# The salt stress-induced LPA response in *Chlamydomonas* is produced via $PLA_2$ hydrolysis of DGK-generated phosphatidic acid[S]

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**Abstract** **The unicellular green alga *Chlamydomonas* has frequently been used as a eukaryotic model system to study intracellular phospholipid signaling pathways in response to environmental stresses. Earlier, we found that hypersalinity induced a rapid increase in the putative lipid second messenger, phosphatidic acid (PA), which was suggested to be generated via activation of a phospholipase D (PLD) pathway and the combined action of a phospholipase C/diacylglycerol kinase (PLC/DGK) pathway. Lysophosphatidic acid (LPA) was also increased and was suggested to reflect a phospholipase $A_2$ ($PLA_2$) activity based on pharmacological evidence. The question of PA's and LPA's origin is, however, more complicated, especially as both function as precursors in the biosynthesis of phospho- and galactolipids. To address this complexity, a combination of fatty acid-molecular species analysis and in vivo $^{32}$P-radiolabeling was performed. Evidence is provided that LPA is formed from a distinct pool of PA characterized by a high α-linolenic acid (18:3*n*-3) content. This molecular species was highly enriched in the polyphosphoinositide fraction, which is the substrate for PLC to form diacylglycerol.[JLR] Together with differential $^{32}$P-radiolabeling studies and earlier PLD-transphosphatidylation and $PLA_2$-inhibitor assays, the data were consistent with the hypothesis that the salt-induced LPA response is primarily generated through $PLA_2$-mediated hydrolysis of DGK-generated PA and that PLD or de novo synthesis [via endoplasmic reticulum - or plastid-localized routes] is not a major contributor.**—Arisz, S. A. and T. Munnik. **The salt stress-induced LPA response in *Chlamydomonas* is produced via $PLA_2$ hydrolysis of DGK-generated phosphatidic acid. *J. Lipid Res.* 2011.** 52: **2012–2020.**

**Supplementary key words** diacylglycerol • lysophosphatidic acid • phospholipase $A_2$ • signal transduction Chlamydomonas • $^{32}$P-radiolabeling • α-linolenic acid • hypersalinity

The unicellular green alga *Chlamydomonas* has proven to be useful model organism to study phospholipid-signaling pathways in response to osmotic and salt stress (1, 2). Recently, particular interest has been focusing on the accumulation of phosphatidic acid (PA) as a lipid second messenger in plant and animal systems (3).

In *Chlamydomonas moewusii*, PA is a minor lipid, composing 0.67 mol% of the total phospholipid pool (4). In response to 150 mM NaCl, PA levels rapidly increase 3- to 4-fold within minutes of application (5). In a recent study, also lysophosphatidic acid (LPA) was shown to accumulate in *C. moewusii* under conditions of salt and nonionic hyperosmotic stress (6, 7). The response was dose-dependent within the range of 150 to 400 mM NaCl, reaching a maximum at 300 mM. Because of a pharmacological inhibitor, the LPA response was suggested to be generated via a phospholipase $A_2$ ($PLA_2$) (6).

The origin of PA and LPA is, however, more complicated, because several pathways can be involved in their synthesis or breakdown. For example, the primary function of LPA and PA as precursors for de novo glycerolipid biosynthesis at the endoplasmic reticulum (ER) and plastidial membranes (**Fig. 1**) is often ignored. This pathway starts with two consecutive acylations of glycerol-3-phosphate (G3P) to generate LPA and PA, by the activities of G3P

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Abbreviations: CDP-Etn, CDP-ethanolamine; CMP-PA, cytidine monophosphate-PA; DAG, diacylglycerol; DGK, DAG kinase; DGPP, diacylglycerol pyrophosphate; ER, endoplasmic reticulum; FAME, fatty acid methyl ester; G3P, glycerol-3-phosphate; GPAT, G3P acyltransferase; InsPP, inositolpolyphosphate; JA, jasmonic acid; α-LA, α-linolenic acid; LPA, lysophosphatidic acid; LPAAT, LPA acyltransferase; LPI, lysophosphatidylinositol; MGDG, monogalactosyldiacylglycerol; NPC, nonspecific PLC; PA, phosphatidic acid; PAK, PA kinase; PAP, PA phosphatase; PBut, phosphatidylbutanol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; $PIP_2$, phosphatidylinositol 4,5-bisphosphate; PPI, polyphosphoinositide; PLA ($A_2$/C/D), phospholipase A ($A_2$/C/D); UDP-Gal, UDP-galactose.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of two figures.

