Positional specificity of lysosomal phospholipase A_2

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Abstract **Lysosomal phospholipase A_2 (Lpla2) is highly expressed in alveolar macrophages and may mediate the phospholipid metabolism of surfactant. Studies on the properties of this phospholipase are consistent with the presence of both phospholipase A_1 and phospholipase A_2 activities. These activities were studied through the production of *O*-acyl compounds, produced by the transacylase activity of Lpla2. Liposomes containing POPC and *N*-acetylsphingosine (NAS) were incubated with the soluble fraction obtained from MDCK cells stably transfected with the mouse Lpla2 gene. Two 1-*O*-acyl-NASs, 1-*O*-palmitoyl-NAS and 1-*O*-oleoyl-NAS, were produced by Lpla2. The formation rate of 1-*O*-oleoyl-NAS was 2.5-fold that of 1-*O*-palmitoyl-NAS. When 1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (OPPC) was used, the formation rate of 1-*O*-oleoyl-NAS was 5-fold higher than that of 1-*O*-palmitoyl-NAS. Thus, Lpla2 can act on acyl groups at both *sn*-1 and *sn*-2 positions of POPC and OPPC. When 1-palmitoyl-2-unsaturated acyl-*sn*-glycero-3-phosphocholines were used as acyl donors, the transacylation of the acyl group from the *sn*-2 position to NAS was preferred to that of the palmitoyl group from the *sn*-1 position. An exception was observed for 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PAPC), for which the formation rate of 1-*O*-palmitoyl-NAS from PAPC was 4-fold greater than that of 1-*O*-arachidonoyl-NAS. Thus, Lpla2 has broad positional specificity for the *sn*-1 and *sn*-2 acyl groups in phosphatidylcholine and phosphatidylethanolamine.**—Abe, A., M. Hiraoka, and J. A. Shayman. **Positional specificity of lysosomal phospholipase A_2. *J. Lipid Res.* 2006.** 47: **2268–2279.**

Supplementary key words alveolar macrophage • phosphatidylcholine • phosphatidylethanolamine • transacylation • surfactant

We previously identified a lysosomal phospholipase A_2 (Lpla2) with specificity toward phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (1). This phospholipase is calcium-independent, is localized to lysosomes, has an acidic pH optimum, and transacylates short-chain ceramides (1–3). Lpla2 was purified from bovine brain and characterized as a water-soluble glycoprotein consisting of a single peptide chain of 45 kDa (2). The primary structure of Lpla2, deduced from DNA sequences coding Lpla2, is highly conserved between mammals, including mouse, rat, human, and cow (3, 4). In addition, Lpla2 is highly homologous with lecithin:cholesterol acyltransferase, another enzyme with both phospholipase and transacylase activities.

Lpla2 is highly expressed in alveolar macrophages (AMs) compared with peritoneal macrophages, peripheral blood monocytes, and other tissues in both mouse and rat (4). Other macrophage-associated phospholipase A_2s, 1-cys-peroxiredoxin and Ca^{2+}-independent phospholipase A_2 ($iPLA_2$), do not show a comparable distribution (4). Granulocyte macrophage colony-stimulating factor (GM-CSF)-deficient mice are known to be a model of impaired surfactant catabolism and develop progressive accumulation of surfactant lipids and proteins in lung (5). Lpla2 expression was evaluated in mice in which GM-CSF was transgenically expressed under the control of the surfactant protein C promoter (SP-C-GM mice) in a background of GM-CSF deficiency (6). Lpla2 expression in the transgenic mice was increased compared with that in wild-type mice. By contrast, Lpla2 expression in GM-CSF-deficient mice was markedly lower than in both wild-type and SP-C-GM mice (4).

Lung surfactant is the surface-active agent composed of phospholipids and proteins that lines pulmonary alveoli. Surfactant stabilizes alveolar volume by reducing surface tension. Acidic phospholipase A activity has been suggested to play an important role in pulmonary surfactant phospholipid catabolism (7). A rare disorder of surfactant catabolism, alveolar proteinosis, is associated with defects in AM differentiation consistent with an important role for the AM in surfactant turnover. To better understand the biological function of Lpla2 and its potential role in surfactant catabolism, Lpla2-deficient ($Lpla2^{-/-}$) mice were created by double conditional gene targeting using Cre/loxP and Flp/FRT systems (8). $Lpla2^{-/-}$ mice were generated by systemic deletion of exon 5 of the Lpla2 gene, which encodes the Lpla2 catalytic site. Lpla2 activity was systemically absent. $Lpla2^{-/-}$ mice were viable and fertile and demonstrated a time-dependent increase in surfactant levels. In addition, the null mice developed significant splenomegaly.

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Lpla2 activity was assayed in the AMs of wild-type and null mice. Unesterified ^{14}C-labeled oleic acid was detected in wild-type mouse AM treated with liposomes consisting of 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphorylcholine/dicethyl phosphate but was trivially produced in the *Lpla2*$^{-/-}$ mouse AM. A marked accumulation of phospholipid, particularly PE and PC, was found in AM and peritoneal macrophages in 3 month old *Lpla2*$^{-/-}$ mice. Electron micrographs revealed extensive lamellar inclusion bodies, a hallmark of cellular phospholipidosis, in the *Lpla2*$^{-/-}$ mouse AM and peritoneal macrophages. These results suggested that Lpla2 plays an important role in cellular phospholipid metabolism and is involved in surfactant phospholipid degradation in AMs (8).

Using AMs obtained from *Lpla2*$^{-/-}$ mouse, it could be demonstrated that the acidic phospholipase A_2 (PLA_2) activity against PC was almost entirely attributable to Lpla2 present in the soluble fraction of the cells obtained as the supernatant from a 100,000 *g* centrifugation. For these experiments, phospholipase A activity was measured as the release of ^{14}C-labeled oleic acid from liposomes consisting of 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphocholine and dicetyl phosphate, assayed under acidic conditions. Although the observed release of oleic acid from the *sn*-2 position supported the conclusion that Lpla2 has PLA_2 activity, an accumulation of 1-hydroxy-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphocholine in *Lpla2*$^{+/+}$ mouse AMs was also observed, consistent with the presence of phospholipase A_1 (PLA_1) activity as well. Thus, the positional specificity of Lpla2 for substrate remained unclear. In this study, a more exhaustive evaluation of the substrate specificity for Lpla2 was undertaken.

MATERIALS AND METHODS

Reagents

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (PLPC), 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PAPC), 1-palmitoyl-2-docosahexanoyl-*sn*-glycero-3-phosphocholine (PDPC), 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC), 1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (OPPC), 1-oleoyl-2-steraroyl-*sn*-glycero-3-phosphocholine (OSPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine (PLPE), 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine (PAPE), 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (1-Pal-lysoPC), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (1-Ole-lysoPC), 1-*O*-1′-(*Z*)-octadecenyl-2-oleoyl-*sn*-glycero-3-phosphocholine [C18 (Plasm)-18:1 PC], and 1-*O*-1′-(*Z*)-octadecenyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine [C18 (Plasm)-20:4 PC] were purchased from Avanti (Alabaster, AL). *N*-Acetyl-D-*erythro*-sphingosine (NAS) was obtained from Matreya (Pleasant Gap, PA); 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphocholine (25 μCi/ml) was from Amersham Bioscience (Piscataway, NJ); BCA protein assay reagent was from Pierce (Rockford, IL); dicetyl phosphate was from Sigma (St. Louis, MO); and HPTLC silica gel plates (10 × 20 cm) were from Merck KGaA (Darmstadt, Germany).

Preparation of soluble fraction from mouse AM

The soluble fractions of AMs were prepared as described previously (4). The AMs were allowed to adhere on the culture dishes (35 mm dish), washed three times with 2 ml of cold phosphate-buffered saline, scraped with a small volume of phosphate-buffered saline, and transferred into a 15 ml plastic tube. The cells were collected by centrifugation at 800 *g* for 10 min at 4°C and resuspended with 0.4–1.0 ml of cold 0.25 M sucrose, 10 mM HEPES (pH 7.4), and 1 mM EDTA. The cell suspension was disrupted with a probe-type sonicator five times for 10 s each at 0°C to obtain the cell homogenate. The homogenate was centrifuged for 1 h at 100,000 *g* at 4°C. The resulting supernatant was passed through a 0.2 μm filter and used as a soluble fraction.

