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# Inhibition of phospholipase D by lysophosphatidylethanolamine, a lipid-derived senescence retardant

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**ABSTRACT** **Phospholipid signaling mediated by lipid-derived second messengers or biologically active lipids is still new and is not well established in plants. We recently have found that lysophosphatidylethanolamine (LPE), a naturally occurring lipid, retards senescence of leaves, flowers, and postharvest fruits. Phospholipase D (PLD) has been suggested as a key enzyme in mediating the degradation of membrane phospholipids during the early stages of plant senescence. Here we report that LPE inhibited the activity of partially purified cabbage PLD in a cell-free system in a highly specific manner. Inhibition of PLD by LPE was dose-dependent and increased with the length and unsaturation of the LPE acyl chain whereas individual molecular components of LPE such as ethanolamine and free fatty acid had no effect on PLD activity. Enzyme-kinetic analysis suggested noncompetitive inhibition of PLD by LPE. In comparison, the related lysophospholipids such as lysophosphatidylcholine, lysophosphatidylglycerol, and lysophosphotidylserine had no significant effect on PLD activity whereas PLD was stimulated by lysophosphatidic acid and inhibited by lysophosphatidylinositol. Membrane-associated and soluble PLD, extracted from cabbage and castor bean leaf tissues, also was inhibited by LPE. Consistent with acyl-specific inhibition of PLD by LPE, senescence of cranberry fruits as measured by ethylene production was more effectively inhibited according to the increasing acyl chain length and unsaturation of LPE. There are no known specific inhibitors of PLD in plants and animals. We demonstrate specific inhibitory regulation of PLD by a lysophospholipid.**

Lipids previously were thought to play important roles only in membrane structure and energy reserves. It is now evident that lipids and their metabolites have many other critical cellular functions particularly as mediators in signal transduction, cell activation, and cell proliferation (1, 2). Studies in animal systems have demonstrated that phospholipases, a group of phospholipid-hydrolyzing enzymes, are key enzymes capable of generating lipids that can act as second messengers in signal transduction (2–4).

We recently have found that lysophosphatidylethanolamine (LPE), a hydrolysis product of PE by phospholipase $A_2$, is able to retard plant senescence. LPE-treated attached and detached leaves had a higher chlorophyll content and a lower rate of both respiration and ethylene production than the controls (5). Tomato fruits at the red stage of physiological maturity were detached with their pedicles and dipped in LPE (50 mg/L) solution. After 5 days of treatment, LPE-treated fruits had a much lower production of ethylene as well as lower electrolyte leakage than the controls (5). In support of this, the vase-life of LPE-treated cut flowers was prolonged to 7 days compared with 2 days in the control (6). LPE-treated flowers also had lower ion leakage and lower ethylene production. When LPE was sprayed on tomato leaves together with ethephon, an ethylene-releasing compound, LPE mitigated the defoliating action of ethephon (7). These results taken together suggest that LPE may be a biologically active lipid that regulates a certain key process during plant senescence and aging.

Increased leakage of electrolytes during plant senescence has been ascribed to the breakdown of membrane phospholipids (8, 9). Reduced leakage of electrolytes in LPE-treated leaves, flowers, and postharvest fruits suggests that LPE may protect membrane integrity by inhibiting membrane lipid degradation (5). Based on the kinetics of release of various lipolytic products *in vivo* and *in vitro*, phospholipase D (PLD) has been proposed to mediate the selective degradation of membrane phospholipids, which is a rapid and early event occurring in senescing tissues (10–15). An increase in PLD expression was observed in senescing leaf tissues, and the expression of PLD was characterized by complex modes including an increase in membrane-associated PLD, differential expression of PLD variants, and changes in amounts of PLD protein and mRNA (16).