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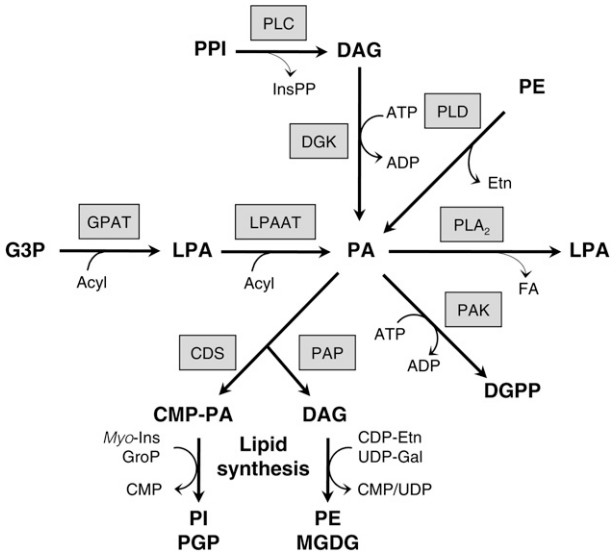


**Fig. 1.** Complexity of PA and LPA metabolism. PA is formed by successive acylations of G3P and LPA for de novo synthesis of glycerolipids, but it can also be formed through the stress-induced activities of PLC/DGK and PLD. For the synthesis of PI and PG(P), PA is converted to cytidine monophosphate-phosphatidic acid (CMP-PA) by CMP-PA synthase (CDS), and for the synthesis of PE and MGDG, it is dephosphorylated by PAP. Note that the PA/DAG substrates in the synthesis of PE and MGDG are different pools localized at the ER and plastidial envelope membrane, respectively. During salt stress, *Chlamydomonas* cells accumulate the alternative PA metabolites DGPP and LPA. The latter is suggested to originate from PA-hydrolyzing $PLA_2$ activity.

acyltransferase (GPAT) and LPA acyltransferase (LPAAT), respectively. For the synthesis of phosphatidylinositol (PI) and its phosphorylated derivatives, phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate ($PIP_2$) or phosphatidylglycerol (PG), PA is converted to cytidine monophosphate-PA (CMP-PA), which can react with *myo*-inositol or glycerolphosphate. Alternatively, for the synthesis of phosphatidylethanolamine (PE) or monogalactosyldiacylglycerol (MGDG), PA is dephosphorylated by PA phosphatase (PAP) to diacylglycerol (DAG), and acquires its headgroup via a reaction with CDP-ethanolamine (CDP-Etn) or UDP-galactose (UDP-Gal), respectively (Fig. 1).

While the PA formation by LPAAT may be prevalent under steady-state conditions, upon environmental stress, other pathways can be activated, generating distinct and transient PA responses that are likely to play a role in signaling (3, 8–14). These pathways include phospholipase D (PLD), which hydrolyzes structural phospholipids, like PE to generate PA, and DAG kinase (DGK), which produces PA by phosphorylation of DAG (4). Under stimulatory conditions, this DAG can be provided by PLC-mediated hydrolysis of polyphosphoinositides (PPI), i.e., PIP and $PIP_2$ (3, 9, 15). Metabolically, DAG can also be generated through hydrolysis of phosphatidylcholine (PC) via nonspecific PLC, called NPC (16–18).

Here, the complexity of the salt stress-induced PA and LPA response in *Chlamydomonas* was studied in more detail to unravel their pathway of synthesis. Based on fatty acid molecular species analyses, in addition to in vivo $^{32}$P-labeling studies, we suggest that the salt stress-induced LPA response is produced via $PLA_2$ hydrolysis of DGK-generated PA.

## MATERIALS AND METHODS

### Cell cultures

*Chlamydomonas moewusii* strain UTEX 10 (mating type minus) from the Culture Collection of Algae, University of Texas (Austin) was autotrophically grown as described before (19). Petri dishes containing cultured cells of ~18 days old were flooded with 20 ml HMCK (10 mM HEPES, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM KCl; pH 7.4), and after 16 h, suspensions of swimming gametes were harvested (4).

### $^{32}$P-labeling and lipid extraction

Cell suspensions were labeled with $^{32}P_i$, and treated ± NaCl in the appropriate concentrations for the indicated times, and the lipids were extracted. To each 100 μl sample, 375 μl $CHCl_3$/MeOH/HCl (50:100:1, by vol) was added to stop all reactions. After vigorous shaking, 375 μl $CHCl_3$ and 100 μl 2 M HCl were added. The resultant upper phase was washed with 400 μl $CHCl_3$/MeOH/1M HCl (3:48:47). Aliquots of concentrated lipid extracts were analyzed by TLC using silica gel 60 plates (Merck) in a solvent of $CHCl_3$-MeOH-$NH_4OH$-$H_2O$ (90:70:4:16, by vol) (20, 21). Radioactivity was quantified by phosphoimaging.

### Fatty acid analysis

For fatty acid analyses, treatments were conducted in separation funnels containing 60 ml cells (density $2.0 \times 10^7$ cells/ml) to which 20 ml buffered NaCl solution (or only buffer) was added. Reactions were stopped by the addition of perchloric acid to a final concentration of 5% (w/v), and lipids were extracted by a previously described method with adjustment for the larger extraction volume (5).

PA and LPA were purified by column-adsorption chromatography on a 2 g silica column (Sep-Pak Plus). The lipid extract was dissolved in hexane and applied to the column. Elution solvents were (*i*) hexane-$Et_2O$ (99:1, 18 ml); (*ii*) hexane-$Et_2O$ (4:1, 15 ml); (*iii*) $CHCl_3$ (10 ml); (*iv*) $Me_2CO$-$CHCl_3$ (2:1, 25 ml); (*v*) $Me_2CO$-MeOH (29:1, 10 ml); (*vi*) $Me_2CO$-MeOH (19:1, 30 ml); (*vii*) $Me_2CO$-MeOH (2:1, 25 ml); and (*viii*) $CHCl_3$-MeOH-$H_2O$ (1:2:0.8, 19 ml). The last eluate was collected in 10 ml tubes in four portions of 4.75 ml. To extract lipids from these aqueous eluates, 3.75 ml $CHCl_3$ and 1 ml 0.9% (w/v) NaCl was added, and then tubes were vigorously shaken and centrifuged to separate two phases of which the lower one, containing LPA and PA, was dried down in a gyrovap at 50°C. The concentrated extracts were further purified by TLC as described. $^{32}$P-lipid markers served to calculate the recovery of each lipid after purification and for their localization on the TLC plate.

