:3 v/v]) TLC plates using 80 mL of an alkaline solvent system (CHCl₃/MeOH/25% NH₄OH/H₂O [45:35:2:8 v/v]) as described previously (Munnik et al., 1994a). For the separation of PtdBut (and other phosphatidylalcohols, such as PtdEt) from phosphatidic acid (PtdOH) and the rest of the phospholipids, lipids were chromatographed in an ethyl acetate solvent system that was the organic upper phase of ethyl acetate/*iso*-octane/HAc/H₂O (13:2:3:10 [v/v]) as described by Liscovitch and Amsterdam (1989).

Radiolabeled phospholipids were detected by autoradiography. Individual spots were scraped from the TLC plate and quantified by liquid scintillation counting. Alternatively, spots were quantified by scanning the autoradiograph with an XRS Omnimedia scanner (Torrance, CA), and the density of each spot was expressed in arbitrary units. Unlabeled phospholipid standards (~10 µg) were visualized by exposure to iodine vapor.

Deflagellation of *C. eugametos* by PtdOH

A stock solution of 2.5 mM PtdOH (1,2-dilauroylglycerol-*sn*-3-phosphatidic acid) was kept in CHCl₃ under nitrogen gas at -20°C. An aqueous solution of PtdOH was prepared daily by evaporating the CHCl₃ under a stream of nitrogen and dispersing the lipid into water by sonication for 1 min in a sonicator bath (no. 2200; Branson Ultrasonics, Danbury, CT) to give a final concentration of 10 mM PtdOH. Routinely, 90 µL cells (1 to 2 × 10⁷ cells per mL) were treated with 10 µL PtdOH of different concentrations for 30 sec and then fixed by adding an equal volume of 1.5% formaldehyde, 0.5% glutaraldehyde. Cells were examined for the loss of their flagella by phase-contrast microscopy. At least 125 cells were scored for each sample, and only cells that had lost both flagella were scored as deflagellated.

Measurement of in Vivo PLD Activity in Carnation Petals

Petal discs of *Dianthus caryophyllus* were prepared as described previously (Munnik et al., 1994b) and labeled with 3.5 µCi ³H-palmitic acid in 250 µL of 25 mM Mes buffer, pH 5.5, in 0.05% Tween 20 by vacuum infiltration. After 5 min, the vacuum was released and 250 µL of the same buffer containing 1% *n*-butyl alcohol (*n*-ButOH) and mastoparan was added. Incubations were stopped after 60 min and lipids extracted as described previously (Munnik et al., 1994b).

Each sample was mixed with a nonradioactive mixture of PtdBut, phosphatidylcholine, and PtdOH (~10 µg each), applied to the TLC plate, and developed using three solvents consecutively, with intermediate drying. First, CHCl₃ was used to remove the free ³H-palmitic acid. Second, acetone removed the ³H-labeled glycolipids, and third, the upper phase of a mixture of ethyl acetate/*iso*-octane/HAc/H₂O (13:2:3:10 [v/v]; Liscovitch and Amsterdam, 1989) was used to separate PtdBut and PtdOH from the rest of the phospholipids. Lipid standards were visualized by iodine vapor, and corresponding regions were scraped from the TLC plate and their radioactivity quantified by liquid scintillation counting.

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