Stably transfected MDCK cells

MDCK cells were transiently transfected with pcDNA3 containing the entire open reading frame of Lpla2 as described previously to obtain Lpla2-overexpressing cells (3). MDCK cells were grown in Dulbecco's modified Eagle's medium-F12 (Gibco BRL) supplemented with 10% fetal calf serum. For transient expression, MDCK cells were cultured in 35 mm dishes. When the cells reached 80% confluence, the cells were transfected with 1 μg/ml purified plasmid using LipofectAMINE Plus™ (Invitrogen) in 1 ml of opti MEM medium (Gibco BRL). One milliliter of DMEM containing 20% fetal calf serum was added after 3 h of incubation at 37°C and 5% CO_2. Twenty-four hours after transfection, the cells were trypsinized and transferred into a 10 cm dish with 8 ml of DMEM-F12 supplemented with 10% fetal calf serum. The transiently transfected cells were treated with medium containing 500 μg/ml G418 (G418 medium) for 2 weeks to establish stable transfectants. G418 medium was replaced with fresh G418 medium every 3 days. The soluble fraction of the stably transfected cells was prepared as described above.

Argentation of the HPTLC plate

An HPTLC plate was immersed into 10% (w/v) $AgNO_3$ in acetonitrile and incubated for 10 min. The plate was dried in a fume hood and baked in an oven for 30 min at 100°C.

Transacylase activity of Lpla2

NAS was used as an acyl group acceptor. The reaction mixture consisted of 40 mM sodium citrate (pH 4.5), 10 μg/ml BSA, 40 μM NAS incorporated into phospholipid liposomes (PC or PE/sulfatide/NAS, 10:1:3 molar ratio), and the soluble fraction (2 μg) in a total volume of 500 μl. The reaction was initiated by addition of the soluble fraction, incubated for 5–30 min at 37°C, and terminated by the addition of 3 ml of chloroform-methanol (2:1) plus 0.3 ml of 0.9% (w/v) NaCl. The mixture was centrifuged for 5 min at room temperature. The resulting lower layer was transferred into another glass tube and dried down under a stream of nitrogen gas. The dried lipid was dissolved in 40 μl of chloroform-methanol (2:1), applied to an HPTLC plate or an argentation HPTLC plate, and developed in a solvent system consisting of chloroform-acetic acid (9:1) or chloroform-acetic acid-methanol (90:5:1). The plate was dried down and soaked in 8% (w/v) $CuSO_4$, $5H_2O$, 6.8% (v/v) H_3PO_4, and 32% (v/v) methanol. The uniformly wet plate was briefly dried down by a hair dryer and charred for 15 min in a 150°C oven. Silver nitrate has an incompatibility with phosphate, which is contained in the copper solution for charring. Therefore, to prevent the silica gel from peeling off the argentation plate during incubation with the copper solution, the dried plate was incubated in 20% (v/v)

methanol containing 0.5% (v/v) acetic acid to remove the silver nitrate until the background of the plate became homogeneous. The plate was then charred as described above. The plate was scanned, and the content of the product (1-*O*-acyl-NAS) was estimated by NIH Image 1.63.

RESULTS

Degradation of 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphocholine by Lpla2$^{+/+}$ and Lpla2$^{-/-}$ mouse AMs

The soluble fraction of AMs from *Lpla2*$^{+/+}$ mice was assayed under acidic conditions with liposomes consisting of 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphocholine and dicetyl phosphate (**Fig. 1A–D**). 1-Hydroxy-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphocholine (2-[^{14}C]oleoyl-lysoPC) as well as ^{14}C-labeled oleic acid were produced. The formation rates of oleic acid (Fig. 1C) and 2-oleoyl-lysoPC (Fig. 1D) were 18.6 and 13.5 nmol/min/mg protein, respectively. By contrast, when using the soluble fraction obtained from *Lpla2*$^{-/-}$ mouse AMs, the rates of formation of oleic acid and 2-oleoyl-lysoPC were 0.428 and 1.91 nmol/min/mg protein, respectively. The difference in phospholipase A activity between *Lpla2*$^{+/+}$ and *Lpla2*$^{-/-}$ mouse AMs is thought to be primarily attributable to Lpla2, because the null mouse has a systemic deficiency of Lpla2. These results suggest that Lpla2 is able to deacylate the acyl groups at both the *sn*-1 and *sn*-2 positions of the POPC molecule, although the specificity of Lpla2 for the *sn*-2 position is preferred over the *sn*-1 position.

Transacylase activity of the soluble fraction obtained from Lpla2-overexpressing MDCK cells

Lpla2 has dual enzyme activities: PLA_2 and transacylase activities (1, 2). The most common assay for transacylase activity by Lpla2 measures the transacylation of a fatty acid between phospholipid (PC and PE) and a short-chain ceramide such as NAS as an acceptor. This transacylase activity measurement has been used to distinguish Lpla2

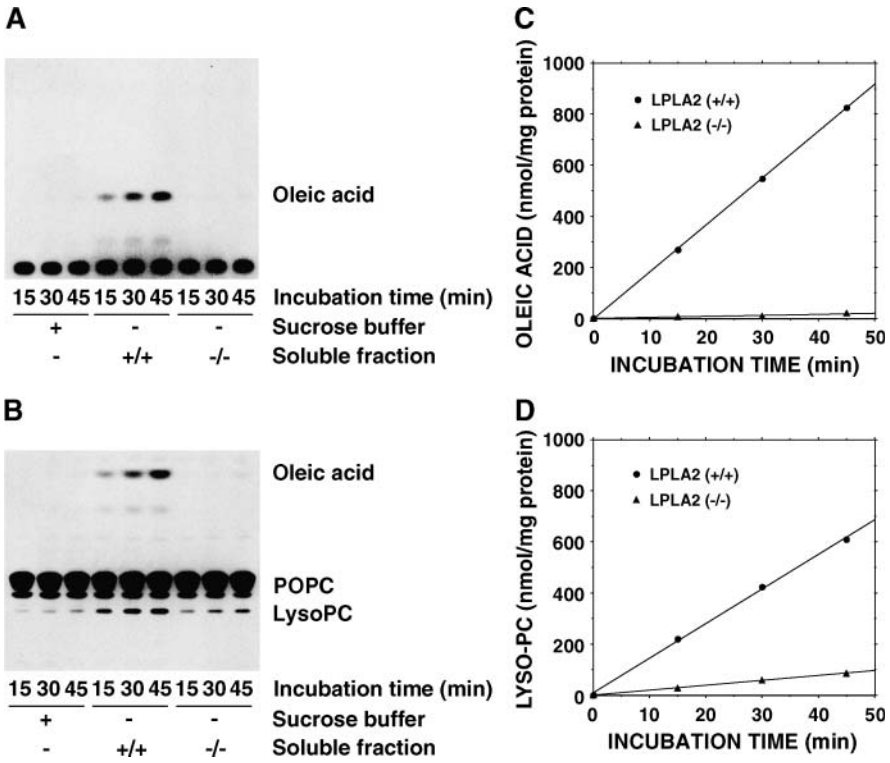

Fig. 1. Degradation of POPC by the soluble fraction obtained from lysosomal phospholipase A_2 (Lpla2)-deficient (*Lpla2*$^{-/-}$) mouse alveolar macrophages (AMs). The reaction mixture contained 48 mM sodium citrate (pH 4.5), 10 μg/ml BSA, liposomes (130 μM phospholipid), and 1.77 μg/ml of the soluble fraction obtained from *Lpla*$^{+/+}$ or *Lpla2*$^{-/-}$ mouse AMs in a total volume of 500 μl. The liposomes consisted of POPC and dicetyl phosphate in a molar ratio of 10:1 with a trace of 1-palmitoyl-2-[1-^{14}C]oleoyl-l-*sn*-3-glycerophosphocholine (2.8×10^5 cpm/assay). The reaction was initiated by adding 20 μl of each soluble fraction or sucrose buffer, maintained for 15, 30, and 45 min at 37°C, and terminated by adding 3 ml of chloroform-methanol (2:1) plus 300 μl of 0.9% NaCl. The resulting lower layer was transferred into a small glass tube and dried down under a stream of nitrogen gas. The dried lipid was redissolved with chloroform-methanol (2:1) and applied to an HPLTC plate. The plate was developed in a solvent system consisting of chloroform-methanol-pyridine (98:2:0.5) (A) or chloroform-methanol-water (60:35:8) (B). After developing, the plate was dried down, sprayed with ENHANCE, and exposed on X-ray film at −80°C for 20 h. Radioactive products, oleic acid and 2-oleoyl-lysophosphatidylcholine (2-oleoyl-lysoPC), were scraped from the plate and counted. Time courses for the release of oleic acid and the formation of 2-oleoyl-lysoPC are shown in C and D, respectively.

from other phospholipases that lack transacylase activity (1–4, 8). The transacylase activity may also be useful to resolve the positional specificity of Lpla2. Using asymmetric diacyl PC or PE as an acyl group donor, the reaction product generated by the transfer of the *sn*-1 should be distinguishable from that of the *sn*-2 by a simple method such as TLC without using radioactive substrates.