To generate fatty acid methyl esters (FAME), lipid spots were scraped from the TLC plates into 3% $H_2SO_4$ in MeOH. Known concentrations of heneicosanoic acid (21:0) methyl esters served as internal standard. Concentrated FAME extracts were analyzed by GC (Varian Chrompack, Bergen op Zoom, The Netherlands) using a 50 m WCOT fused silica column and FID with carrier gas $N_2$ at 30 ml/min. Operating conditions were either 180°C isothermal or the temperature was programmed from 180°C to 220°C at 0.5°C/min with injector and detector temperatures at 250°C and 270°C, respectively.

### In vitro $PLA_2$ digestion of phospholipids

TLC-separated lipids were recovered from the silica and dissolved by sonication in 1 ml ethylether/MeOH (98:2, by vol).

Five units of bee venom $PLA_2$ was added in 100 μl 100 mM Tris HCl buffer (pH 8.9) containing 9.1 mM $CaCl_2$. After 3 h incubation at 25°C with frequent shaking, reactions were stopped by adding 20 μl 0.5 M EDTA. Lipid products were extracted after evaporation of the ether phase by three consecutive extractions of the aqueous phase. Pooled extracts were dried, and the precipitate was resolved into $CHCl_3$ for TLC purification and analysis. For the purpose of accurate quantitation of LPA in fatty acid analyses, a C17-LPA standard was prepared from the corresponding, commercially available di-C17-PA (Sigma) by in vitro $PLA_2$ digestion.

### Quantitation and statistics

Calculation of the original lipid fatty acid masses was based on GC peak areas of algal FAMEs divided by the internal standard area, and subsequently corrected for variation in recovery, using $^{32}P$- and C17-lipid markers. Presented charts of fatty acid profiles show typical results, representative of three experiments showing similar trends. The data in Fig. 4D represent average values (n = 3). Statistical differences were determined using an unpaired *t*-test.

## RESULTS

When *Chlamydomonas* cells were metabolically labeled with $^{32}P_i$ and subsequently treated with 300 mM NaCl for only 5 min, several changes in the $^{32}P$-phospholipid pattern could be observed (**Fig. 2**), confirming earlier reports. Thus, typical $^{32}P$-increases were found in PA (by 1.5 to 14 times, depending on the prelabeling time), LPA (by 1.8 to 96 times, depending on the prelabeling time), diacylglycerol pyrophosphate (DGPP), PIP, PI(3,5)$P_2$, and PI(4,5)$P_2$ (2, 6, 22–25).

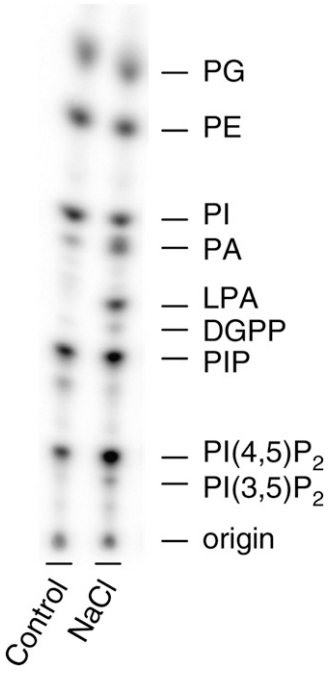


**Fig. 2.** Effect of salt stress on $^{32}P$-phospholipid patterns of *Chlamydomonas*. Cells were labeled for 3 h and then treated for 5 min with buffer (Control) or 300 mM NaCl. Lipids were extracted, separated by TLC, and then visualized by PImaging. Results of a typical experiment are shown (n > 30).

The increase in $^{32}P$-PA was suggested to result from both a PLD pathway in which the structural phospholipid PE is hydrolyzed and the combined activities of the PLC/DGK pathway (5, 22). PLC hydrolyzes PI4P and PI(4,5)$P_2$ into inositolpolyphosphates (InsPP) and DAG, and the latter can be converted to PA via DGK (Fig. 1). DGPP is the phosphorylated product of PA, which may represent an attenuation of PA as a signaling molecule, but it could also be a phospholipid signal itself (23, 26–28).

Like PA, the study of LPA is complicated by the multiplicity of pathways that synthesize it (Fig. 1). LPA is formed in the pathway of de novo PA synthesis, but it can also result from acyl hydrolysis of PA by a $PLA_2$ activity. The first possibility implies that LPA is precursor to PA, whereas the latter implies that LPA is the product of PA. To study these product-precursor relationships, fatty acid analyses were performed, as distinct phospholipid classes and pools can have characteristic fatty-acid fingerprints that are inherited by their metabolites (5, 29, 30).

