The acylation of lipophilic alcohols by lysosomal phospholipase A_2

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Abstract **A novel lysosomal phospholipase A_2 (LPLA2) with specificity toward phosphatidylethanolamine and phosphatidylcholine was previously purified and cloned. LPLA2 transfers *sn*-1 or *sn*-2 acyl groups of phospholipids to the C1 hydroxyl of the short-chain ceramide *N*-acetylsphingosine (NAS) under acidic conditions. The common features of lipophilic alcohols serving as acceptor molecules in the transacylase reaction were examined. 1-*O*-Hexadecyl-2-acetyl-*sn*-glycerol (HAG) was acylated by LPLA2 similar to NAS. HAG competed with NAS and inhibited NAS acylation. The transacylation of 1-*O*-hexadecyl-glycerol (HG), 1-*O*-palmityl-2-*O*-methyl-*sn*-glycerol (PMG), and monoacylglycerols was also investigated. HG, PMG, 1- or 3-palmitoyl-*sn*-glycerol, and 2-palmitoylglycerol were converted to 1,3-alkylacylglycerol, 1,2-dialkyl-3-acylglycerol, 1,3-diacylglycerol, and 1,2- or 2,3-diacylglycerol, respectively. HG and monoacylglycerol inhibited the acylation of NAS by the enzyme with IC_{50} values of 35 and 45 μM, respectively. Additionally, the enzyme acylated glycerol to produce 1- or 3-acyl-*sn*-glycerol but not 2-acylglycerol. Therefore, the preferred acceptor molecules for LPLA2 are primary alcohols with one long carbon chain and one small nonpolar residue linked to the C2 position of ethanol. The enzyme acylated other natural lipophilic alcohols, including anandamide and oleoylethanolamide. Thus, LPLA2 may function to remodel acyl groups and modulate the biological and pharmacological activities of some lipophilic alcohols.—**Abe, A., M. Hiraoka, and J. A. Shayman. **The acylation of lipophilic alcohols by lysosomal phospholipase A_2. *J. Lipid Res.* 2007.** 48: **2255–2263.**

Supplementary key words *N*-acetylsphingosine • ceramide • anandamide • 1-*O*-hexadecyl-2-acetyl-*sn*-glycerol • D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-propanol • amiodarone

A novel acidic phospholipase A_2 (PLA2) activity was found in MDCK cell homogenates (1). This activity was recovered in the soluble fraction and localized to lysosomes (2). The enzyme displayed both transacylase and PLA2 activities and a preference for phosphatidylethanolamine (PE) and phosphatidylcholine (PC) as substrate compared with other phospholipids (1). Lysosomal phospholipase A_2 (LPLA2) was purified from bovine brain and was characterized as a water-soluble glycoprotein consisting of a single polypeptide chain with a molecular mass of 45 kDa (3). The purified protein retained the characteristics of an acidic Ca^{2+}-independent PLA2 with transacylase. Protein and nucleotide sequencing demonstrated that LPLA2 is encoded by the same gene as lecithin: cholesterol acyltransferase-like lysophospholipase, and that the primary structure of LPLA2 is highly preserved between mammals (2, 4). Because the lysophospholipase activity of the enzyme is very low compared with the PLA2 activity, we have proposed LPLA2 as the preferred nomenclature.

LPLA2 is posttranslationally modified by both signal peptide cleavage and *N*-glycosylation (2). The amino acid sequence of LPLA2 has 49% identity to LCAT and belongs to the αβ-hydrolase superfamily. The catalytic triad and four cysteine residues conserved in LPLA2 are preserved in LCAT (2, 5). LPLA2 is now termed the group XV PLA2 (6).

LPLA2 is most highly expressed in alveolar macrophages (AMs) (7). The LPLA2 activity and protein expression are extremely low in AMs from granulocyte macrophage colony stimulating factor$^{-/-}$ mice, a model of pulmonary alveolar proteinosis. *LPLA2*$^{-/-}$ mice generated by systemic deletion of LPLA2 gene exon 5, which encodes the catalytic site, lost LPLA2 activity systemically (8). A marked accumulation of phospholipids, in particular PE and PC, was found in AM, peritoneal macrophages (PMs), and spleen in *LPLA2*$^{-/-}$ mice at an early stage. Ultrastructural analysis revealed extensive lamellar inclusion bodies, a hallmark of cellular phospholipidosis, in the *LPLA2*$^{-/-}$ mouse AM and PM. Therefore, LPLA2 may play a critical role in cellular phospholipid homeostasis in those macrophages.

The fact that LPLA2 possesses transacylase activity as well as PLA2 activity suggests an alternative role for LPLA2 besides phospholipid homeostasis. There are many biosynthetic and synthetic bioactive lipophilic alcohols, some of whose activities may be modulated via acylation of the

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hydroxyl group in those alcohols. Ceramide is one of the natural bioactive lipophilic alcohols and is involved in cell growth, proliferation, differentiation, development, and apoptosis (9, 10). *N*-Acetylsphingosine (NAS), also known as C2 ceramide, has been used to mimic the effect of ceramide on cells for a long time among many researchers. Our previous report showed that NAS is a favorable acceptor in the transacylation reaction by LPLA2 (1). On the contrary, a long acyl chain ceramide was acylated by the enzyme but behaved poorly as an acceptor in an in vitro assay. Other bioactive lipophilic alcohols, such as diolein and sphingosine, were also observed to be poor acceptors. These results suggest that the acceptor molecules used in the transacylation reaction by LPLA2 have a common chemical structure. Based on an ethanol structure, it was proposed that at least one long carbon chain group and one small neutral residue linked to the C2 position of ethanol are requisite to be a preferred acyl acceptor for transacylation by the enzyme. In this study, we confirm the existence of this structural requirement for acceptor molecules in the transacylation reaction. We investigated the acylation of biosynthetic and synthetic lipophilic alcohols using recombinant mouse LPLA2. We also observed that LPLA2 can not only deacylate acyl groups of acylated lipophilic alcohols but also transacylate acyl groups of acylated lipophilic alcohols to acceptor lipophilic alcohols. In addition, the accumulation of a certain lipophilic alcohol was found in MDCK cells treated with cationic amphiphilic drugs (CADs), which inhibit the transacylase activity of LPLA2 (11). This study suggests another role for LPLA2 in addition to phospholipid catabolism.

MATERIALS AND METHODS

Reagents

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1-*O*-hexadecyl-2-acetyl-*sn*-glycerol (HAG) were purchased from Avanti. Amiodarone, 1-*O*-hexadecyl-*rac*-glycerol (HG), oleoylethanolamide (OEA), anandamide (AEA), 1-*O*-palmityl-2-*O*-methyl-*sn*-glycerol (PMG), 1-palmitoyl-*rac*-glycerol, 2-palmitoylglycerol, and 3-palmitoyl-*sn*-glycerol were from Sigma. NAS was obtained from Matreya. The BCA protein assay reagent was from Pierce. High-performance thin-layer chromatography (HPTLC) silica gel plates, 10 × 20 cm, were from Merck. D-*Threo*-1-phenyl-2-decanoylamino-3-morpholino-propanol (PDMP) was synthesized previously in our laboratory (12).