The soluble fraction prepared from Lpla2-overexpressing MDCK cells was incubated in the presence of POPC liposomes, and the products were separated by TLC. Two major compounds were produced by this reaction (**Fig. 2A**). Both compounds were alkaline-unstable, and the alkaline methanolysis of these products resulted in the generation of fatty acid methyl esters and NAS (data not shown). The mobility of the faster moving compound corresponded to that of synthetic 1-*O*-palmitoyl-NAS. Because the mobility of lipids containing one or more unsaturated acyl groups is delayed on an argentation plate, the other alkaline-unstable compound was 1-*O*-oleoyl-NAS. A similar assay of the soluble fraction obtained from control pcDNA-transfected MDCK cells lacking the Lpla2 gene showed that the endogenous Lpla2 activity and transacylase activities by other enzymes in MDCK cells are negligible in this assay system (Fig. 2A). Therefore, the soluble fraction obtained from Lpla2-overexpressing MDCK cells was used as the enzyme source in subsequent experiments.

When POPC liposomes were used as the acyl donor, the formation of 1-*O*-oleoyl-NAS by Lpla2 was greater than that of 1-*O*-palmitoyl-NAS (Fig. 2A). This observation is consistent with the specificity of Lpla2 for the *sn*-2 over the *sn*-1 position as shown above. The initial velocity of 1-*O*-oleoyl-NAS formation was 2.5-fold higher than that of 1-*O*-palmitoyl-NAS formation (Fig. 2B, C). However, the initial velocity of 1-*O*-oleoyl-NAS formation was 5-fold higher

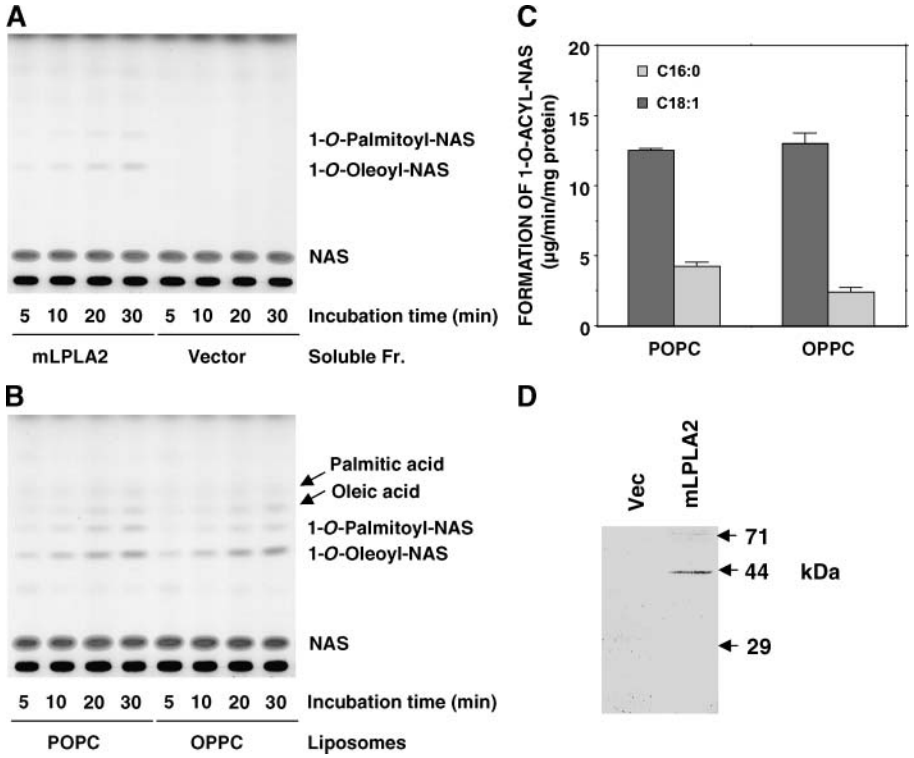

Fig. 2. Transacylase activity by the soluble fraction obtained from mouse Lpla2-overexpressing MDCK cells in the presence of POPC or 1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (OPPC). The soluble fraction was prepared from MDCK cells stably transfected with pcDNA conjugated with or without the Lpla2 gene. A: Liposomes consisting of POPC, sulfatide, and *N*-acetylsphingosine (NAS) were incubated with each soluble fraction for 5, 10, 20, and 30 min at 37°C. The reaction products were extracted and separated by an argentation HPTLC plate using a solvent system consisting of chloroform-acetic acid (9:1). B: Liposomes consisting of POPC or OPPC, sulfatide, and NAS were incubated with the soluble fraction obtained from Lpla2-overexpressing MDCK cells for 5, 10, 20, and 30 min at 37°C. The reaction products were extracted and separated by an argentation HPTLC plate using a solvent system consisting of chloroform-acetic acid-methanol (90:5:1). C: The reaction products 1-*O*-palmitoyl-NAS and 1-*O*-oleoyl-NAS were quantified by scanning the plate, and the initial velocity was estimated. D: Confirmation of the expression of Lpla2 by Western blotting. The soluble fraction was prepared from MDCK cells stably transfected with pcDNA3 (Vec) or pcDNA3-Lpla2 as described in Materials and Methods. For Western blotting, 18.5 μg of protein of each soluble fraction was separated by SDS-PAGE and subjected to immunoblotting with an anti-mouse Lpla2 peptide (^{100}RTSRATQFPD) in rabbit serum. The antigen-antibody complex on the membrane was visualized with an anti-rabbit IgG HRP-conjugated goat antibody using diaminobenzidine and hydrogen peroxide. Error bars denote standard deviation (n = 3).

than that of 1-*O*-palmitoyl-NAS formation when OPPC was used as an acyl donor. At 5 and 10 min in both cases, the formation of oleoyl-lysoPC and palmitoyl-lysoPC corresponded to the formation of 1-*O*-palmitoyl-NAS and 1-*O*-oleoyl-NAS, respectively, as confirmed by a different TLC system (data not shown). The presence of Lpla2 in the MDCK cells was confirmed by Western blotting (Fig. 2D).

When the 1-acyl-lysoPCs, 1-pamitoyl- and 1-oleoyl-lysoPC, were used as acyl donors, the transacylation of the acyl group by the soluble fraction was extremely low compared with that from POPC and OPPC (**Fig. 3A**, C). In addition, the formation rate of 1-*O*-oleoyl-NAS from C18 (Plasm)-18:1 PC liposomes by Lpla2 was 81% of that from POPC liposomes (data not shown). These results indicate that the transacylase reaction originates from the PLA_1 and PLA_2 activities in Lpla2 itself.

The carbon chain length of oleic acid [9-*cis*-octadecenoic acid, 18:1 (n-9)] is greater than that of palmitic acid. To evaluate the effect of the fatty acyl chain length and the double bond of the acyl group in PC on the positional specificity of Lpla2, SOPC and OSPC were used as acyl donors (Fig. 3B, D). The chain length of stearic acid is longer than that of oleic acid. The positional specificity of Lpla2 for SOPC and OSPC was comparable to that for POPC and OPPC. However, the total formation of 1-*O*-acyl-NAS from SOPC or OSPC was slightly less than that from POPC or OPPC. These data suggest that the positional specificity of Lpla2 is not solely dependent on the fatty acid chain length and that the enzyme acts on the unsaturated acyl group in preference to the saturated acyl group.