There are no known specific inhibitors of PLD in plants and animals as stated by Cockcroft (4). Whereas PLD-activating mechanisms have been widely studied (17), the negative regulation of PLD is poorly understood. Several stimulators of PLD activity have been described, including protein kinase (18–20), small molecular weight G proteins including Arf, Rho, and Ras (18, 20, 21), and a 50-kDa cytosolic protein (22). This stimulatory effect on PLD activity has been reported to be inhibited by ceramide (23) and akylphosphate esters (24) depending on the nature of activation. Recently, some brain cytosolic proteins have been found to inhibit PLD activity (25–27). All of these studies were conducted using animal systems, and similar information on plant PLD is lacking. In the present study, we demonstrate that LPE, a lipid-derived senescence retardant, can inhibit the activity of partially purified PLD in a highly specific manner in plants.

## MATERIALS AND METHODS

**Chemicals and Plant Materials.** Natural LPE purified from egg yolk and synthetic LPE with different acyl chains (14:0, 16:0, 18:0, and 18:1) were obtained from Avanti Polar Lipids. All other phospholipids, chemicals, and materials were obtained from Sigma. Phospholipids and fatty acid were dissolved in chloroform/methanol/KOH (1 N) (95:5:1, vol/vol). After water was added, organic solvents were expelled by flowing nitrogen gas. Stock solution concentrations were adjusted to 1 mM with water before being added to the reaction mixture.

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Abbreviations: LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEOH, phosphatidylethanol; PLD, phospholipase D.

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Bulk preparation of LPE solution for dipping cranberry fruits was done by sonicating LPE powder suspended in water without predissolving in organic solvents.

Partially purified cabbage PLD, which commonly has been used for investigating the biochemical and physiological aspects of PLD (28, 29), was dissolved in 50 mM Tris (pH 8.0) and added to a reaction mixture with a final concentration of 0.6 $\mu$g/ml to examine the effect of LPE on PLD activity.

In addition to the partially purified cabbage PLD, we also investigated the effect of LPE on the activities of membrane-associated PLD and soluble PLD, which were obtained from two plant sources, i.e., cabbage (*Brassica oleracea* L. Blue Vintage) and castor bean (*Ricinus communis* L. cv Hale). Castor bean plants were grown in plastic pots containing a mixture of vermiculite and perlite (1:1, vol/vol), which were subirrigated at 22°C with Hoagland nutrient solution under cool-white fluorescent lights (150 $\mu$mol·min$^{-1}$·m$^{-2}$) with a 14-h photoperiod (16). Cabbage was obtained from fresh market.

**Tissue Fractionation.** Fully expanded leaves from 2-month-old castor bean plants and cabbage were harvested, quickly frozen in liquid nitrogen, and homogenized with a mortar and pestle chilled on ice (30). An extraction buffer containing 50 mM Tris·HCl (pH 8.0), 10 mM KCl, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 2 mM DTT was added to the powdered samples. After grinding for 5 min, the homogenate was centrifuged at 6,000 × *g* for 10 min to remove debris. The supernatant was centrifuged at 100,000 × *g* for 30 min to fractionize the extract into soluble and membrane-associated PLD. The resultant supernatant was collected as the soluble fraction and the pellet as the membrane fraction, which had been washed once with extract buffer to remove soluble contaminants. The soluble PLD and membrane-associated PLD samples were added to the reaction mixture at final concentrations of 100 $\mu$g/ml and 10 $\mu$g/ml, respectively.