As shown in **Fig. 3**, salt stress induced major increases in 16:0 and 18:1*n*-9 in both LPA and PA pools. On the basis of quantitation of fatty acids, PA was increased by 4.2 ± 1.2 times (average ± SD, n = 4), and LPA by 7.2 ± 2.2 times (average ± SD, n = 4), the respective control levels. However, the LPA pool generated in response to salt also contained 18:3*n*-3 (α-linolenic acid, α-LA) (Fig. 3B, D, peak 8). Because this species was hardly detectable under steady-state conditions and increased in response to salt stress to become one of LPA's major constituents (Fig. 3B, D), these results strongly suggest an activation of a novel metabolic pathway rather than an upregulation of de novo synthesis. Subsequently, this possibility was investigated further.

Interestingly, α-LA was not particularly abundant in the pool of PA under either condition (Fig. 3A, C). To explain this difference, it was hypothesized that the α-LA-enriched LPA was perhaps derived from a preceding, short-lived part of the PA increase with a similar α-LA enrichment in its fatty acid composition. To test this hypothesis, cells were treated for a shorter period (i.e., 30 s and 5 min) with 300 mM NaCl, and then the fatty acid compositions of LPA and PA quantitatively were analyzed (**Fig. 4**).

Within 30 s of NaCl stimulation, PA increased substantially and was characterized by the abundance of palmitic (16:0) and oleic acids (18:1), whereas α-LA, the hallmark of stimulated LPA, was relatively minor at both time points (Fig. 4A, B). Although this seems to argue against α-LA-enriched LPA resulting from a preceding phase of PA formation, it could be derived from a metabolically separated subpool of PA containing a relatively high α-LA content. Indeed, the increase in α-LA was found in the same order of magnitude in both lipids when plotted along the same axis (Fig. 4C). The PA increase could, therefore, underlie the significantly α-LA-enriched fatty acid spectrum of newly formed LPA (Fig. 4D). Simultaneously, the LPA increment displayed an enrichment in 18:2, whereas 18:1*n*-9 was diminished (Fig. 4D).

As salt stress-induced PA in *Chlamydomonas* is of mixed origin, it was hypothesized that LPA formation selectively drew upon one of the PA metabolic pools, explaining the

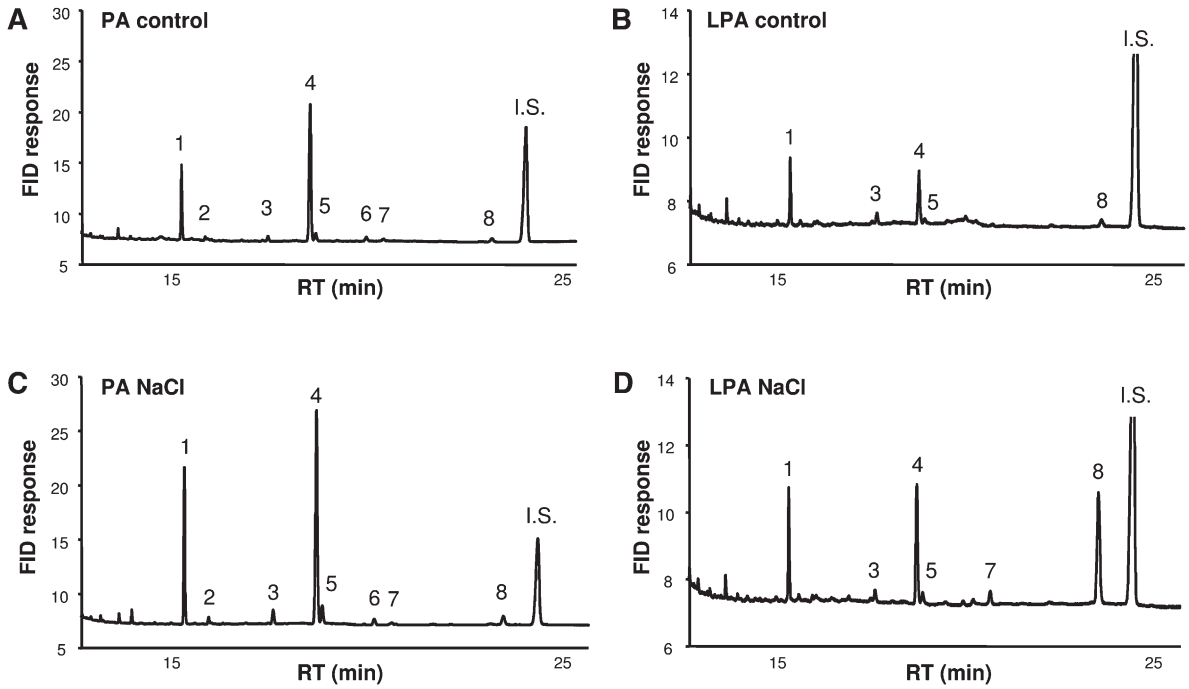


**Fig. 3.** Fatty acid composition analyses of PA and LPA in salt-stressed *Chlamydomonas* cells under control conditions (A, B) and treated with 300 mM NaCl for 5 min (C, D). FAMEs were made from purified phospholipids and analyzed by GC. Peak identification: 1, 16:0; 2, 16:1; 3, 18:0; 4, 18:1*n*-9; 5, 18:1*n*-7; 6, 18:2*n*-9; 7, 18:2*n*-6; 8, 18:3*n*-3 (α-LA); and IS, internal standard (21:0).