Positional specificity of Lpla2

To further characterize the positional specificity of Lpla2, various 1-palmitoyl-2-unsaturated acyl-PCs were used as acyl donors (**Fig. 4**, **Table 1**). One of the 1-*O*-acyl-NASs produced by Lpla2 from PLPC liposomes was 1-*O*-linoleoyl-NAS (Fig. 4A). 1-*O*-Linoleoyl-NAS, containing a C18:2 acyl chain, was clearly separable from 1-*O*-palmitoyl-NAS and 1-*O*-oleoyl-NAS by use of the argentation TLC plate. The formation rates of both 1-*O*-acyl-NASs from PLPC were slightly higher than those from POPC (Table 1).

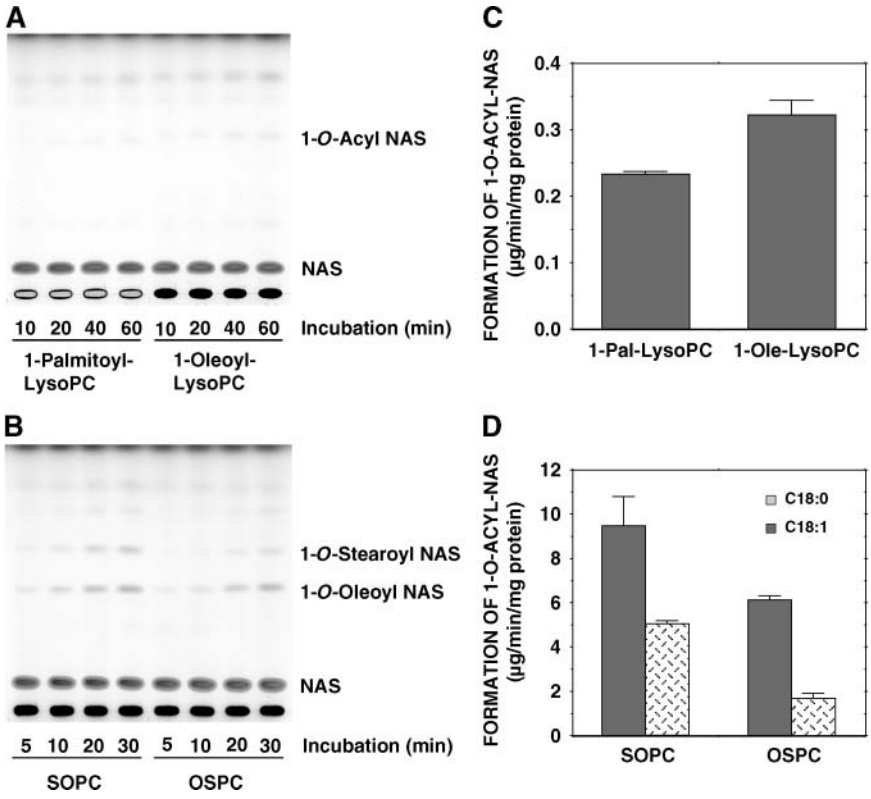

Fig. 3. Transacylase activity by the soluble fraction obtained from Lpla2-overexpressing MDCK cells in the presence of 1-acyl-lysoPC, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC), or 1-oleoyl-2-steraroyl-*sn*-glycero-3-phosphocholine (OSPC). A: Suspensions consisting of 1-palmitoyl-lysoPC or 1-oleoyl-lysoPC, sulfatide, and NAS (10:1:1.7 molar ratio) were incubated with the soluble fraction obtained from Lpla2-overexpressing MDCK cells for 20, 40, and 60 min at 37°C. The reaction products were extracted and separated by a regular HPTLC plate using a solvent system consisting of chloroform-acetic acid (9:1). The initial velocity for each product is shown in C. B: Liposomes consisting of SOPC or OSPC, sulfatide, and NAS were incubated with the soluble fraction obtained from Lpla2-overexpressing MDCK cells for 5, 10, 20, and 30 min at 37°C. The reaction products were extracted and separated by an argentation HPTLC plate using a solvent system consisting of chloroform-acetic acid (9:1). The initial velocity for each product is shown in D. For more details, see Materials and Methods. Error bars denote standard deviation (n = 3).

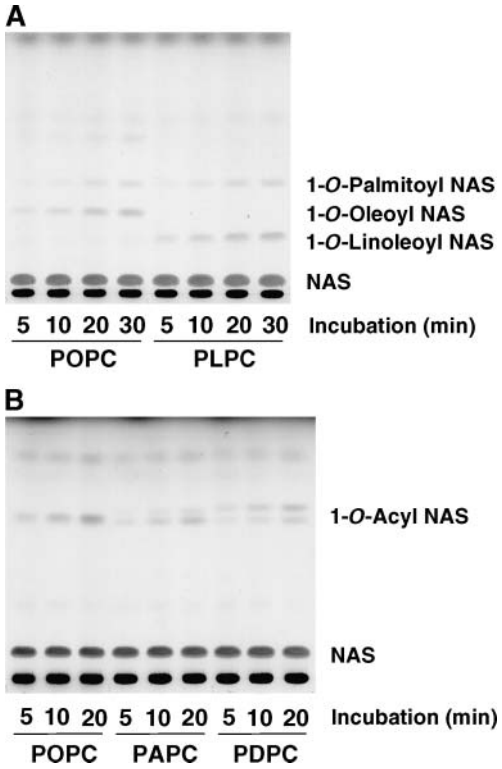

Fig. 4. Transacylase activity by the soluble fraction obtained from Lpla2-overexpressing MDCK cells in the presence of various 1-palmitoyl-2-unsaturated acyl-PCs. A: Liposomes consisting of POPC or 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (PLPC), sulfatide, and NAS were incubated with the soluble fraction obtained from Lpla2-overexpressing MDCK cells for 5, 10, 20, and 30 min at 37°C. The reaction products were extracted and separated by an argentation HPTLC plate using a solvent system consisting of chloroform-acetic acid-methanol (90:5:1). B: Liposomes consisting of 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PAPC) or 1-palmitoyl-2-docosahexanoyl-*sn*-glycero-3-phosphocholine (PDPC), sulfatide, and NAS were incubated with the soluble fraction obtained from Lpla2-overexpressing MDCK cells for 5, 10, 20, and 30 min at 37°C. The reaction products were extracted and separated by a regular HPTLC plate using a solvent system consisting of chloroform-acetic acid (9:1). For more details, see Materials and Methods.

1-*O*-Acyl-NASs including a polyunsaturated long chain acyl group such as arachidonoyl and docosahexanoyl groups were distinguishable from 1-*O*-palmitoyl-NAS using a regular HPTLC plate (Fig. 4B). In general, the mobility of 1-*O*-acyl-NAS with a long acyl chain group is greater than that of 1-*O*-palmitoyl-NAS. Interestingly, the specificity of Lpla2 for the *sn*-2 position was strikingly reversed when PAPC was used as an acyl group donor (Fig. 4B, Table 1). The rate of 1-*O*-palmitoyl-NAS formation from PAPC by Lpla2 was significantly higher than that from other 1-palmitoyl-2-unsaturated acyl-PCs (Table 1). In contrast, the rate of 1-*O*-arachidonoyl-NAS formation was noticeably lower than that of 1-*O*-acyl-NAS formation from the *sn*-2 acyl group transfer of other 1-palmitoyl-2-unsaturated acyl-PCs (Table 1). Consistent with the use of PAPC as substrate, the formation rate of 1-*O*-arachidonoyl-NAS from

TABLE 1. Positional specificity of Lpla2 on 1-palmitoyl-2-unsaturated phosphatidylcholines

Liposome	PLA_2 Activity	PLA_1 Activity	*sn*-2/*sn*-1
POPC	1-*O*-Oleoyl-NAS	1-*O*-Pal-NAS	
	100 ± 1.06	40.2 ± 1.18	2.49
PLPC	1-*O*-Linoleoyl-NAS		
	116 ± 5.14	55.9 ± 4.51	2.08
PAPC	1-*O*-Arachidonoyl-NAS		
	22.2 ± 4.01	91.1 ± 1.75	0.24
PDPC	1-*O*-Docosahexanoyl-NAS		
	68.9 ± 2.01	44.6 ± 2.35	1.54

Lpla2, lysosomal phospholipase A_2; NAS, *N*-acetylsphingosine; PAPC, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; PDPC, 1-palmitoyl-2-docosahexanoyl-*sn*-glycero-3-phosphocholine; PLA_2 and PLA_1, phospholipase A_2 and phospholipase A_1; PLPC, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine. The enzyme activity is somewhat different from time to time. The initial velocity in each assay was obtained as described in Materials and Methods. POPC, PLPC, PAPC, and PDPC indicate liposomes consisting of POPC, PLPC, PAPC, and PDPC, respectively/sulfatide/NAS (10:1:3). The PLA activities shown were calculated as follows: PLA_2 activity = 100 × [transacylase activity of Lpla2 to *sn*-2 of phosphatidylcholine (PC)]/(transacylase activity of Lpla2 to *sn*-2 of POPC); PLA_1 activity = 100 × (transacylase activity of Lpla2 to *sn*-1 of PC)/(transacylase activity of Lpla2 to *sn*-2 of POPC); *sn*-2/*sn*-1 = (PLA_2 activity)/(PLA_1 activity). Error indicates SD (n = 3).