Transacylase activity of LPLA2

The reaction mixture consisted of 40 mM sodium citrate (pH 4.5), 10 μg/ml BSA, 40 μM acceptor incorporated into phospholipid liposomes (DOPC/sulfatide/acceptor, 10:1:3.2 in molar ratio), and recombinant mouse LPLA2 (29 ng) in a total volume of 500 μl. The reaction was initiated by adding the enzyme, incubating for 5–30 min at 37°C, and terminating the reaction by adding 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 resultant lower organic 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) and applied on an HPTLC plate or an argentation HPTLC plate and developed in a solvent system consisting of chloroform-acetic acid (90:10, 95:5, or 96:4, v/v) or chloroform-methanol-acetic acid (98:2:1 or 90:1:9, v/v). The plate was dried and soaked in 8% (w/v) $CuSO_4$, $5H_2O$, 6.8% (v/v) H_3PO_4, and 32% (v/v) methanol until uniform. The wet plate was briefly dried with a hair dryer and charred for 15 min in a 150°C oven. For production of an argentation HPTLC plate, the HPTLC plate was immersed into 10% (w/v) $AgNO_3$ in acetonitrile and incubated for 10 min. The plate was dried in a hood and baked in an oven for 30 min at 100°C. When using an argentation plate, to prevent the silica gel peeling off the plate during incubation with the copper solution, the dried plate was once incubated in 20% (v/v) methanol containing 0.5% acetic acid until the background of the plate became homogeneous. The plate was subsequently treated as described above. The plate was scanned, and the content of the product (1-*O*-acyl-NAS) was estimated by the use of NIH Image 1.63.

Cell culture

MDCK cells seeded on a six-well plate were grown in 2 ml of DMEM-F12 medium containing 10% FCS with 1× antibiotic-antimyotic (Invitrogen). The cells were maintained in an incubator with 5% CO_2 at 37°C. After reaching 90% confluence, the cells were treated with or without CADs, as indicated in the figure legends.

After the treatment, the cells in each well were washed three times with 2 ml of cold PBS, scraped with 1 ml of cold PBS, and transferred into a glass tube (12.5 × 130 mm). Another 1 ml of PBS was used to recover the remaining cells in the dish. Five milliliters of methanol plus 2.5 ml of chloroform were added into the tube and sonicated briefly with a water-bath-type sonicator. The mixture stood for 1 h and was centrifuged for 30 min at 2,000 *g* at room temperature. The supernatant was transferred into a glass tube (12.5 × 160 mm), mixed with 2.5 ml of chloroform plus 2.5 ml of 0.9% NaCl, and centrifuged for 5 min at 800 *g* at room temperature. The resulting lower layer was washed twice with 2.5 ml of methanol plus 2 ml of 0.9% NaCl and then once with 2.5 ml of methanol plus 2 ml of water. The lower organic layer was transferred into a glass tube (10 × 130 mm), dried down under a stream of nitrogen gas, and used for lipid analysis.

RESULTS

Acylation of HAG and inhibition of the formation of 1-*O*-acyl-NAS by HAG

The transacylase reaction by LPLA2 was originally found when NAS was present with phospholipid in the reaction mixture (1). NAS, a synthetic short acyl chain ceramide, was noted to be a better acceptor of the transacylase reaction with PC or PE as donor than long acyl chain ceramides. NAS is a lipophilic alcohol with one primary alcohol residue and one small neutral residue (acetyl group) linked to the C2 position of a sphingol backbone. However, NAS is less hydrophobic than natural ceramides that are characterized by the presence of a long acyl carbon chain. Conceivably, there are a number of natural and nonnatural lipophilic alcohols that have a similar structure to NAS. Some of these lipophilic alcohols are bioactive and could serve as the acceptor in the transacylation reaction by LPLA2.

Purified recombinant mouse LPLA2 was used in the present study to evaluate this possibility. Both the release

of fatty acid and the formation of 1-*O*-acyl-NAS were observed when the recombinant LPLA2 was incubated with liposomes containing NAS (**Fig. 1A**). Because DOPC was used as a substrate in the assay system, the released fatty acid and the formed acyl-NAS were oleic acid and 1-*O*-oleoyl-NAS, respectively. HAG is an alkylacylglycerol with a primary alcohol group at the C3 position. HAG is structurally very similar to NAS. As expected, the enzyme produced two main products when HAG coexisted with DOPC in the reaction mixture (Fig. 1A). One product possessed the same mobility as free fatty acid on the TLC plate (Fig. 1A). The other, a nonpolar product, was characterized by the highest mobility on the TLC plate and was degraded to HAG and methyl ester by alkaline methanolysis (data not shown). These data indicate that LPLA2 acylates the hydroxyl group at the C3 position of HAG. The acyl product must be 1-*O*-hexadecyl-2-acetyl-3-oleoyl-*sn*-glycerol. In this assay system, the rate of formation of the acyl compounds produced by the enzyme was linear for 5 min (data not shown). The initial velocity of acyl-HAG formation by the enzyme was two times higher than the formation of acyl-NAS. As expected, HAG inhibited the formation of 1-*O*-acyl-NAS when HAG was present with NAS in the liposomes (Fig. 1A). HAG inhibited the acylation of NAS in a dose-dependent manner with an IC_{50} of 25 μM (Fig. 1B).

Acylation of HG, PMG, and monoacylglycerols

To more generally characterize the structural requirements of other alcohols as acceptors in the transacylation by LPLA2, structurally similar lipophilic alcohols, including 1-*O*-monoalkylglycerol, 1,2-dialkylglycerol, and monoacylglycerols to HAG, were investigated. HG is an ether-lipid monoalkylglycerol with two hydroxyl groups located at the C2 position and at the C3 position. Free fatty acid and nonpolar product were produced when the enzyme was incubated with liposomes containing HG (**Fig. 2A**). The nonpolar product was degraded to methyl ester and the parent compound by alkaline methanolysis (data not shown). These findings suggest that the enzyme acylates the hydroxyl group at the C2 or C3 position of HG. The mobility of the alkaline labile product [relative mobility (R_f) ~ 0.74] was lower than that of triacylglycerol (TAG) (R_f ~ 0.90) but slightly higher than that of 1,3-diacylglycerol (DAG) (R_f ~ 0.70) in the TLC assay (Fig. 2A). The mobility of 1,3-alkylacylglycerol is always greater than that of 1,3-DAG in the solvent system used in Fig. 2A. By contrast, the mobility of 1,2-alkylacylglycerol is lower than that of 1,3-DAG. Thus, the product produced from HG by the enzyme is most likely to be 1-*O*-hexadecyl-3-oleoyl-*rac*-glycerol. These data indicate that the enzyme acylates the hydroxyl group at the C3 position in HG but not at the C2 position. The initial velocity of acyl-HG formation by the enzyme was 50% of that of acyl-NAS. HG also inhibited the transacylation of NAS by LPLA2 in a dose-dependent manner with an IC_{50} of 35 μM (Fig. 2D).