**PLD Activity Assay.** The activity of partially purified cabbage PLD was assayed by measuring the phosphorus content contained in phosphatidylethanol (PEOH) and phosphatidic acid (PA) released from the substrate phosphatidylcholine (PC) (30). For this assay, 20 $\mu$mol of PC from egg in chloroform was dried under a stream of nitrogen gas. The lipid was emulsified in 1 ml of $H_2O$ by sonication at room temperature. A standard enzyme assay mixture contained 100 mM Mes/NaOH (pH 6.5), 50 mM $CaCl_2$, 0.5 mM SDS, 20 $\mu$l substrate (0.4 $\mu$mol), 1% ethanol, and 20 $\mu$l enzyme solution in a total volume of 200 $\mu$l (31). The assay mixture was then incubated at 30°C for 30 min in the water bath. The reaction was stopped by adding 750 $\mu$l of chloroform/methanol (1:2). Chloroform (200 $\mu$l) was added to the mixtures followed by 200 $\mu$l of KCl (2 M). After vortexing, the chloroform and aqueous phases were separated by centrifugation at 12,000 × *g* for 5 min. The chloroform phase was collected and dried. The dried samples were dissolved in 50 $\mu$l of chloroform before they were spotted onto a TLC plate (silica gel G). The plate was developed with the solvent of chloroform/methanol/$NH_4OH$ (65:35:5). Lipids on plates were visualized by exposure to iodine vapor. The spots corresponding to lipid standards PEOH, PA, and PC were scraped into vials, and the amounts were quantitated by measuring phosphorus content as described in Rouser *et al.* (32). PEOH, the product of transphosphatidylation reaction, was used as an indicator of PLD activity rather than PA, the product of hydrolytic reaction, because the former is not readily metabolized.

The PLD activities associated with membrane and soluble fractions obtained from cabbage and castor bean tissues were measured by quantifying the release of radiolabeled PEOH and PA from the substrate PC (16). For this purpose, 0.4 $\mu$Ci of L-3-phosphatidylcholine, 1,2-di[1-$C^{14}$]palmitoyl (Amersham) was mixed with 20 $\mu$mol of PC from egg in chloroform. The assay condition and reaction product separation were the same as described above. Radioactivity in PEOH, PA, and PC scraped from the TLC was quantitated by scintillation spectroscopy.

**LPE Treatment and Fruit Ethylene Production.** Postharvest treatment of fruit tissues with LPE (egg yolk) has been found to retard senescence and enhance shelf life of fruits (5, 7). However, the impact of different acyl chains of LPE on fruit senescence has not been investigated. In the present study, complementary to the effect of different acyl chains of LPE on PLD activity, we investigated the effect of different acyl chains of LPE on ethylene production of cranberry fruits. Fully ripened cranberry fruits (*Vaccinium macrocarpon* Ait. "Stevens") were harvested during the fall season and kept in a cold room. Randomly selected postharvest cranberry fruits (15 berries per sample) were dipped into LPE solutions with the different acyl chains (100 $\mu$M) for 30 min, then air-dried and left at room temperature (26 ± 2°C). After 2 days, berries were incubated in a sealed glass jar for 24 h to measure ethylene production. Ethylene was quantified with a gas chromatograph equipped with a flame ionization detector (Shimadzu 9AM, Shimadzu) (5).

## RESULTS AND DISCUSSION

**Inhibition of PLD Activity by LPE.** We studied if LPE, a naturally occurring phospholipid, acts as a biologically active lipid mediator by inhibiting PLD activity *in vitro* in a specific manner. The inhibitory effects of LPE on partially purified cabbage PLD were assayed using PC as substrate. The PLD activity was inhibited by LPE with different acyl chains at the concentrations of 40 and 200 $\mu$M (Fig. 1). The extent of inhibition increased with the length and the unsaturation of acyl chains. LPE with an acyl chain of 18:1 was the most effective inhibitor among the tested species, and resultant PLD activity was 16% and 11% of the control at the LPE concentrations of 40 and 200 $\mu$M, respectively. On the other hand, LPE 14:0, which seldom is present in plant tissues, had very little effect. The effects of LPE with other acyl chains including 18:2 and 18:3 would be interesting to test, but these forms of LPE are not commercially available at the present time. A dramatic inhibition of PLD by LPE (18:1), as compared with other LPE molecules tested, suggests that a specific configuration of LPE is needed for this inhibitory effect.