disparate fatty acid compositions of PA and LPA. The LPA molecular species would then reflect the composition of either the PLD substrate, PE, or PLC's substrate, the PPIs. PE has been shown to contain mainly 18:1*n*-9 (92 mol%), and in the MS spectrum, the $(18:1)_2$ species was predominant, accounting for over 95% of the total PE (5). Although a minor amount of α-LA (2 mol%) was present in PE (4), it probably is absent from the PA produced by PLD-mediated PE hydrolysis during salt stress (5). Although this suggested that PLD-derived PA could not lead to the increase in α-LA-containing LPA, it could still be precursor to the 18:1-containing LPA.

In contrast, PPIs contain both α-LA and 18:1*n*-7, a hallmark-fatty acid of PI and its derivatives (4). So their hydrolysis by PLC and subsequent phosphorylation of DAG could generate these molecular species of PA (Fig. 3A, C and Fig. 4) (5). If this PA pool could be a substrate to $PLA_2$, it might account for the increase in α-LA and 18:1*n*-7 species of LPA.

$PLA_2$ hydrolysis of PA from a mixed PLD- and PLC/DGK-derived origin could thus account for the production of the corresponding LPA species, provided that these fatty acids are esterified to the *sn*-1-OH position of the respective substrate lipids (i.e., PE and PPI). To test this hypothesis, a positional study of the fatty acids in PE and PI, which is similar to the PPIs (4, 5), was performed (**Table 1**). The lipids were purified from a total lipid extract, and then digested in vitro using $PLA_2$ from bee venom to generate the corresponding lysophospholipids, which were purified on TLC and analyzed for their fatty acid content by GC.

As shown in Table 1, the α-LA and 18:1*n*-7 content was not decreased in lyso-phosphatidylinositol (LPI) compared with its parent PI, suggesting that these fatty acids were predominantly linked to the *sn*-1 position, which is resistant to $PLA_2$ digestion. These results are in agreement with the suggested pathway in *Chlamydomonas* involving $PLA_2$-hydrolysis of PA derived from PPIs.

Some additional $^{32}$P-labeling experiments were performed that confirmed our hypothesis. This included a differential $^{32}$P-labeling technique (9, 21) that takes advantage of the extremely fast $^{32}$P-labeling of the cellular ATP pool when cells are only briefly incubated with $^{32}$P-$P_i$. Consequently, lipids that are direct products of ATP-dependent phosphorylation, such as DGK-generated PA, are quickly labeled, whereas PA that is derived from PLD activity is slowly labeled because of the relatively slow labeling kinetics of PE, which takes from hours to days (9, 12, 21). Salt-induced PA and LPA in *Chlamydomonas* were found to exhibit rapid labeling kinetics, similar to PPI, consistent with their formation through a DGK-$PLA_2$ route (supplementary Fig. I). Conversely, a long-term labeling protocol designed to visualize the gradual label incorporation into structural lipids showed steady increases (up to 2 days) in $^{32}$P-PE, whereas salt-stimulated $^{32}$P-LPA declined, arguing against a major role for PLD in providing the $PLA_2$ substrate (supplementary Fig. II).

## DISCUSSION

The rapid accumulation of PA (in minutes) seems to be an early hallmark of various plant stress responses (3, 9, 13, 31). Being the product of multiple pathways, however, the origin of this PA is complex. In *Chlamydomonas* cells, salt stress activates both PLD and DGK, generating a PA increment of mixed origin. In addition, $^{32}$P-LPA