C18 (Plasm)-20:4 PC liposomes by Lpla2 was 28% of that of 1-*O*-oleoyl-NAS from POPC (data not shown). In addition, when PDPC having a C22:6 acyl chain at the *sn*-2 position was used, Lpla2 acted on the *sn*-2 position preferentially but maintained sizable activity toward the *sn*-1 fatty acid (Fig. 4B, Table 1).

PE is not only a good substrate for Lpla2 but also a better substrate than PC (1, 2). Three separate 1-palmitoyl-2-unsaturated PEs were used as acyl group donors to confirm the positional specificity of Lpla2 for PE (**Fig. 5B**). Lpla2 was able to transfer both *sn*-1 and *sn*-2 acyl groups of PE to NAS, as observed when using 1-palmitoyl-2-unsaturated acyl-PCs (Fig. 5A). The positional specificity of Lpla2 for PE was similar to that for PC, although the ratio and rate of each produced 1-*O*-acyl-NAS were slightly different between PC and PE with the same asymmetric diacyl groups (Fig. 5A, B). The formation rates of 1-*O*-palmitoyl-NAS and 1-*O*-oleoyl-NAS from POPE were slightly lower than those from POPC. On the contrary, the transacylation rates of 1-*O*-linoleoyl-NAS and 1-*O*-palmitoyl-NAS from PLPE to NAS were obviously higher than those from POPC (**Table 2**). Although Lpla2 showed a preference for 1-*O*-palmitoyl-NAS formation from PAPE liposomes, the formation rate of 1-*O*-arachidonoyl-NAS was close to that of 1-*O*-palmitoyl-NAS (Table 2).

These results indicate that Lpla2 recognizes the unsaturated acyl chain at the *sn*-2 position preferentially but shows some activity toward the saturated acyl chain at the *sn*-1 position of most 1-palmitoyl-2-unsaturated acyl-PCs and PEs, with the exception of PAPC and PAPE. The positional specificity of Lpla2 for the *sn*-2 of phospholipid appears to be lessened by the polyunsaturated long-chain acyl group at the *sn*-2 position, particularly when an arachidonoyl group is present.

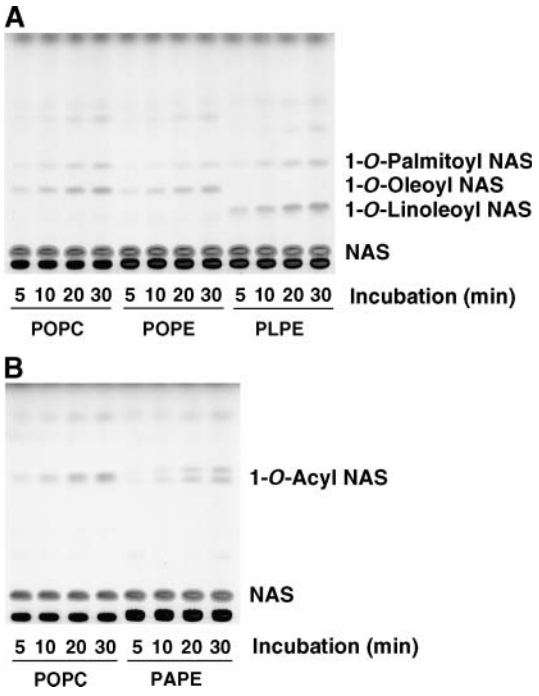

Fig. 5. Transacylase activity of the soluble fraction obtained from Lpla2-overexpressing MDCK cells in the presence of various 1-palmitoyl-2-unsaturated acyl-phosphatidylethanolamines. A: Liposomes consisting of POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), or 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine (PLPE), sulfatide, and NAS were incubated with the soluble fraction obtained from Lpla2-overexpressing MDCK cells for 5, 10, 20, and 30 min at 37°C. The reaction products were extracted and separated by an argentation HPTLC plate using a solvent system consisting of chloroform-acetic acid-methanol (90:5:1). B: Liposomes consisting of POPC or 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine (PAPE), sulfatide, and NAS were incubated with the soluble fraction obtained from Lpla2-overexpressing MDCK cells for 5, 10, 20, and 30 min at 37°C. The reaction products were extracted and separated by a regular HPTLC plate using a solvent system consisting of chloroform-acetic acid (9:1). The initial velocities for each product were estimated as described in the legend to Fig. 2 and are provided in Table 1.

TABLE 2. Positional specificity of Lpla2 on 1-palmitoyl-2-unsaturated phosphatidylethanolamines

Liposome	PLA_2 Activity	PLA_1 Activity	*sn*-2/*sn*-1
POPC	1-*O*-Oleoyl-NAS	1-*O*-Pal-NAS	
	100 ± 12.1	40.8 ± 7.70	2.45
POPE	1-*O*-Oleoyl-NAS		
	71.7 ± 14.1	26.3 ± 9.22	2.73
PLPE	1-*O*-Linoleoyl-NAS		
	185 ± 30.4	53.8 ± 9.83	3.44
PAPE	1-*O*-Arachidonoyl-NAS		
	45.6 ± .791	58.4 ± 2.13	0.78

PAPE, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine; PLPE, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine. The initial velocity in each assay was obtained as described in Materials and Methods. POPC, POPE, PLPE, and PAPE indicate liposomes consisting of POPC, POPE, PLPE, and PAPE, respectively/sulfatide/NAS (10:1:3). The transacylase activity of Lpla2 to *sn*-2 of POPC is 13.9 ± 1.68 μg/min/mg protein. The PLA activities shown were calculated as follows: PLA_2 activity = 100 × (transacylase activity of Lpla2 to *sn*-2 of PC or phosphatidylethanolamine)/(transacylase activity of Lpla2 to *sn*-2 of POPC); PLA_1 activity = 100 × (transacylase activity of Lpla2 to *sn*-1 of PC or phosphatidylethanolamine)/(transacylase activity of Lpla2 to *sn*-2 of POPC); *sn*-2/*sn*-1 = (PLA_2 activity)/(PLA_1 activity). Error indicates SD (n = 3).

Change of positional specificity of Lpla2 for PAPE and PAPC

In the previous study, Lpla2 purified from bovine brain showed that the PLA_1 activity of Lpla2 on PE is 7% of the total phospholipase A activity of Lpla2 (2). In that study, liposomes consisting of DOPC/1-palmitoyl-2-[^{14}C]arachidonoyl-*sn*-glycero-3-phosphoethanolamine/dicetyl phosphate/NAS (7:3:1:0.86 molar ratio) were used. The present study shows that up to 50% of the total transacylase activity of Lpla2 might be attributable to PLA_1 activity when liposomes consisting of PAPE/sulfatide/NAS (10:1:3 molar ratio) are used. Lpla2 activity is greatly affected by the lipid composition of liposomes (1) and is markedly inhibited by detergents such as Triton X-100, suggesting that Lpla2 requires a lipid bilayer to express its enzyme activity (2). In general, unsaturated PE has an inverted corn shape and forms hexagonal II structures in membranes. Therefore, when PAPE molecules are packed in liposomes with a lipid bilayer structure, the positional specificity of Lpla2 for PAPE may be improved.