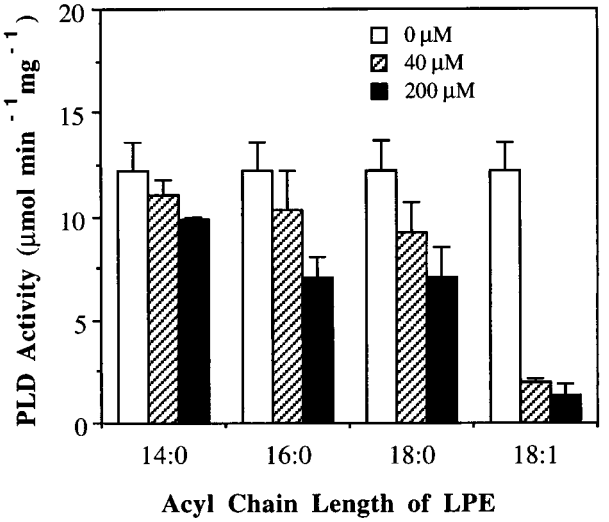


FIG. 1. Inhibition of partially purified cabbage PLD activity by LPE with different acyl chains. PLD activity was assayed by measuring the amounts of phosphatidylethanol, the product of transphosphatidylation reaction, with quantitation of phosphorus. Values are means ± SE of three separate measurements.

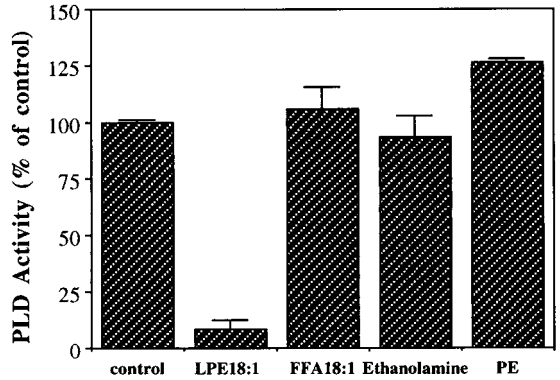


FIG. 2. Structural specificity of LPE (18:1) for its inhibition of partially purified cabbage PLD activity. Final concentrations of LPE (18:1), free fatty acid 18:1 (FFA18:1), ethanolamine, and PE were 100 mg/L in the reaction mixture. Values are means ± SE of three separate measurements.

**Structural Specificity of LPE.** The effect of different components of LPE molecules on PLD activity was tested to determine if any structural specificity was necessary for LPE inhibition. The head group (ethanolamine) and acyl chain (18:1 fatty acid) by themselves had no inhibitory effect on PLD activity (Fig. 2). These results indicate that only the intact LPE molecule is capable of inhibiting PLD, and a loss of any structural components results in complete ineffectiveness, thus indicating its structural specificity. On the other hand, PE had some stimulatory effect on PLD activity. In the presence of 200 $\mu$M PE, the PLD activity was 126% of the control (Fig. 2). Because PE is itself a preferential substrate of PLD (33), the increase in PLD activity could be explained by its direct stimulating effect on PLD and/or a preferential hydrolysis of PE by PLD.

**Dose Dependency and Kinetics of PLD Inhibition.** Inhibition of PLD by LPE was dose-dependent (Fig. 3). LPE (18:1) showed a dramatic inhibitory effect at the 10-$\mu$M concentration resulting in 50% activity of the control and a gradual increase of inhibition with increased concentrations up to 200 $\mu$M. LPE concentrations of 10 and 200 $\mu$M reflect 0.5 and 10 mol percentages of total phospholipids in reaction mixture, respectively. Cellular content of LPE has been found to be ≈2 mol % of total phospholipids in both the plasma membrane of potato leaves (34) and the microsomes of Zucchini seedlings (35). The inhibitory affect of PLD at an *in vivo* concentration

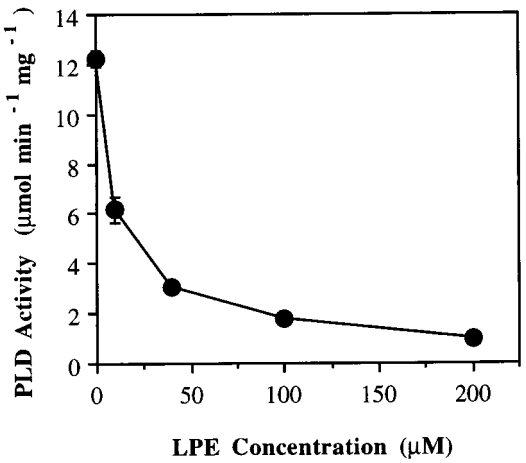


FIG. 3. Inhibition of partially purified cabbage PLD activity as a function of LPE concentration. Values are means ± SE of three separate experiments.