PMG is a 1,2-dialkylglycerol, and the hydrophobicity of the side chain at the C2 position of PMG appears to be between that of HAG and HG. As observed in the HAG and NAS studies, the enzyme converted PMG to a more nonpolar product, which was alkaline unstable (data not shown). The nonpolar product of the transacylation of PMG was 1-*O*-palmityl-2-*O*-methyl-3-oleoyl-*sn*-glycerol (Fig. 2B). The initial velocity of acyl-PMG formation by the enzyme was 80% of that of acyl-NAS.

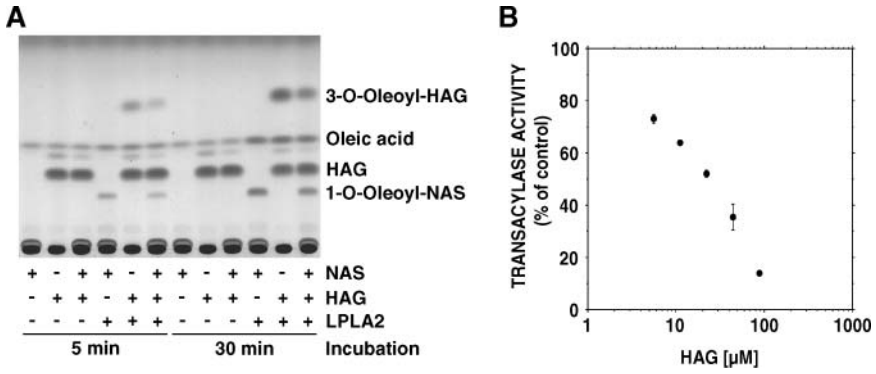

Fig. 1. Acylation of 1-*O*-hexadecyl-2-acetyl-*sn*-glycerol (HAG) by lysosomal phospholipase A_2 (LPLA2) and inhibition of the acylation of *N*-acetylsphingosine (NAS) by HAG. A: Acylation of HAG by LPLA2. The reaction mixture consisted of 48 mM Na-citrate (pH 4.5), 58 ng/ml purified mouse LPLA2, and liposomes containing 123 μM 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 12.3 μM sulfatide plus 40 μM acceptor (NAS, HAG, or NAS and HAG) in a total volume of 500 μl. The reaction was initiated by the addition of enzyme and kept for 5 and 30 min at 37°C. After the incubation, total lipids were extracted, applied to a high-performance thin-layer chromatography (HPTLC) plate, and developed in a solvent system consisting of chloroform-acetic acid (96:4, v/v). 3-*O*-Oleoyl-HAG denotes 1-*O*-hexadecyl-2-acetyl-3-oleoyl-*sn*-glycerol. B: Inhibition of esterification of NAS by HAG. The reaction mixture consisted of 48 mM Na-citrate (pH 4.5), 58 ng/ml purified mouse LPLA2, and liposomes containing 123 μM DOPC, 12.3 μM sulfatide, and 10 μM NAS plus different concentrations of HAG in a total volume of 500 μl. The mixture was incubated for 5 min at 37°C. A minor band on the plate with a slightly slower mobility than oleic acid is attributable to a small contamination contained in HAG. Error bars indicate SD (n = 3).

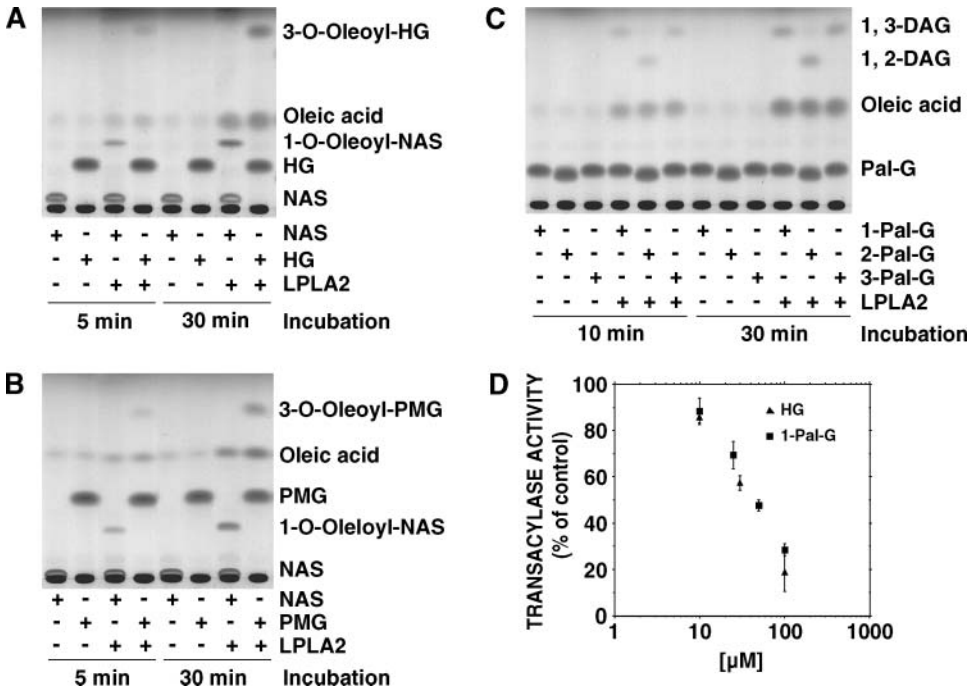

Fig. 2. Acylation of lipophilic alcohols by LPLA2 and inhibition of the acylation of NAS by lipophilic alcohols. A–C: Acylation of lipophilic alcohols by LPLA2. The reaction mixture consisted of 48 mM Na-citrate (pH 4.5), 58 ng/ml purified mouse LPLA2, and liposomes containing 123 μM DOPC and 12.3 μM sulfatide plus 40 μM 1-*O*-hexadecyl-*rac*-glycerol (HG), 40 μM 1- or 3-palmitoyl-*sn*-glycerol (1- or 3-Pal-G), 40 μM 2-palmitoylglycerol (2-Pal-G), or 40 μM 1-*O*-palmityl-2-*O*-methyl-*sn*-glycerol (PMG) in a total volume of 500 μl. The mixture was incubated for 5, 10, or 30 min at 37°C. After the incubation, total lipids were extracted, applied to an HPTLC plate, and developed in a solvent system consisting of chloroform-methanol-acetic acid (98:2:1, v/v). 3-*O*-Oleoyl-HG and 3-*O*-oleoyl-PMG denote 1-*O*-hexadecyl-3-oleoyl-*rac*-glycerol and 1-*O*-palmityl-2-*O*-methyl-3-oleoyl-*sn*-glycerol, respectively. D: Inhibition of the esterification of NAS by HG or 1-Pal-G. The reaction mixture consisted of 48 mM Na-citrate (pH 4.5), 58 ng/ml purified mouse LPLA2, and liposomes containing 123 μM DOPC, 12.3 μM sulfatide, and 10 μM NAS plus different concentrations of HG or 1-Pal-G in a total volume of 500 μl. The mixture was incubated for 5 min at 37°C. Error bars indicate SD (n = 3).