To test this possibility, liposomes consisting of DOPC/PAPE/sulfatide/NAS (7:3:1:3 molar ratio) were examined (**Fig. 6**). Sulfatide was used as a negatively charged lipid instead of dicetyl phosphate because dicetyl phosphate is not distinguishable from 1-*O*-oleoyl-NAS in TLC analysis. The net amount of 1-*O*-arachidonoyl-NAS formed was estimated by subtracting the amount of 1-*O*-palmitoyl-NAS and 1-*O*-oleoyl-NAS formed from the total amount of 1-*O*-acyl-NAS formed, because the 1-*O*-arachidonoyl-NAS band overlaps with the NAS band in argentation TLC (Fig. 6B). Accordingly, the formation rate of 1-*O*-arachidonoyl-NAS produced from DOPC/PAPE/sulfatide/NAS liposomes was 11.3 μg/min/mg protein (Fig. 6C). The ratio of the 1-*O*-arachidonoyl-NAS formation rate to the 1-*O*-palmitoyl-NAS formation rate (6.65 μg/min/mg protein) was 1.7:1. When liposomes consisting of DOPC/PAPC/sulfatide/NAS (7:3:1:3 molar ratio) were used, a similar enhancement of 1-*O*-arachidonoyl-NAS formation was observed. However, the formation rate of 1-*O*-arachidonoyl-NAS produced, 1.18 μg/min/mg protein, was much less than that observed in DOPC/PAPE/sulfatide/NAS liposomes (Fig. 6C). The ratio of the formation rate of 1-*O*-arachidonoyl-NAS to 1-*O*-palmitoyl-NAS produced (1.77 μg/min/mg protein) was 0.66:1. These data indicate that the presence of PAPC or PAPE with DOPC in liposomes, and not as single phospholipids, promotes the deacylation of the arachidonoyl group at the *sn*-2 position of PAPC or PAPE rather than the deacylation of the palmitoyl group at the *sn*-1 position. The amounts of 1-*O*-palmitoyl-NAS and 1-*O*-arachidonoyl-NAS formed by Lpla2 from DOPC/PAPE/sulfatide/NAS liposomes were greater than those from DOPC/PAPC/sulfatide/NAS liposomes. On the other hand, the formation of 1-*O*-oleoyl-NAS by Lpla2 was

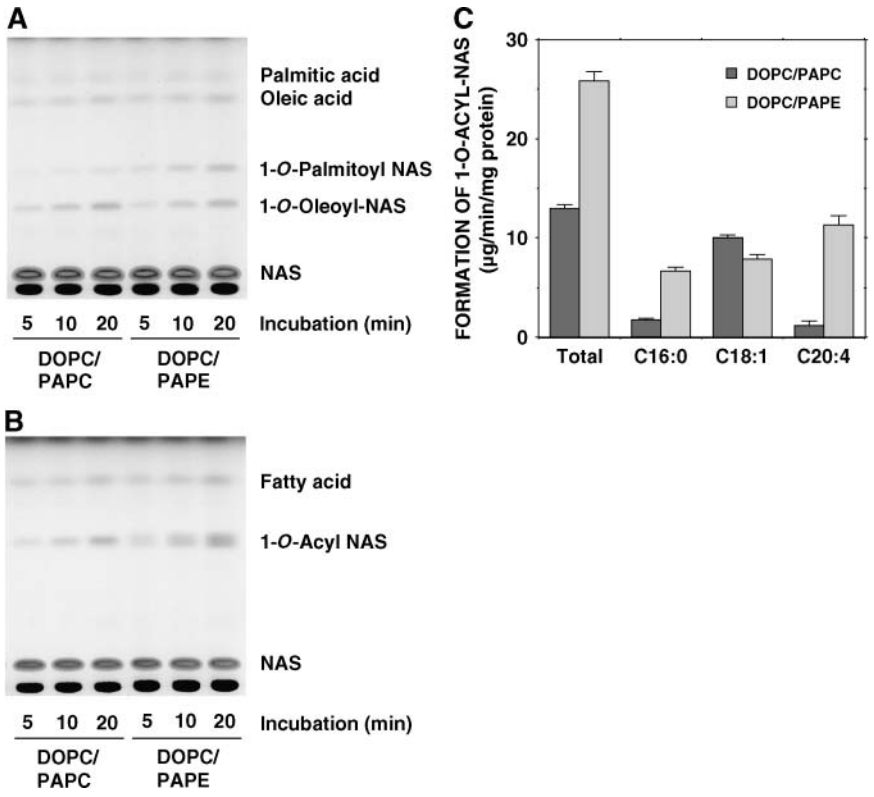

Fig. 6. Change of the positional specificity of Lpla2 for PAPE and PAPC by coexistence with 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC). Liposomes consisting of DOPC, PAPC, sulfatide, and NAS (7:3:1:3 molar ratio) or DOPC, PAPE, sulfatide, and NAS (7:3:1:3) were incubated with the soluble fraction obtained from Lpla2-overexpressing MDCK cells for 5, 10, and 20 min at 37°C. The reaction products were extracted and separated by an argentation HPTLC plate (A) or a regular HPTLC plate (B) using a solvent system consisting of chloroform-acetic acid (9:1). Individual 1-*O*-acyl-NASs are separable on the argentation plate with the exception of 1-*O*-arachidonoyl-NAS, which comigrates with NAS. The initial velocity for each product generated from DOPC/PAPC liposomes or DOPC/PAPE liposomes was estimated as described for Fig. 2 (C). The histogram represents means ± SD (n = 3). Error bars denote standard deviation.

not much different between the two types of liposomes. Therefore, Lpla2 may act on oleoyl groups of DOPC in both liposomes similarly. These results also indicate that PAPE is a better substrate for Lpla2 than PAPC and that the deacylation of the arachidonoyl group at the *sn*-2 position of PAPC and PAPE by Lpla2 is primarily regulated by the lipid composition of liposomes.

The transacylase activity of Lpla2 on PAPE was furthered characterized by changing the ratio of PAPE to DOPC in liposomes (**Fig. 7**). In this experiment, the total phospholipid concentration in the reaction mixture was maintained at the same concentration in each case. As expected, the addition of DOPC not only increased the Lpla2 activity on PAPE as its substrate but also changed the positional preference of Lpla2 on PAPE (Fig. 7). Both 1-*O*-palmitoyl-NAS and 1-*O*-arachidonoyl-NAS formation rates by Lpla2 showed bell-shaped curves (Fig. 7C). By contrast, the 1-*O*-oleoyl-NAS formation rate increased as the mol% of DOPC in the liposomes was increased, although a slight lag phase in the rate was observed at lower mol% of DOPC (Fig. 7C). An apparent decrease in the formation rate of 1-*O*-palmitoyl- and 1-*O*-arachidonoyl-NASs observed at higher mol% of DOPC in liposomes was likely attributable to a decreased PAPE concentration in the reaction mixture. On the other hand, the 1-*O*-palmitoyl- and 1-*O*-arachidonoyl-NASs formed at lower mol% of DOPC in liposomes could be related to the ability of PAPE to form a hexagonal phase in membranes.

After the initiation of this project, purified recombinant Lpla2 was successfully produced from stably transfected HEK cells. Therefore, the presence of both PLA_1 and PLA_2 activities in purified Lpla2 was verified. The formation of two 1-*O*-acyl-NASs, 1-*O*-palmitoyl-NAS and 1-*O*-oleoyl-NAS, by the purified Lpla2 was observed when the argentation HPTLC plate was used in the present assay system (**Fig. 8**). The ratio of PLA_2 activity to PLA_1 activity of the purified Lpla2 is 2.5:1. These results firmly support the view that Lpla2 itself is able to deacylate both acyl chains of phospholipid and that the recombinant protein exhibits the same properties seen in stably transfected MDCK cells.