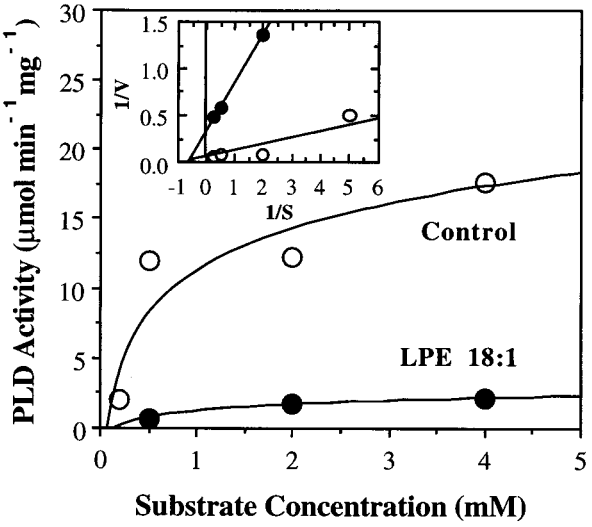


FIG. 4. Effect of substrate concentration on the inhibition of partially purified cabbage PLD by LPE (18:1). Final concentration of LPE in the reaction mixture was 200 $\mu$M. (*Inset*) Lineweaver–Burk plot suggesting noncompetitive inhibition of PLD by LPE. These experiments were repeated three times, and similar results were obtained each time. Data are from one experiment.

of LPE suggests that LPE could be a lipid mediator in cellular responses involving PLD.

To characterize the inhibition of PLD, effects of substrate concentration on PLD inhibition were analyzed in the presence and absence of LPE (Fig. 4). Normal assay conditions use the saturating concentration of substrate (2 mM PC). The inhibitory effect of LPE(18:1) was maintained even at the 4-mM substrate concentration (Fig. 4). The apparent $K_m$ for PLD was 1.7 mM and did not change in the presence of LPE. However, the presence of LPE(18:1) resulted in a dramatic decrease in $V_{max}$ (2.9 $\mu$mol·min$^{-1}$·mg$^{-1}$ protein) compared with the control ($V_{max}$ of 20.0 $\mu$mol·min$^{-1}$·mg$^{-1}$ protein). These results suggest noncompetitive inhibition of PLD by LPE. The $V_{max}$ and apparent $K_m$ of cabbage PLD were comparable to the values reported for castor bean PLD, a $K_m$ of 3.3 mM and a $V_{max}$ of 12.5 $\mu$mol·min$^{-1}$·mg$^{-1}$ protein (36).

***In Situ* Inhibition of PLD by LPE.** PLD is present not only in soluble form in cytosol but also in membrane-associated form, so we determined *in situ* inhibition of LPE on membrane-associated PLD extracted from cabbage and castor bean leaves. Specific activities of membrane-associated and soluble cabbage PLD were decreased to 59% and 51% of the control in the presence of LPE (18:1), respectively (Table 1). Membrane-associated and soluble castor bean PLD activities also decreased to 31% and 30% of the control, respectively. These results indicate that both membrane-associated and soluble PLD activities are inhibited by LPE. The inhibition of PLD associated with membrane and soluble fractions was, however,

Table 1. Inhibition of soluble and membrane-associated PLD activities (nmol·min$^{-1}$·mg$^{-1}$ protein) by LPE (18:1)

| | Soluble PLD | | Membrane-associated PLD | |
|---|---|---|---|---|
| | Cabbage | Castor bean | Cabbage | Castor bean |
| Control | 45.2 ± 3.5 | 10.2 ± 0.1 | 368.8 ± 6.5 | 153.8 ± 8.5 |
| LPE (18:1), 200 $\mu$M | 23.1 ± 1.6 | 3.1 ± 0.1 | 217.0 ± 13.0 | 47.0 ± 3.6 |
| Ratio, LPE/control | 0.51 | 0.30 | 0.59 | 0.31 |