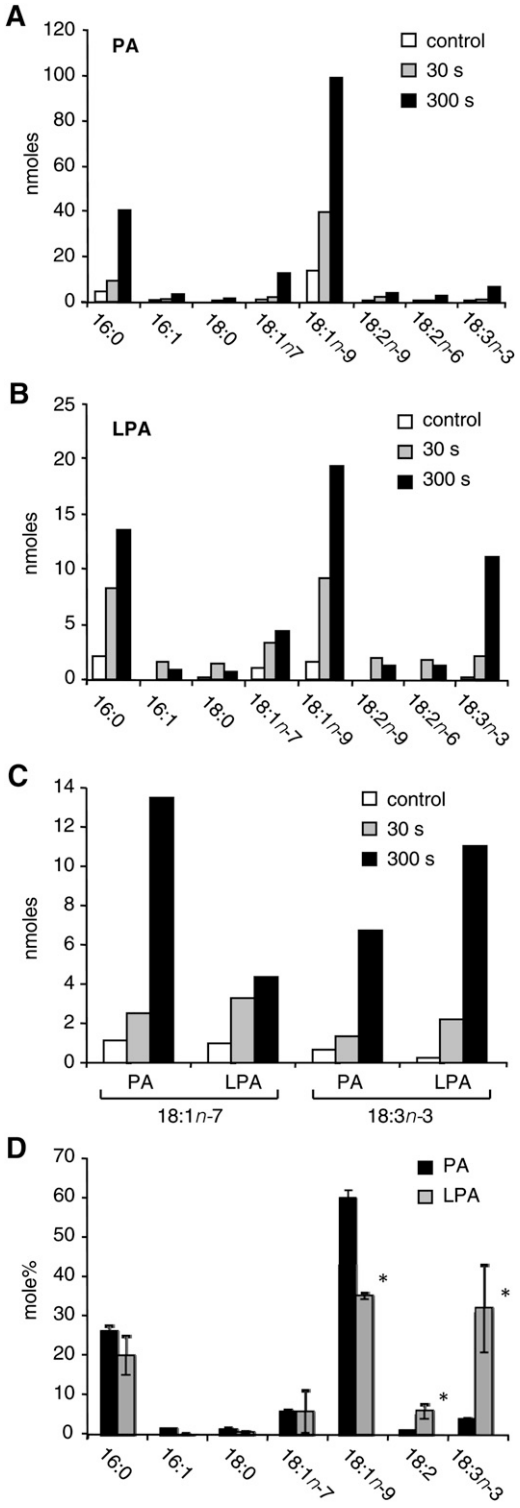


**Fig. 4.** Comparison of PA (A) and LPA (B) fatty acid signatures after 30 s or 5 min of hyperosmotic stress. Lipids were isolated from *Chlamydomonas* after challenge with 300 mM NaCl for the respective times. Increments in 18:1*n*-7 and 18:3*n*-3, characteristic fatty acids of PPIs, were registered in both lipids after 30 s and 5 min (C). The fatty acid spectra of newly formed PA and LPA after 5 min of salt treatment (D) show LPA's significant enrichment in 18:3*n*-3 and 18:2, and a decrease in 18:1*n*-9 (mean ± SD, n = 3; $*P < 0.003$).

TABLE 1. Fatty acid composition of PE and PI and their lyso-derivatives acquired by in vitro $PLA_2$-catalyzed digestion of *C. moewusii* lipids

| Lipid | 16:0 | 18:1*n*-9 | 18:1*n*-7 | 18:2*n*-6 | 18:3*n*-3 |
|---|---|---|---|---|---|
| Parent[a] | | | | | |
| PE | 5 ± 1 | 92 ± 4 | 0 | 1 | 2 |
| PI | 50 ± 4 | 14 ± 1 | 20 ± 2 | 9 ± 2 | 7 ± 1 |
| Daughter[b] | | | | | |
| LPE | 5 ± 1 | 92 ± 2 | 0 | 0 | 3 |
| LPI | 5 ± 1 | 19 ± 8 | 57 ± 10 | 6 ± 1 | 13 ± 2 |

Values are mol% average ± SD.
[a] n = 4.
[b] n = 2.

accumulated, which was proposed to be generated through $PLA_2$ activity on PA (6). The present work used fatty acid analysis to provide evidence for a pathway connecting PLC/DGK signaling to the PA and LPA response. The suggested pathway was confirmed using differential radiolabeling techniques. A model is presented that maps different metabolic PA and LPA pools and their precursors and shows where the NaCl-induced LPA response fits in (**Fig. 5**).

### LPA accumulates under salt stress, not via GPAT, but through $PLA_2$ activity

The enzymes GPAT and LPAAT are responsible for de novo synthesis of LPA and PA, respectively (Fig. 1). This route prevails at basal conditions, but its activity might be induced under stress conditions, similar to observations of de novo synthesized DAG in hypo-osmotically stressed *Dunaliella salina* (32). However, the evidence presented indicates that the salt-induced LPA response in *Chlamydomonas* is not a consequence of stimulated GPAT activity. First, the α-LA-rich LPA under salt stress is clearly different from de novo synthesized LPA molecular species (Fig. 3B, D and Fig. 4B, D). Second, under basal conditions, $^{32}$P-radiolabeling of LPA occurred slowly, reaching equilibrium after days, whereas salt-stimulated LPA showed a contrary labeling trend (supplementary Fig. II). As a product of enhanced GPAT activity, LPA would display the slow equilibration kinetics of its precursors, which is not the case.

Not only did the results argue against de novo synthesis as the source of the LPA response, but several lines of evidence also suggested that it was derived from PA through $PLA_2$ activity. Salt stress triggers a rapid increase in PA (Fig. 2), which has been shown previously to consist of different subpools (5, 22). LPA's peculiar fatty acid composition (Figs. 3 and 4) and its labeling kinetics (supplementary Figs. I and II) were consistent with its formation, through $PLA_2$, from one of these PA subpools (discussed below). Accordingly, the increase in PA sets in a bit earlier than the LPA increase (supplementary Fig. I-A) (6). The results are also in agreement with a previous study, showing that pharmacological inhibition of $PLA_2$ abrogated the LPA response (6). This treatment simultaneously increased the $^{32}$P-level of PA and its metabolite DGPP, again indicating that LPA, like DGPP, is derived from salt-induced PA.