DISCUSSION

This study, using the soluble fraction obtained from *Lpla2*$^{+/+}$ and *Lpla2*$^{-/-}$ mouse AMs, is consistent with the

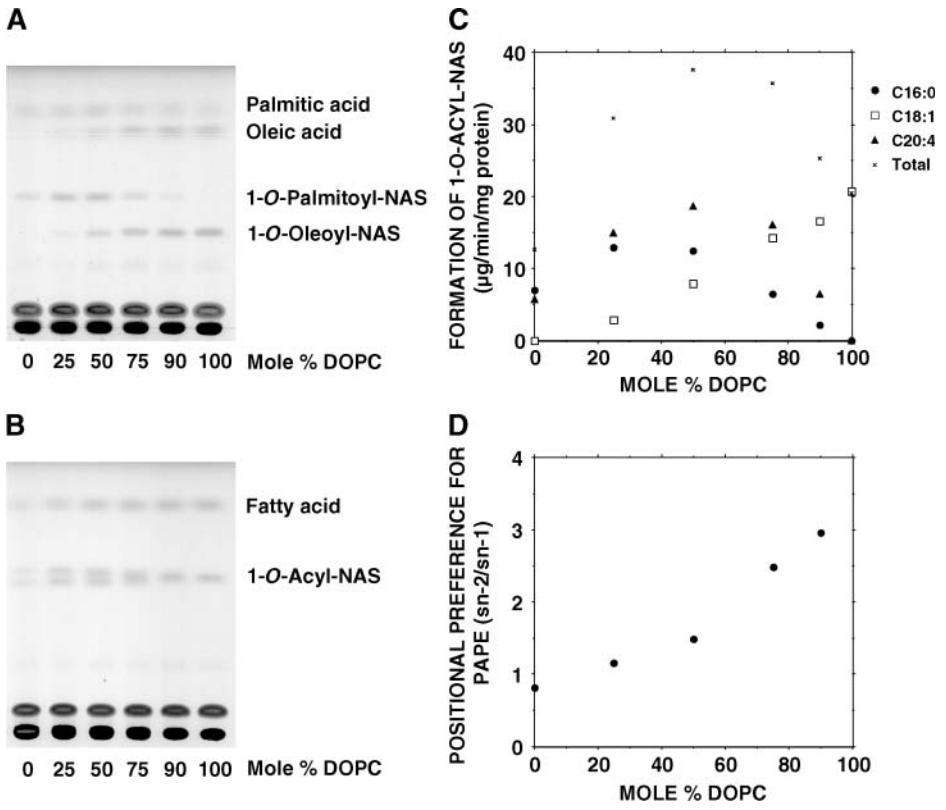

Fig. 7. Effect of DOPC added to PAPE liposomes on Lpla2 activity. Liposomes consisting of DOPC, PAPE, sulfatide, and NAS were incubated with the soluble fraction obtained from Lpla2-overexpressing MDCK cells for 10 min at 37°C. Under these conditions, 1-*O*-acyl-NAS formation was linear for 10 min. The molar ratio of phospholipid/sulfatide/NAS in liposomes was 10:1:3. The molar ratio of DOPC and PAPE was varied, although the phospholipid concentration in the reaction mixture was kept constant. The reaction products were extracted and separated by an argentation HPTLC plate (A) or a regular HPTLC plate (B) using a solvent system consisting of chloroform-acetic acid (9:1). C: The rate for each product generated from DOPC/PAPE liposomes determined by scanning the plate as described in Materials and Methods. D: Positional preference of Lpla2 for PAPE. *sn*-2/*sn*-1 = [phospholipase A_2 (PLA_2) activity]/[phospholipase A_1 (PLA_1) activity]. 1-*O*-Palmitoyl-NAS and 1-*O*-arachidonyl-NAS formation rates in C were used as measurements of PLA_1 and PLA_2 activities, respectively.

conclusion that Lpla2 has PLA_1 activity as well as PLA_2 activity. The transacylase activity of Lpla2 in the soluble fraction prepared from Lpla2-overexpressing MDCK cells was examined by thin-layer chromatography using an argentation HPTLC plate to confirm the positional specificity of Lpla2 for the phospholipids PC and PE. The soluble fraction containing Lpla2 was suitable for the transacylase activity measurement of Lpla2, because little 1-*O*-acyl-NAS was detected in the extracts of the vector control-transfected cells under acidic conditions. Therefore, 1-*O*-acyl-NAS formed by the soluble fraction in the presence of liposomes results from the transacylase activity of Lpla2.

Both the release of fatty acid and the formation of 1-*O*-acyl-NAS by Lpla2 were simultaneously observed when the reaction mixture contained PC and/or PE and NAS. In the initial reaction by the enzyme, the formation of 1-*O*-acyl-NAS was linear. As shown on most TLC plates in this study, the formation of 1-*O*-acyl-NAS was always preferred to the release of fatty acid. In our assay system, it was considerably more difficult to obtain accurate and precise values for the released fatty acids by scanning the plate, because the fatty acid bands on the plate are typically more diffuse and have lower density and higher background than the 1-*O*-acyl-NAS bands. In spite of such difficulties, however, in some instances (Fig. 2B) the release of fatty acid can be quantified. In this example, the reaction is linear and the formation rate of 1-*O*-oleoyl-NAS is four times higher than the release rate of oleic acid.

We previously reported that the fatty acid release and 1-*O*-acyl-NAS formation are linear in the initial reaction when purified bovine Lpla2 is incubated with liposomes containing DOPC, 1-palmitoyl-2-labeled-arachidonoyl-PE, and NAS (2). The relative level of PLA_2 versus transacylase activity is dependent on NAS concentration (1). We currently view Lpla2 as primarily a phospholipase A with moderate transacylase activity. As reported previously, Lpla2 is completely inhibited by DPF (9), cannot transfer free fatty acid or fatty acyl-CoA to NAS (1), and has the catalytic triad amino acid residues (serine, aspartate, and histidine) in common with some hydrolases (10). Site-directed mutagenesis at the putative catalytic serine residue of Lpla2 abolishes both the PLA_2 and transacylase

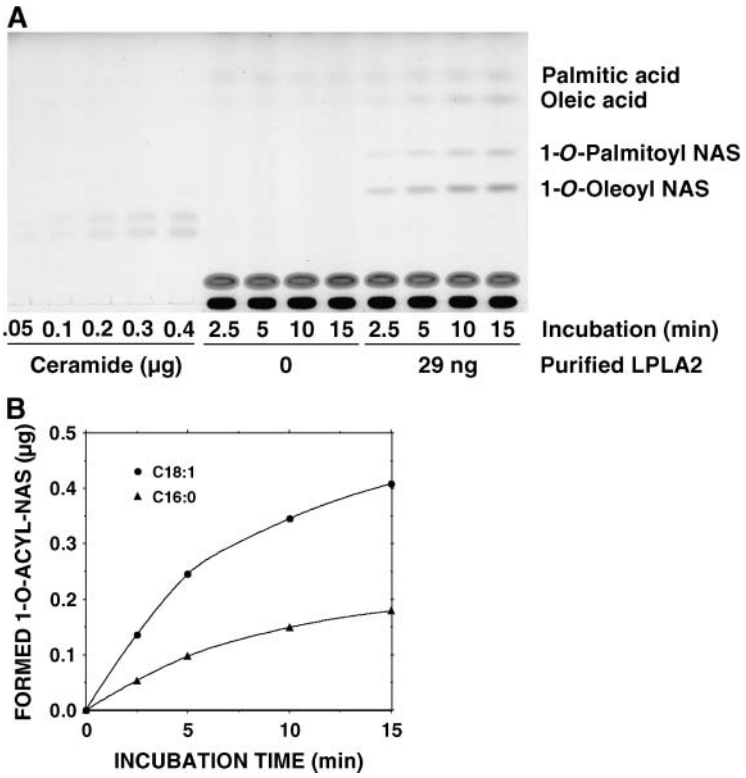

Fig. 8. Transacylase activity by purified recombinant mouse Lpla2 on POPC. The soluble fraction was prepared from HEK293 cells stably transfected with pcDNA3.1 histidine-tagged Lpla2. The enzyme was purified from the soluble fraction with a nickel Sepharose column. A: Liposomes consisting of POPC, sulfatide, and NAS were incubated with or without the purified enzyme (29 ng) for 2.5, 5, 10, and 15 min at 37°C. The reaction products were extracted and separated by an argentation HPTLC plate using a solvent system consisting of chloroform-acetic acid (9:1). B: Time course of the transacylation shown in A.

activities of Lpla2. These data support the interpretation that the mechanism of action of Lpla2 proceeds through an acyl enzyme intermediate during the enzyme reaction, as proposed for other hydrolytic enzymes with a similar catalytic triad. Thus, an acceptor of the acyl group such as NAS must compete with water in the deacylation by Lpla2. The linearity of both 1-*O*-acyl-NAS formation and fatty acid release in the initial reaction by Lpla2 demonstrates that the competition is constant in the initial reaction. Thus, the formation of 1-*O*-acyl-NAS or the release of fatty acid reflects the preference of deacylation between *sn*-1 and *sn*-2 positions in the initial reaction. Therefore, we used 1-*O*-acyl-NAS formation as the basis of the positional specificity of Lpla2, because in this assay system 1-*O*-acyl-NAS formation can be measured more accurately and precisely than free fatty acid release.