As expected from the results with HG and PMG, LPLA2 produced free fatty acid and a nonpolar product when the reaction mixture liposomes consisted of phospholipid and monoacylglycerol (MAG) (Fig. 2C). The nonpolar product was converted to methyl ester and glycerol by alkaline methanolysis (data not shown). The mobility of the nonpolar products produced from 1-palmitoylglycerol and 3-palmitoylglycerol were the same as that observed with 1,3-DAG but not with 1,2-DAG by TLC analysis. On the contrary, the mobility of the nonpolar product produced from 2-palmitoylglycerol was the same as that observed with 1,2-DAG. Both primary alcohol groups at C1 and C3 in 2-palmitoylglycerol, on stereochemical grounds, have an equal chance to conjugate the acyl group in the transacylase reaction by LPLA2. Therefore, the resultant product in that reaction is most likely a mixture of 1,2- and 2,3-DAGs. No formation of TAG was observed in either case. The initial velocity of any DAG formed by the enzyme was 30% of that observed for 1-*O*-acyl-NAS. The preliminary study showed that each monoacylglycerol has comparable ability to inhibit 1-*O*-acyl-NAS formation by the enzyme (data not shown). 1-Palmitoyl-*rac*-glycerol inhibited the transacylation of NAS by the enzyme in a dose-dependent manner with an IC_{50} of 45 μM (Fig. 2D). These data suggest that a preferred acceptor molecule consists of a primary alcohol with one long carbon chain plus one small neutral residue with a weak hydrophobicity linked to the core carbon.

Acylation of glycerol by LPLA2

To confirm the selectivity of the hydroxyl group in the acceptor molecule, a simpler alcohol was examined. Glycerol, unlike other simple alcohols such as methanol and ethanol, stabilizes protein in solution even when used at high concentrations. A preliminary study demonstrated that glycerol is very poor as an acceptor. However, the acylation of glycerol by LPLA2 was observed when a high concentration of glycerol (30%, w/v) is used in the reaction mixture. The enzyme produced two compounds when it was incubated with liposomes in the presence of 30% glycerol (**Fig. 3A**). One product was free fatty acid. The other product was an alkaline unstable compound that showed the same mobility as 1-MAG or 3-MAG, but not 2-MAG, by TLC analysis. These results indicate that the alkaline unstable compound produced from glycerol by the enzyme is 1-MAG or 3-MAG. Free fatty acid is not

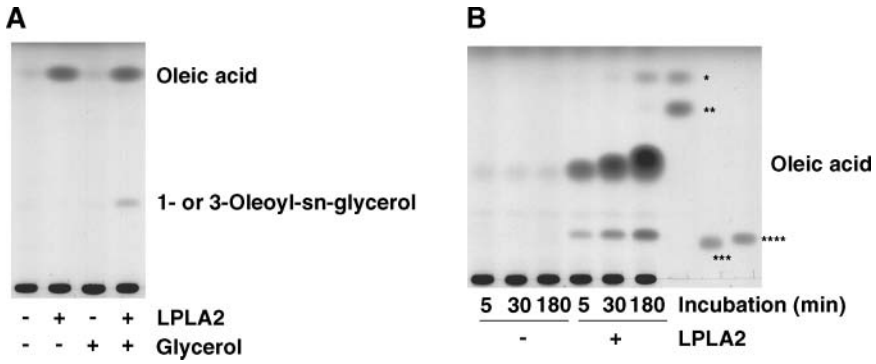

Fig. 3. A: Acylation of glycerol by LPLA2. The reaction mixture consisted of 48 mM Na-citrate (pH 4.5), 30% (w/v) glycerol, 116 ng/ml purified mouse LPLA2, and liposomes containing 123 μM DOPC and 12.3 μM sulfatide in a total volume of 500 μl. The mixture was incubated for 5 min at 37°C. After the incubation, total lipids were extracted, applied to an HPTLC plate, and developed in a solvent system consisting of chloroform-methanol-acetic acid (90:1:9, v/v). B: The same reaction mixture was incubated for 5, 30, and 180 min at 37°C. The plate was developed in a solvent system consisting of chloroform-methanol-acetic acid (98:2:1, v/v). Single, double, triple, and quadruple asterisks indicate 1,3-DAG, 1,2-DAG, 2-palmitoylglycerol, and 1- or 3-palmitoyl-*sn*-glycerol, respectively.

separated from DAG in the solvent system used in Fig. 3A. The study in Fig. 2B demonstrated that 1-MAG or 3-MAG is converted to 1,3-DAG by the enzyme. With the use of a different solvent system, we observed that 1-MAG or 3-MAG formed from glycerol by the enzyme is further acylated to 1,3-DAG but not 1,2-DAG with time (Fig. 3B). These data suggest that the acylation of the acceptor molecule in the transacylase reaction occurs at a primary alcohol group but not a secondary alcohol group. Consistent with this finding is the prior observation that cholesterol is a very poor acceptor in the transacylase reaction by LPLA2, in distinction to LCAT (2, 4).

Structural requirement of acceptors

A common structural feature between NAS and other lipophilic alcohols that act as an acceptor of the acyl group in the transacylation emerges from the present studies (**Fig. 4**). Position X in Fig. 4 is a long carbon chain group such as an aliphatic group and acyloxy group, which contributes to the hydrophobicity of the molecule. Position Y is a small neutral residue that is not bulky. Introduction of an *O*-acetyl group or an *N*-acetyl group to position Y renders the alcohol molecule more active as an acceptor of the acyl group. On the contrary, the addition of a long carbon acyl chain group at position Y provides a negative effect on transacylase activity by LPLA2 (1). Actually, natural ceramides and DAGs with a long carbon acyl chain at the Y position are extremely poor as acceptors. Thus, the preferential acceptor molecule may be generalized to consist of derivatives of ethanol that possess one long carbon chain group and one small neutral residue linked at the C2 position of an ethanol molecule.

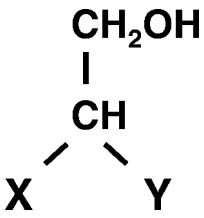

Fig. 4. Common features of lipophilic alcohol as an acyl acceptor. X, a long carbon chain group; Y, -H, -OH, -CH_2OH, -OCH_3, -$OCOCH_3$, or -$NHCOCH_3$.