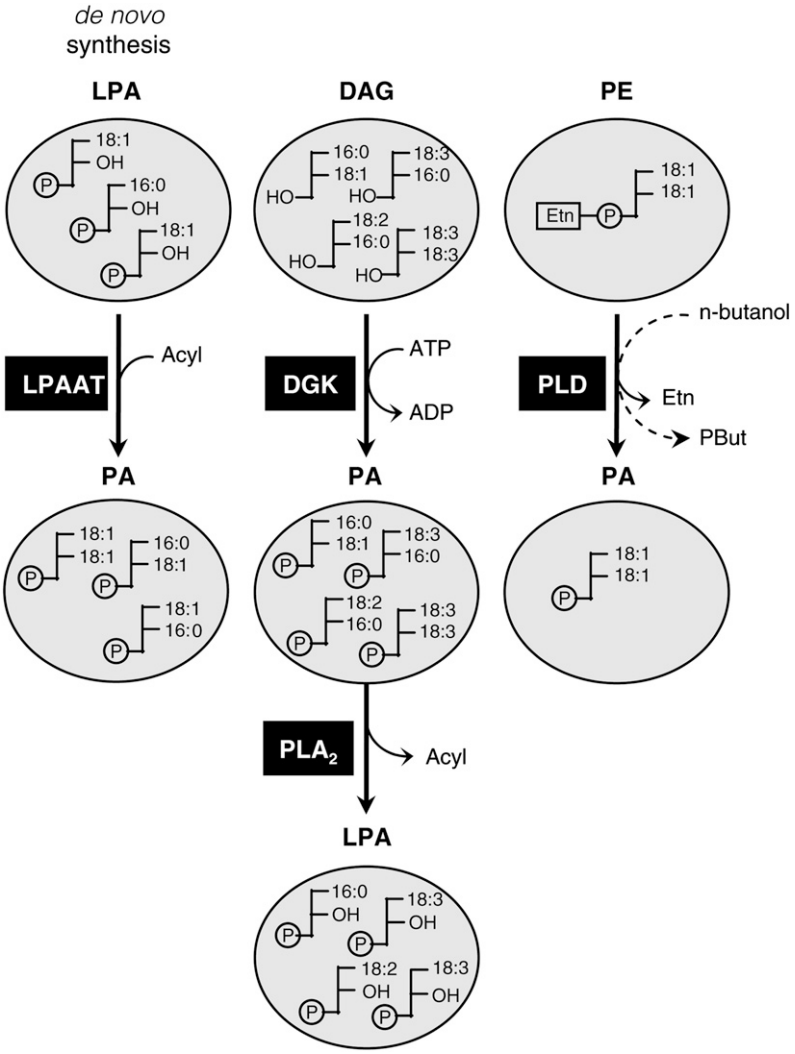


**Fig. 5.** Schematic representation of LPA and PA pools with distinct molecular species compositions, based on data from this and previous reports (5, 9). The fatty acids in de novo synthesized PA and LPA differ from the fatty acids in PA and LPA generated in the DGK and PLD signaling pathways. The accumulation of α-LA-enriched LPA in the salt stress response in *Chlamydomonas* is suggested to be derived from DGK-generated PA. In the presence of n-butanol, PLD can catalyze a transphosphatidylation reaction by which the artificial lipid PBut is formed, which is used as marker of PLD activity. The fatty acid composition of PBut matches that of the PLD substrate lipid pool (PE), and of PLD's normal product, PA.

**Salt stress-induced $PLA_2$ hydrolyses a subpool of PA that is generated via the PLC/DGK route**

As summarized in Fig. 1 and represented in Fig. 5, several potential pathways could generate PA during salt stress. De novo synthesis of PA implicates LPA as precursor, whereas the PLC/DGK and PLD pathways may provide PA as substrate to $PLA_2$, generating LPA as product. Previous studies have implicated the PLC/DGK and PLD pathways in the salt response in *Chlamydomonas* (5, 22). From fatty acid analyses of the artificial PLD product phosphatidylbutanol (PBut) (33), it was inferred that the enzyme hydrolyzed PE and that this did not produce α-LA-containing PA species (5), which is in agreement with our present conclusion that $PLA_2$'s substrate is provided by another pathway (Fig. 5). NaCl-stimulated LPA species contain several fatty acids that are characteristic of $PIP_2$, PIP, and PI: α-LA, 18:1*n*-7, and 18:2*n*-6 (4). Each is present at the *sn*-1 position of PI (Table 1), suggesting that sequential PIP and/or $PIP_2$ hydrolysis by PLC and subsequent phosphorylation of DAG could provide the PA substrate to $PLA_2$. This was further supported by our $^{32}$P-radiolabeling experiments. Under pulse-labeling conditions, which favor labeling of the products of DGK, salt induced rapid and large $^{32}$P-PA and $^{32}$P-LPA increments (supplementary Fig. I). Conversely, under conditions that favor label incorporation into PLD's substrate, $^{32}$P-LPA levels declined, likely following the decreased specific radioactivity of ATP (supplementary Fig. II). Interestingly, hyperosmotic stress in *Arabidopsis* seedlings induced the formation of DAG and PA species with a high 18:2*n*-6 and α-LA content, which was speculated to reflect a PLC/DGK pathway, drawing on a PPI pool with increased PUFA levels (33).

The finding that pharmacological inhibition of $PLA_2$ not only decreased salt-induced LPA formation in *Chlamydomonas* but also simultaneously increased the levels of PA and DGPP (6) has interesting implications, as it suggests that $PLA_2$ and PA kinase (PAK) compete for the same PA substrate pool. According to the present data, this is the PA pool generated through the PLC/DGK pathway. As DGK and PAK are predicted to be mainly localized at the plasma membrane (9, 34), $PLA_2$ might be active there as well.