Data are mean ± SE of two separate extractions (duplicate experiments from each extraction) prepared from cabbage and castor bean leaves.

less pronounced than the inhibition of partially purified cabbage PLD by LPE (Fig. 1 and Table 1). This is perhaps caused by the presence of some interfering factors or the other forms of PLD that are less sensitive to LPE. Partial purification of PLD, therefore, has been suggested as a critical step in the characterization of the regulatory mechanism of PLD (25). For this reason, in the present study, we have used partially purified cabbage PLD, which is commercially available. However, these observations of the inhibitory effect of LPE on membrane-associated and soluble PLD extracted from leaf tissues support the results obtained with partially purified PLD.

**Inhibition of Fruit Ethylene Production by LPE.** Previously, LPE (extracted from egg yolk) had been found to delay fruit senescence as indicated by lowered rates of ethylene production when compared with the control (5). Because we found that inhibitory effectiveness of LPE on PLD was dependent on the length and unsaturation of acyl chain of LPE (Fig. 1), the effects of LPE with different acyl chains on fruit senescence were tested. Cranberry fruits were treated with LPE with 14:0, 16:0, 18:0, and 18:1 chain lengths, and ethylene production by these fruits was monitored. The inhibition of ethylene production increased with acyl chain length and the unsaturation of LPE (Table 2). LPE (18:1) resulted in the most dramatic decrease (40%) in ethylene production 2 days after treatment. Of interest, this pattern of inhibition of ethylene production by various types of LPE was similar to the pattern of inhibition of PLD by various types of LPE (Fig. 1). These results indicate that inhibition of PLD activity and ethylene production is consistently dependent on the acyl chain length and the unsaturation of LPE. These results taken together suggest that LPE is a biologically active lipid mediator.

**Structurally Selective Regulation of PLD by Lysophospholipids.** To address whether the inhibition of PLD could occur by a wide range of lysophospholipids, the inhibitory effect of LPE on PLD was compared with related lysophospholipids present in plant cells (Fig. 5). Lysophosphatidylcholine (LPC), lysophosphatidylglycerol, and lysophosphatidylserine did not show a significant effect on PLD activity. However, lysophosphatidylinositol (LPI) showed inhibitory effects somewhat similar to that of LPE. Whereas lysophosphatidic acid (LPA) significantly increased PLD activity (Fig. 5). For example, at 200-$\mu$M concentrations of LPI and LPA, the PLD activity was 31% and 169% of the control, respectively. The only synthetic lysophospholipid used in Fig. 5 was LPA. All other lysophospholipids were from natural sources containing primarily 16:0 and 18:0 fatty acids and a small amount of 18:1. In addition to LPA (16:0) (Fig. 5), we also tested LPA (18:1) and found similar results from the two types of LPA. LPC and LPE have been found to stimulate the activities of plasma membrane $H^+$-ATPase (35, 37) and protein kinase (38). In the present study, LPE but not LPC had a strong inhibitory effect on PLD (Fig. 5). These results indicate that the regulatory effect of individual lysophospholipids on PLD enzyme is very specific and structurally selective.

In addition to LPE, the results suggest that LPI and LPA are also lipid mediators for regulating PLD in plants. LPA has been identified as a hormone- and growth factor-like lipid serving multiple physiological functions, at least in vertebrate cells, including platelet aggregation, smooth muscle contraction, and fibroblast proliferation (39). In addition, LPA has already been found to be a stimulator of mammalian PLD activity (40, 41). The present study suggests that LPA may also