Acylation of other biosynthetic lipophilic alcohols by LPLA2

Acylethanolamides such as OEA and AEA are biosynthetic and neutral products that have one primary alcohol group and one long carbon chain group at carbon positions 1 and 2, respectively. According to the hypothesis for the structural requirement shown in Fig. 4, it was predicted that LPLA2 would acylate the hydroxyl group of acylethanolamides. Indeed, the enzyme converted OEA and AEA to nonpolar compounds with a higher mobility than their parent compounds in the TLC assay (**Fig. 5A**). The nonpolar products produced from OEA and AEA were converted to OEA and methyl ester and AEA and methyl ester, respectively, by alkaline methanolysis (data not shown). In this study, the nonpolar products produced from OEA and AEA are *O*-oleoyl-OEA and *O*-oleoyl-AEA, respectively. Both acylethanolamides competed with NAS in the transacylase reaction by LPLA2 (data not shown), as did HAG and other lipophilic glycerols. AEA inhibited the transacylation of NAS by the enzyme in a dose-dependent manner with an IC_{50} of 45 μM (Fig. 5B). These results further support the hypothesis for the structural requirement of the lipophilic alcohol as an acyl acceptor in the transacylase reaction by LPLA2.

Deacylation and transacylation of the acyl group of alcohols acylated by LPLA2

In previous studies, we observed that unlike fatty acids released from phospholipids by LPLA2, the levels of acyl

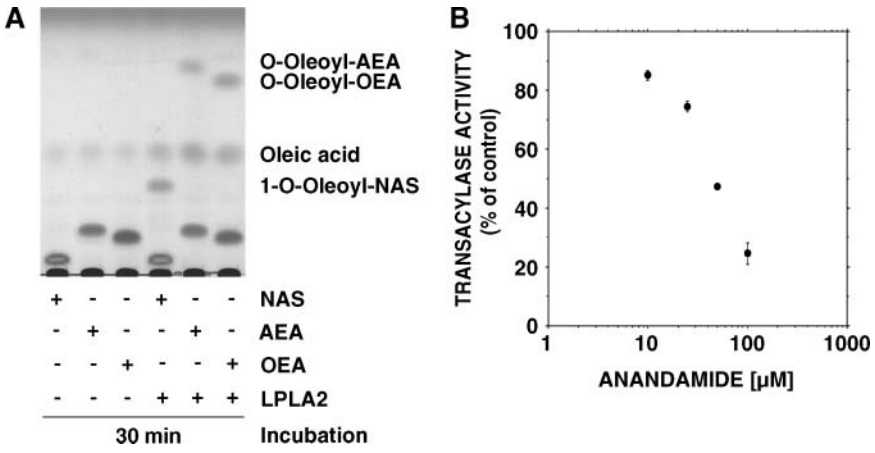

Fig. 5. Acylation of acylethanolamide by LPLA2. A: Acylation of anandamide (AEA) and oleoylethanolamide (OEA) by LPLA2. The reaction mixture consisted of 48 mM Na-citrate (pH 4.5), 58 ng/ml purified mouse LPLA2, and liposomes containing 123 μM DOPC and 12.3 μM sulfatide plus 40 μM NAS, 40 μM AEA, or 40 μM OEA in a total volume of 500 μl. The reaction was initiated by the addition of the enzyme and kept for 30 min at 37°C. After the incubation, total lipids were extracted, applied to an HPTLC plate, and developed in a solvent system consisting of chloroform-methanol-acetic acid (98:2:1, v/v). B: Inhibition of the esterification of NAS by AEA. The reaction mixture consisted of 48 mM Na-citrate (pH 4.5), 58 ng/ml purified mouse LPLA2, and liposomes containing 123 μM DOPC, 12.3 μM sulfatide, and 10 μM NAS plus different concentrations of ANA in a total volume of 500 μl. The mixture was incubated for 5 min at 37°C. Error bars indicate SD (n = 3).

compounds produced by LPLA2 always reached a certain plateau and gradually declined with time in the transacylase reaction (1, 3). This observation suggested that there may be degradation of the acylated lipophilic alcohol produced in the reaction mixture. Therefore, the acyl compound produced by LPLA2 was possibly deacylated by the same enzyme. To confirm the presence of the reverse reaction, the deacylation and transacylation of the acyl group of transacylated products produced by LPLA2 was investigated.

1-*O*-Palmitoyl-NAS, one of the acyl compounds produced by LPLA2, was synthesized chemically and incorporated into liposomes. When liposomes containing DOPC and 1-*O*-palmitoyl-NAS were incubated with LPLA2, a substantial decrease of 1-*O*-palmitoyl-NAS and the generation of several products were noted by TLC assay using an argentation plate (**Fig. 6A**). The main product in the reaction corresponded to oleic acid. The other products corresponded to palmitic acid and 1-*O*-oleoyl-NAS. Although it was difficult to see all products in this assay, the band corresponding to NAS was identified as one of the reaction products by another TLC assay (data not shown). These results indicate that oleic acid was released from DOPC and that palmitic acid and NAS were produced from 1-*O*-palmitoyl-NAS by LPLA2 activity. In addition, the formation of 1-*O*-oleoyl-NAS is interpreted to suggest that NAS produced from 1-*O*-palmitoyl-NAS by the enzyme can act as an acceptor of the oleoyl group of DOPC in the transacylase reaction.

When the enzyme was incubated with diolein in the presence of NAS, three products were detected (Fig. 6A, B). One major product corresponded to 1-*O*-oleoyl-NAS. Although the other two products were not obvious on the plate in the system in Fig. 6A, they corresponded to fatty acid and MAG by the use of another solvent system (Fig. 6B). Fatty acid was detected as a diffused band between the 1,2- and 1,3-diolein bands and MAG was detected as diffused doublet bands around MAG (Fig. 6B). Therefore, fatty acid and MAG are oleic acid and monooleoylglycerol, respectively. Additionally, the mono-oleoylglycerol produced by the enzyme appears to correspond primarily to 2-oleoylglycerol from its mobility. As expected from a broad positional specificity of LPLA2 to phospholipid acyl groups (13), the acyl group at the C2 position as well as the C1 position of MAG was hydrolyzed or transferred to NAS by the enzyme (Fig. 6B). In addition, LPLA2 catalyzes neither deacylation nor transacylation of the acyl group of TGA and cholesteryl ester (data not shown). Despite the ability of LPLA2 to deacylate MAG and DAG, LPLA2 failed to form DAG from MAG or TAG from DAG in the presence of NAS (Fig. 6B).