**LPA and $PLA_2$ signaling**

In animal systems, LPA is an intercellular signaling molecule that can be produced by a highly PA-selective $PLA_2$ (35), and it functions as ligand for G-protein coupled

endothelial differentiation gene (EDG) receptors (36). In plants, however, these receptors are missing, and in a unicellular alga, it is not very likely that LPA is secreted and sensed by others. Nonetheless, other functions for $PLA_2$ products have been described, and during the last few years, several advances in plant $PLA_2$ signaling in higher plants have been made (37–40).

The PLA superfamily includes a broad range of enzymes. Although it is not always clear which substrate is used, both lysophospholipids and free fatty acids have been implicated in signaling (38, 40, 41). Literature on PA-specific enzymes is limited, however. From *Arabidopsis*, a putative PA-$PLA_1$ gene (*SRG1*) has been cloned in which the knockout mutant displayed reduced gravitropic responses (42). In *Arabidopsis*, a novel $PLA_2$, *SOBER1*, with no homology to any of the plant PLA families, was suggested to suppress the elicitor-induced induction of a hypersensitive response by reducing the accumulation of PA (43). However, based on in vitro activity of recombinant SOBER1, its substrate was speculated to be PC rather than PA. Other recent plant studies implicating a role for $PLA_2$ includes auxin signaling (39), pH regulation (44, 45), plant defense (37, 43–46), stomatal opening (47), pollen development and germination (48), and vesicular transport (39, 48). For PA, putative protein targets have been identified in a proteomics screen using PA-affinity beads (49). It will be interesting to test LPA as a competitor in those assays, as some of the proteins may be LPA targets.

### Salt stress responses in *Chlamydomonas* and other green algal systems

While a similar salt-induced LPA response seems to be lacking in higher plants, it has been reported in *Dunaliella salina* under hypersalinity stress (50). In this system also, $^{32}$P-lyso-PC increased and $^{32}$P-PA decreased. The distinct response may be related to the fact that *D. salina* lives in saline environments and is extremely salt tolerant. Phospholipid changes were only found when salt concentrations increased from 1.7 to 3.4 M NaCl, which may induce effects of NaCl toxicity, in addition to cell volume decrease, plasma membrane infolding, and shrinking of internal membranes. In the green alga *Micrasterias denticulata*, salt stress induced similar ultrastructural changes and a rapid (5 min) accumulation of reactive oxygen species, followed by symptoms of programmed cell death (51), which in higher plants has been associated with PA signaling (52, 53). With respect to osmoregulation, *Chlamydomonas* has a unique mechanism based on the function of its contractile vacuole, which has been implicated in the elimination of water in a low osmotic potential environment (54); however, its function in hyperosmotic stress has yet to be evaluated.

### Possible functions of free fatty acids and their metabolites

In *Chlamydomonas*, the major molecular species of PI that have 18:3 are 18:3/16:0 and 18:3/18:3 (5). Upon $PLA_2$ hydrolysis, they would generate free palmitic acid and α-LA. Free fatty acids and their metabolites may have functions in the regulation of enzymes, such as PLDδ, whose activity is enhanced in the presence of oleate and, to a lesser extent, α-LA (55). Moreover, α-LA can be metabolized to octadecanoids like jasmonic acid (JA), which acts as growth factor and modulator of stress resistance (56). Wounding-induced JA accumulation was shown to be generated by two chloroplast-localized galactolipases/PLAs, *At*DAD1 and *At*DGL1 (57).

In an evolutionary distant diatom, *Thalassiosira rotula*, wounding has been reported to trigger $PLA_2$ activity, releasing C20 polyunsaturated fatty acids within minutes. These fatty acids were further metabolized to the defensive aldehydes 2,4-decadienal and 2,4,7-decatrienal (58). In *Arabidopsis* leaves, wounding leads to the formation of hexanal through a lipid-hydrolyzing activity, releasing α-LA as its precursor (59). Apart from the role of these aldehydes in toxic defense against grazers, they have been speculated to be chemical signals of unfavorable growth conditions, inducing programmed cell death within phytoplankton communities (1).

### Biophysical aspects of $PLA_2$-mediated LPA formation

Due to an intramolecular hydrogen bond between the headgroup of LPA and the *sn*-2-hydroxyl on the glycerol backbone, LPA carries more negative charge than PA (60). Moreover, at neutral pH, LPA has the shape of an inverted cone, whereas PA behaves as a cone shape, which could be even more pronounced if it contains PUFAs like α-LA (61). Hence, the interconversion of PA and LPA by $PLA_2$ and LPAAT may affect local membrane charge and curvature depending on the membrane environment. Both effects may contribute to the function of the mammalian LPAATs C-terminal-binding protein/brefeldin A-ADP ribosylated substrate (CtBP/BARS) (62) and endophilin 1 (63) in vesicle formation. The response to hyperosmotic stress in *Chlamydomonas* may require membrane surface reduction by endocytosis to adapt to the decreased cell volume.

The question of where the different pools of PA and LPA characterized in this study (Fig. 5) are located in the cell will be important. Lipid biosensors for PA and LPA may yield valuable information about where and when PA and LPA are formed (64).

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