As confirmed in this study, Lpla2 is able to deacylate both *sn*-1 and *sn*-2 acyl groups of POPC and OPPC and transfer them to the hydroxyl group at the first carbon of NAS. In both cases, an oleoyl group is used more effectively than a palmitoyl group. In addition, the enzyme favors the oleoyl group at the *sn*-2 position of SOPC or the *sn*-1 position of OSPC. These results suggest that Lpla2 does not always demonstrate specificity for the fatty acid position on the glycerophosphate backbone. These results also suggest that Lpla2 displays specificity for an unsaturated acyl group over a saturated acyl group of PC.

The carbon chain of an oleoyl group containing one double bond is shorter than that of a stearoyl group but longer than that of a palmitoyl group. The transition temperatures of POPC, OPPC, SOPC, and OSPC are −2, −9, 6, and 9°C, respectively. The total activity for 1-*O*-acyl-NAS formation by Lpla2 may be correlated with the transition temperature. At temperatures above these transitions, unsaturated acyl chains in the lipid bilayer are more flexible than saturated acyl chains. Saturated acyl chains are well packed in the membranes. By contrast, unsaturated acyl chains perturb such order. Perturbed packing may increase the frequency at which the unsaturated acyl chains access the enzyme.

The reaction products generated from POPC and OPPC by Lpla2, palmitoyl-lysoPC and oleoyl-lysoPC, are detectable by TLC. The ratio of palmitoyl-lysoPC to oleoyl-lysoPC closely corresponds with that of 1-*O*-oleoyl-NAS to 1-*O*-palmitoyl-NAS. This finding indicates that both the transacylase activity and phospholipase A activity of Lpla2 proceed side by side. Lpla2 has a catalytic triad of amino acids and is thought to form an acyl enzyme intermediate via its catalytic serine residue during the deacylase reaction (3, 11). The constant ratio of 1-*O*-acyl-NAS to 2-acyl-lysoPC suggests that the formation of acyl enzyme is a rate-limiting step in the enzyme reaction. The formation of 1-*O*-oleoyl-NAS from C18 (Plasm)-18:1 PC liposomes demonstrates that Lpla2 has PLA_2 activity, as initially suggested by our previous study (3).

1-Palmitoyl-lysoPC and 1-oleoyl-lysoPC are poor substrates for Lpla2. In addition, lysophospholipids undergo acyl migration naturally, the migration of an acyl group from the *sn*-2 to the *sn*-1 dominating over that from the *sn*-1 to the *sn*-2 (12). Thus, deacylation from the *sn*-1 and *sn*-2 positions of OPPC as well as POPC by Lpla2 results from the direct action of Lpla2 itself and not through deacylation occurring in concert with acyl group migration.

As expected from our earlier work, Lpla2 demonstrated a preference for the *sn*-2 position of various 1-*O*-palmitoyl-

2-unsaturated acyl-PCs and acyl-PEs, with the exception of PAPC and PAPE. Polyunsaturated long-chain acyl groups such as arachidonoyl and docosahexanoyl groups at the *sn*-2 position of a phospholipid weaken the positional specificity of Lpla2 for the *sn*-2 position. Specifically, Lpla2 showed a predominant specificity for the *sn*-1 position over the *sn*-2 position of PAPC, although Lpla2 demonstrated some specificity for the *sn*-2 position of PDPC. Because polyunsaturated acyl groups at the *sn*-2 position in PC result in different packing characteristics from those observed with POPC and PLPC, the polyunsaturated species may reduce the *sn*-2 preference of Lpla2 by affecting the lipid bilayer structure. In support of this interpretation, we observed that an increase in the fraction of PAPE in the DOPC/PAPE liposomes resulted in a decrease in the formation of 1-*O*-acyl-NAS by Lpla2 and a lower preference of Lpla2 for *sn*-2 on PAPE (Fig. 7). By contrast, the addition of DOPC, a bilayer-forming species, improved the PLA_2 activity and positional preference on PAPE as substrate. Thus, Lpla2 acts preferentially on PE species when present in bilayer structures compared with hexagonal structures. Lpla2 needs a proper lipid bilayer structure and fluidity in lipid membranes to fully express its enzyme activity (1, 2). Therefore, the positional specificity of Lpla2 is substantially influenced by the phospholipid composition in liposomes and membranes.

Mouse Lpla2 has 49% homology to human lecithin:cholesterol acyltransferase (hLCAT). Both enzymes belong to the αβ-hydrolase superfamily and have the amino acid residues forming the catalytic triad: serine, aspartic acid/glutamic acid, and histidine. Although hLCAT is generally known to be specific for the *sn*-2 position of PC, hLCAT is able to transfer a significant percentage of the *sn*-1 position of the acyl group of certain PC species to cholesterol (13). Subbaiah and colleagues (13) reported that the contribution of the *sn*-1 acyl group from various 1-palmitoyl-2-acyl PCs for cholesteryl ester synthesis is 1.0% from 1,2-dipalmitoyl-PC, 1.4% from 1-palmitoyl-2-eicosatrinoyl-PC, 7.3% from POPC, 47% from 1-palmitoyl-2-eicosapentaenoyl-PC, 49.9% from PAPC, 54.9% from PDPC, and 72.3% from 1-palmitoyl-2-stearoyl-PC. They concluded that the positional specificity of hLCAT is affected by the chain length of the *sn*-2 acyl group rather than the carbon number or the number and position of double bonds of the acyl group. The positional specificity of Lpla2 is as broad for the *sn*-1 and *sn*-2 positions of phospholipids as hLCAT but is not correlated with the chain length of the acyl group in phospholipids.

As mentioned above, Lpla2 is able to selectively deacylate fatty acid from the *sn*-1 position of PC containing an arachidonoyl group at the *sn*-2 position. At the same time, 2-*O*-arachidonoyl-lysoPC is generated as a side product. Even if PAPC is present with DOPC, Lpla2 can deacylate a significant amount of the fatty acid from the *sn*-1 position of PAPC. The generation pathway of 2-*O*-arachidonoyl-lysoPC via Lpla2 is potentially interesting because 2-*O*-arachidonoyl-lysoPC is a precursor of 2-*O*-arachidonoyl-monoglycerol. 2-*O*-Arachidonoyl-monoglycerol is a natural product in various mammalian tissues other than blood plasma, in particular brain (14–16), an endogenous ligand for the central (CB1) and peripheral (CB2) cannabinoid receptors (17, 18), and a substrate for cyclooxygenase-2 (19). Recently, Yan et al. (20) showed that purified human $iPLA_2$ is characterized by highly selective PLA_1 activity to PAPC and does not significantly hydrolyze 2-*O*-arachidonoyl-lysoPC. Additionally, they demonstrated that 2-*O*-arachidonoyl-lysoPC accumulates after the incubation of purified rat hepatic peroxisomes with $iPLA_2$ and is a natural product in human myocardium, where $iPLA_2$ is expressed strongly (20). These observations indicate that the generation pathway of 2-*O*-arachidonoyl-lysoPC through $iPLA_2$ may link to eicosanoid signaling in mammalian cells and add diversity to the well-known eicosanoid signaling process that is induced via arachidonic acid release from PAPC by cytosolic PLA_2 activation. The potential involvement of Lpla2 in the generation of 2-arachidonoyl-lysoPC is unknown, although Lpla2 can produce 2-arachidonoyl-lysoPC from PAPC in vitro. It will be of interest to investigate the generation and accumulation of 2-*O*-arachidonoyl-lysoPC through Lpla2 in mammalian tissues using *Lpla2*$^{-/-}$ mice.

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