Accumulation of lipophilic alcohol in MDCK cells treated with CADs

Our previous study suggested that the inhibition of LPLA2 activity by CADs such amiodarone and PDMP contributes to the phospholipidosis induced by these CADs in MDCK cells (11). PDMP and amiodarone inhibit the transacylase activity of LPLA2 in a dose-dependent manner. Interestingly, MDCK cells treated with a concentration of CAD that induces cellular phospholipidosis showed an obvious increase of nonpolar lipid as well as phospholipid (**Fig. 7**). The mobility of the nonpolar lipid, which is comparable to that of HG, was slightly higher than that of ceramide in the TLC assay using a regular plate (Fig. 7A). The nonpolar lipid was completely separated

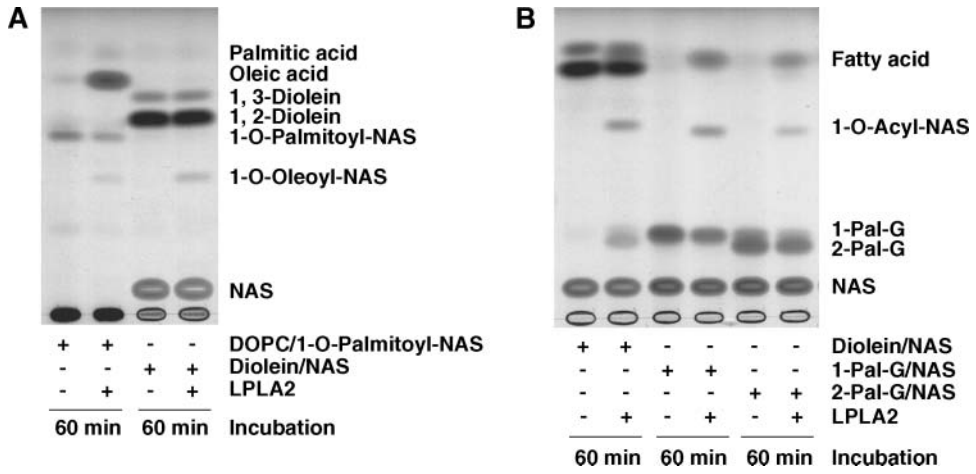

Fig. 6. Deacylation and transacylation of the acyl group of alcohols acylated by LPLA2. The reaction mixture consisted of 48 mM Na-citrate (pH 4.5), 145 ng/ml purified mouse LPLA2, and liposomes containing 123 μM DOPC and 12.3 μM sulfatide plus 2 μM 1-*O*-palmitoyl-NAS, 123 μM 1,2-diolein and 12.3 μM sulfatide plus 40 μM NAS, or 123 μM 1- or 2-palmitoylglycerol (1- or 2-Pal-G) or 12.3 μM sulfatide plus 40 μM NAS in a total volume of 500 μl. The reaction was initiated by the addition of the enzyme and kept for 60 min at 37°C. After the incubation, total lipids were extracted, applied to an HPTLC plate, and developed in a solvent system consisting of chloroform-acetic acid (90:10, v/v). An argentation plate and a regular plate were used in A and B, respectively. 1-*O*-Palmitoyl-NAS was synthesized by an alkylation of NAS with palmitoyl chloride under basic conditions. As seen in A, 1,2-diolein contains a small amount of 1,3-diolein. This is attributable to an equilibration between 1,2-diolein and 1,3-diolein, which occurs during storage.

from ceramide using a borate-treated plate and maintained the same mobility as HG (Fig. 7B). These results demonstrate that the nonpolar lipid corresponds to 1-*O*-alkylglycerol and suggest that an accumulation of a certain lipophilic alcohol induced by CAD in the cell is associated with inhibition of the transacylase activity of LPLA2 by CAD.

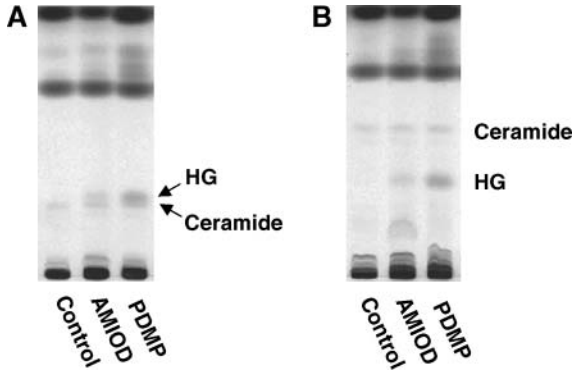

Fig. 7. Accumulation of a lipophilic alcohol in MDCK cells treated with a cationic amphiphilic drug. MDCK cells were treated on six-well plates with or without 15 μM amiodarone (AMIOD) or 50 μM D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-propanol (PDMP) for 7 days. The medium was replaced every other day with 2 ml of fresh DMEM-12F supplemented with 10% FCS and antibiotics plus or minus amiodarone or PDMP. After the treatment, the cells were collected and cellular lipid extraction was carried out as described in Materials and Methods. Each lipid extract corresponded to 200 μg of cellular protein and was applied to an HPTLC plate treated with (B) or without (A) borate and developed in a solvent system consisting of chloroform-acetic acid (90:10, v/v).

DISCUSSION

Previous studies demonstrated that LPLA2 possesses dual enzyme activities, a transacylase and a PLA2 activity (1–3). A site-directed mutagenesis study of LPLA2 showed that the enzyme has a putative catalytic triad structure consisting of three amino acid residues (serine, aspartic acid, and histidine) that is essential for the hydrolysis reaction (5). These studies indicated that the enzyme may form an acyl enzyme intermediate via the catalytic serine residue of the triad in the transition state of deacylation of phospholipid. The acyl group of the intermediate is transferred to a hydroxyl group of water and alcohol to form free fatty acid and ester, respectively (2, 5). A similar enzymatic mechanism has been proposed for cytosolic phospholipase A_2 (cPLA2) (14–16). cPLA2 catalyzes a transacylase reaction as well as a deacylase reaction when the phospholipase assay is carried out in the presence of glycerol. The product of the transacylation reaction is MAG, and the acyl group is predominantly esterified to a primary alcohol group of glycerol, as observed in our study (16).

The present study demonstrates that the acceptor molecules in the transacylase reaction by LPLA2 may be viewed as simple derivatives of ethanol required to possess one long carbon chain group and one small neutral residue linked at the C2 position of ethanol. Indeed, the transacylase activity of LPLA2 was observed when lipophilic alcohols were incubated with phospholipids under acidic conditions. In this reaction, the enzyme had a much greater preference for a primary alcohol as the acceptor of the acyl group than for a secondary alcohol, suggesting that the transacylation of alcohol by the enzyme is

greatly dependent on the nucleophilicity of the hydroxyl group of the acceptor alcohol. Although inhibition of acyl-NAS formation by other lipophilic alcohols in the transacylase assay of LPLA2 occurred, this may be attributable to a nucleophilic competition between NAS and the lipophilic alcohols for the acyl-LPLA2 intermediate.