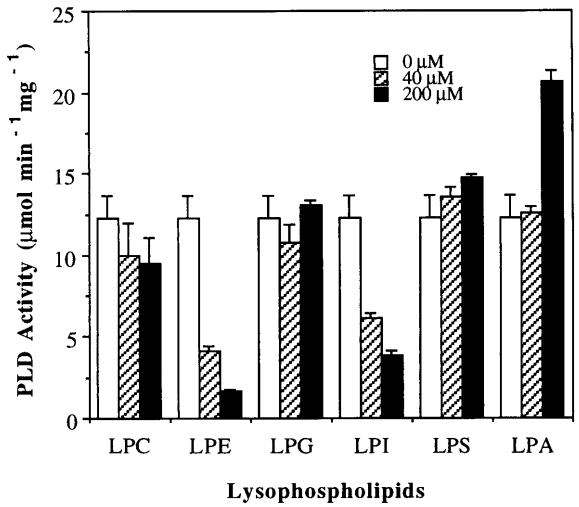


FIG. 5. Effect of different lysophospholipids on partially purified cabbage PLD activity. Final concentrations of the lysophospholipids were 0 (control), 40, and 200 $\mu$M in the reaction mixture. LPA (16:0) was synthetic, whereas LPC, LPE, and lysophosphatidylglycerol were from egg yolk, LPI was from soybean, and lysophosphatidylserine was from bovine brain. Values are mean ± SE of three separate experiments.

be involved in cellular functions in plants, in part, by stimulating phospholipid hydrolysis of PLD.

Our study shows a specific inhibitory regulation of PLD by LPE and LPI, which directly target the activity of PLD enzyme. This is a significant finding because there are no known specific inhibitors of PLD in plants and animals (4). Although a number of stimulators of PLD activity have been studied, including protein kinase (18–20), small molecular weight G proteins including Arf, Rho, and Ras (18, 20, 21) and a 50-kDa cytosolic protein (22), the negative regulation of PLD is poorly understood. In addition, some inhibitors of this stimulation of PLD activity have been described. Ceramide, a sphingolipid, has been reported to inhibit mammalian PLD activation (42) and has recently been found to interfere with protein kinase C-mediated activation of PLD (23). There have been several other potential inhibitors of PLD reported including akylphosphate esters (24) and brain cytosolic proteins (25, 26). Lukowski *et al.* (27) recently claimed that fodrin, a brain cytosolic actin binding protein, is the first described inhibitor of brain PLD; however, the nature of this inhibition is not known. No such information is available on plant PLD. In mammalian systems, PLD has been proposed as a critical component of cellular signal transduction in response to many hormones, neurotransmitters, and growth factors (2, 17). In plants, PLD activity is linked to seed germination and in responses to various stresses including wounding, drought, pathogenesis, frost, and $\gamma$-irradiation (15, 30, 31, 33, 43–46). Thus, information on the negative regulation of PLD could provide valuable insight in several areas of biology.

Lysophospholipids including LPC and LPE have been found to be made and regulated by the cell in response to the treatment of auxin, a growth-stimulating plant hormone, which activates phospholipase $A_2$ (38). The products of phospholipase $A_2$, such as LPC and LPE, have been reported to

Table 2. Inhibition of ethylene production in cranberry fruits by LPE (100 $\mu$M) with different acyl chains

| | Control | LPE (14:0) | LPE (16:0) | LPE (18:0) | LPE (18:1) |
|---|---|---|---|---|---|
| Ethylene, nl·h$^{-1}$·g$^{-1}$ FW | 1.78 ± 0.38 | 1.78 ± 0.11 | 1.65 ± 0.05 | 1.27 ± 0.05 | 1.06 ± 0.13 |
| Relative % | 100 | 100.0 | 92.7 | 71.3 | 59.6 |

Values are mean ± SE of three replications.

stimulate a protein kinase (38). Based on these findings, Scherer and coworkers (38, 47) suggested that the lipid metabolites generated by hormone-activated phospholipase $A_2$ may constitute a new second messenger system in plants. The present study may open new opportunities to determine cellular roles of lipid mediators such as LPE and LPI in the physiological functions involving regulation of PLD in plants and possibly in animals. Future studies of these biologically active lipids may further reveal their functions in growth and development.

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