This study also demonstrates that LPLA2 catalyzes the deacylation and transacylation of the acyl group of lipophilic alcohols acylated by LPLA2. This finding suggests that those acylated lipophilic compounds are available as acyl donors in the transacylase reaction and as substrate in the lipase reaction catalyzed by LPLA2. Although LPLA2 showed a broad positional specificity for the carbon position of the acyl group of MAG and DAG in the deacylase and transacylase reactions, the enzyme appears to prefer the C1 carbon position to the C2 carbon position of the glycerol backbone. The acylation of acceptor alcohols overwhelmingly occurred at a primary alcohol group, as observed in the assay containing phospholipid as the acyl donor. Although cPLA2 also displays significant enzymatic hydrolysis of 1- or 3-arachidonoyl-*sn*-glycerol, cPLA2 does not hydrolyze 1- or 3-α-linolenoyl-*sn*-glycerol and 2-arachidonoyl-*sn*-glycerol detectably (16). cPLA2 appears to possess more restricted positional specificities with regard to the acyl chain of monoacylglycerol compared with LPLA2.

The deacylation and transacylation of the acyl group of MAG, DAG, and 1-*O*-acyl-NAS by LPLA2 is a type of enzymatic reverse reaction. Interestingly, LPLA2 is phylogenetically related to phospholipid:diacylglycerol acyltransferase, an enzyme that catalyzes the acyl-CoA-independent synthesis of TAG in yeast and plants (17). However, LPLA2 does not acylate 1,2-DAG. In addition, neither TAG nor cholesteryl ester is deacylated by LPLA2. Although the deacylation and transacylation of the acyl groups of lipophilic alcohols by LPLA2 might be much weaker than those of phospholipids, such a dual reaction could be involved in the regulation of intracellular lipophilic alcohol levels and the rearrangement and/or remodeling of acyl groups of the lipophilic alcohols within the cell. Recently, it was demonstrated that novel calcium-independent PLA2 (iPLA2) family members that are differently categorized from group XV LPLA2 have TAG lipase and acylglycerol transacylase activities. These activities are acyl-CoA-independent transacylation reactions (18). Based on these observations, the authors suggested that these iPLA2s are involved in TAG liberation, recycling, and lipid homeostasis in the cell. Also, there are some reports for other Ca^{2+}-independent PLA2 with lipase activity as well as PLA2 activity. Phospholipase B/lipase purified from rat small intestines shows PLA2, lysophospholipase, and lipase activities (19), and PLA2/lipase purified from rat testes exhibits PLA2 and MAG lipase activities (20). Both enzymes are different from LPLA2 in substrate specificity, molecular mass, and protein and gene sequences. These are comparable examples of a multifunctional PLA2s.

Many CADs developed and approved as therapeutic drugs have been shown to induce phospholipidosis in human and other mammalian tissues. Generally, CADs tend to accumulate in lysosomes and to inhibit lysosomal enzyme activities. LPLA2 is found in the lysosomal fraction of MDCK cells (2). The CADs amiodarone and PDMP cause the accumulation of phospholipid and the formation of numerous lamellar inclusion bodies in MDCK cells (11). In general, the substantial accumulation of phospholipids in lysosomes is manifest as the formation of multilamellar inclusion bodies in cytoplasm, which is the histological hallmark of phospholipidosis. Furthermore, these CADs inhibit LPLA2 activity in a dose-dependent manner. Our recent study indicated that the impairment of phospholipid catabolism via LPLA2 is substantially involved in cellular phospholipidosis induced by certain CADs. The present study showed that these CADs evoke an accumulation of a certain nonpolar lipid as well as phospholipids. TLC analyses using two different plates showed that the nonpolar lipid corresponds to 1-*O*-alkylglycerol. The inhibition of LPLA2 activity by CADs might contribute to a substantial accumulation of 1-*O*-alkylglycerol in CAD-treated MDCK cells. It was reported that 1-*O*-alkylglycerol inhibits the activity of purified protein kinase C (PKC) in vitro and reduces membrane-associated PKC activity and the translocation of PKC to membrane by phorbol ester (21). A significant increase of monoalkylglycerol and a decrease of PKC activity were observed in quiescent cells. Endogenous monoalkylglycerol has been suggested to play a role as a regulator of PKC. Thus, an accumulation of 1-*O*-alkylglycerol evoked by an impairment of LPLA2 activity by CADs may affect PKC-associated signaling pathways and modulate cell processes such as cell proliferation and growth.

Some acceptor alcohols used in this study are biologically active. NAS, a synthetic short acyl chain ceramide, is a better acceptor of the acyl group deacylated from PC or PE in the transacylase reaction than long acyl chain ceramides (1). NAS has been used in many studies as a substitute for ceramide, which is thought to be an important effector molecule in some signal transduction processes (9, 10). Recently, it was reported that NAS is synthesized from PAF and sphingosine via the transacetylase in HL-60 cells (22). HAG is an alkylacylglycerol with a primary alcohol group and is known as a biosynthetic precursor of platelet-activating factor via remodeling and retroconversion de novo routes (23). HAG induces an inhibition of PKC activation by DAG (24, 25), a stimulation of HL-60 cell differentiation (26), and a delayed aggregation of rabbit platelets (27). HG is found as a constituent of certain ether-linked phospholipids such as PAF. HG is a 1-*O*-alkylglycerol and shows noticeable biological effects, as described above. Acylethanolamides are known to have pharmacological as well as biological effects on mammalian cells and tissues. AEA is an endogenous cannabinoid neurotransmitter in various mammalian tissues, in particular the brain, and an endogenous ligand for the central cannabinoid receptor (CB1) and peripheral cannabinoid receptor (CB2) (28). OEA is also known as an inhibitor of ceramidase (29). The acylation of these lipophilic alcohols must increase their affinity to lipid bilayers, perturb physicochemical properties of the membranes, and affect their affinity to their targets, resulting

in modulation of those lipophilic alcohol-mediated cellular functions.

The recent study of *LPLA2*$^{-/-}$ mice showed that LPLA2 may play a critical role in cellular phospholipid homeostasis in AM, PM, and spleen in young mice (8). The present study shows that some of the biosynthetic lipophilic alcohols whose levels must be precisely regulated in living cells are acylated or deacylated by LPLA2 in vitro assay, raising an alternative functional role of the enzyme in addition to the hydrolysis of the acyl group of glycerophospholipids. Therefore, LPLA2 may be a regulatory enzyme that modulates bioactive lipophilic alcohols in acidic compartments such as endosomes and lysosomes.

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REFERENCES

1. Abe, A., J. A. Shayman, and N. S. Radin. 1996. A novel enzyme that catalyzes the esterification of *N*-acetylsphingosine. Metabolism of C2-ceramides. *J. Biol. Chem.* **271:** 14383–14389.
2. Hiraoka, M., A. Abe, and J. A. Shayman. 2002. Cloning and characterization of a lysosomal phospholipase A2, 1-*O*-acylceramide synthase. *J. Biol. Chem.* **277:** 10090–10099.
3. Abe, A., and J. A. Shayman. 1998. Purification and characterization of 1-*O*-acylceramide synthase, a novel phospholipase A2 with transacylase activity. *J. Biol. Chem.* **273:** 8467–8474.
4. Taniyama, Y., S. Shibata, S. Kita, K. Horikoshi, H. Fuse, H. Shirafuji, Y. Sumino, and M. Fujino. 1999. Cloning and expression of a novel lysophospholipase which structurally resembles lecithin cholesterol acyltransferase. *Biochem. Biophys. Res. Commun.* **257:** 50–56.
5. Hiraoka, M., A. Abe, and J. A. Shayman. 2005. Structure and function of lysosomal phospholipase A2: identification of the catalytic triad and the role of cysteine residues. *J. Lipid Res.* **46:** 2441–2447.
6. Schaloske, R. H., and E. A. Dennis. 2006. The phospholipase A2 superfamily and its group numbering system. *Biochim. Biophys. Acta.* **1761:** 1246–1259.
7. Abe, A., M. Hiraoka, S. Wild, S. E. Wilcoxen, R. Paine, 3rd, and J. A. Shayman. 2004. Lysosomal phospholipase A2 is selectively expressed in alveolar macrophages. *J. Biol. Chem.* **279:** 42605–42611.
8. Hiraoka, M., A. Abe, Y. Lu, K. Yang, X. Han, R. W. Gross, and J. A. Shayman. 2006. Lysosomal phospholipase A2 and phospholipidosis. *Mol. Cell. Biol.* **26:** 6139–6148.
9. Kolesnick, R., and D. W. Golde. 1994. The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signaling. *Cell.* **77:** 325–328.
10. Hannun, Y. A., and C. Luberto. 2000. Ceramide in the eukaryotic stress response. *Trends Cell Biol.* **10:** 73–80.
11. Abe, A., M. Hiraoka, and J. A. Shayman. 2007. A role for lysosomal phospholipase A2 in drug induced phospholipidosis. *Drug Metabolism Letters.* **1:** 49–53.
12. Shayman, J. A., L. Lee, A. Abe, and L. Shu. 2000. Inhibitors of glucosylceramide synthase. *Methods Enzymol.* **311:** 373–387.
13. Abe, A., M. Hiraoka, and J. A. Shayman. 2006. Positional specificity of lysosomal phospholipase A2. *J. Lipid Res.* **47:** 2268–2279.
14. Reynolds, L. J., L. L. Hughes, A. I. Louis, R. M. Kramer, and E. A. Dennis. 1993. Metal ion and salt effects on the phospholipase A2, lysophospholipase, and transacylase activities of human cytosolic phospholipase A2. *Biochim. Biophys. Acta.* **1167:** 272–280.
15. Guenther, M. G., M. R. Witmer, and J. R. Burke. 2002. Cytosolic phospholipase A2 shows burst kinetics consistent with the slow, reversible formation of a dead-end complex. *Arch. Biochem. Biophys.* **398:** 101–108.
16. Gelb, M. H., M. K. Jain, A. M. Hanel, and O. G. Berg. 1995. Interfacial enzymology of glycerolipid hydrolases: lessons from secreted phospholipases A2. *Annu. Rev. Biochem.* **64:** 653–688.
17. Dahlqvist, A., U. Stahl, M. Lenman, A. Banas, M. Lee, L. Sandager, H. Ronne, and S. Stymne. 2000. Phospholipid:diacylglycerol acyltransferase: an enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants. *Proc. Natl. Acad. Sci. USA.* **97:** 6487–6492.
18. Jenkins, C. M., D. J. Mancuso, W. Yan, H. F. Sims, B. Gibson, and R. W. Gross. 2004. Identification, cloning, expression, and purification of three novel human calcium-independent phospholipase A2 family members possessing triacylglycerol lipase and acylglycerol transacylase activities. *J. Biol. Chem.* **279:** 48968–48975.
19. Tojo, H., T. Ichida, and M. Okamoto. 1998. Purification and characterization of a catalytic domain of rat intestinal phospholipase B/lipase associated with brush border membranes. *J. Biol. Chem.* **273:** 2214–2221.
20. Ito, M., U. Tchoua, M. Okamoto, and H. Tojo. 2002. Purification and properties of a phospholipase A2/lipase preferring phosphatidic acid, bis(monoacylglycerol) phosphate, and monoacylglycerol from rat testis. *J. Biol. Chem.* **277:** 43674–43681.
21. Warne, T. R., F. G. Buchanan, and M. Robinson. 1995. Growth-dependent accumulation of monoalkylglycerol in Madin-Darby canine kidney cells. Evidence for a role in the regulation of protein kinase C. *J. Biol. Chem.* **270:** 11147–11154.
22. Lee, T. C., M. C. Ou, K. Shinozaki, B. Malone, and F. Snyder. 1996. Biosynthesis of *N*-acetylsphingosine by platelet-activating factor: sphingosine CoA-independent transacetylase in HL-60 cells. *J. Biol. Chem.* **271:** 209–217.
23. Lee, T. C., B. Malone, M. L. Blank, V. Fitzgerald, and F. Snyder. 1990. Regulation of the synthesis of platelet-activating factor and its inactive storage precursor (1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine) from 1-alkyl-2-acetyl-*sn*-glycerol by rabbit platelets. *J. Biol. Chem.* **265:** 9181–9187.
24. Daniel, L. W., G. W. Small, J. D. Schmitt, C. J. Marasco, K. Ishaq, and C. Piantadosi. 1988. Alkyl-linked diglycerides inhibit protein kinase C activation by diacylglycerols. *Biochem. Biophys. Res. Commun.* **151:** 291–297.
25. Bass, D. A., L. C. McPhail, J. D. Schmitt, S. Morris-Natschke, C. E. McCall, and R. L. Wykle. 1988. Selective priming of rate and duration of the respiratory burst of neutrophils by 1,2-diacyl and 1-*O*-alkyl-2-acyl diglycerides. Possible relation to effects on protein kinase C. *J. Biol. Chem.* **263:** 19610–19617.
26. McNamara, M. J., J. D. Schmitt, R. L. Wykle, and L. W. Daniel. 1984. 1–*O*-Hexadecyl-2-acetyl-sn-glycerol stimulates differentiation of HL-60 human promyelocytic leukemia cells to macrophage-like cells. *Biochem. Biophys. Res. Commun.* **122:** 824–830.
27. Satouchi, K., M. Oda, K. Saito, and D. J. Hanahan. 1984. Metabolism of 1-*O*-alkyl-2-acetyl-*sn*-glycerol by washed rabbit platelets: formation of platelet activating factor. *Arch. Biochem. Biophys.* **234:** 318–321.
28. Di Marzo, V., and D. G. Deutsch. 1998. Biochemistry of the endogenous ligands of cannabinoid receptors. *Neurobiol. Dis.* **5:** 386–404.
29. Sugita, M., M. Willians, J. T. Dulaney, and H. W. Moser. 1975. Ceramidase and ceramide synthesis in human kidney and cerebellum. Description of a new alkaline ceramidase. *Biochim. Biophys. Acta.* **398:** 125–131.