Facile syntheses of acyl dihydroxyacetone phosphates and lysophosphatidic acids having different acyl groups

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Abstract In this study, we report novel and simple chemical syntheses of acyl dihydroxyacetone phosphate (DHAP) and 1-acyl glycero-3-phosphate [lysophosphatidic acid (LPA)], key intermediaries in the formation of glycerolipids containing ester and ether bonds. The synthesis of acyl DHAPs involved acylating the dimethyl ketal of DHAP by acid anhydride using 4-pyrrolidinopyridine as the catalyst, and the resulting product was deketalized by $HClO_4$ in acetone to produce acyl DHAP. The acid anhydride was either added directly or generated in the reaction mixture from the corresponding fatty acid using dicyclohexylcarbodiimide as the condensing agent. Using these methods, a number of acyl DHAPs having short-, medium-, and long-chain saturated and unsaturated acyl groups were synthesized, with overall yields from 37% to 75%. The activities of these acyl DHAPs as substrates for guinea pig liver peroxisomal acyl DHAP:NADPH reductase and alkyl DHAP synthase were then determined. Next, starting from these acyl DHAPs, a variety of LPAs were synthesized by chemical reduction of the ketone group. Biological activities of these LPAs were determined by measuring their relative abilities to release intracellular Ca^{2+} via the LPA receptor. A combined chemical-enzymatic method is also described to prepare the natural LPA from the racemic mixture.—Das, A. K., J. E. Milam, R. C. Reddy, and A. K. Hajra. **Facile syntheses of acyl dihydroxyacetone phosphates and lysophosphatidic acids having different acyl groups.** ***J. Lipid Res.*** **2006.** 47: **1874–1880.**

Supplementary key words acylation • dihydroxyacetone phosphate dimethyl ketal • acyl dihydroxyacetone phosphate reductase • alkyl dihydroxyacetone phosphate synthase • sodium cyanoborohydride reduction

Acyl dihydroxyacetone phosphate (DHAP) is the key intermediate for the biosynthesis of ether and nonether glycerolipids via the peroxisomal pathway (1, 2). Although this phospholipid is not commercially available, a number of methods have been described for its chemical synthesis (3–5). One of these methods, phosphorolysis of acyloxydiazoacetone (5), has been used by different workers to synthesize acyl DHAP (6–8). The synthesis of acyl DHAP via the direct acylation of DHAP, following the method of Gupta, Radhakrishnan, and Khorana (9) for the direct acylation of glycerophosphate, also has been described, with reported yields ranging from 5% (10) to 50–70% (11). Our attempts to chemically acylate DHAP using previously described methods (9–11) produced acyl DHAP in low yields and a number of by-products that were difficult to remove (A. K. Das and A. K. Hajra, unpublished results). In contrast, we found that the dimethyl ketal of DHAP (DHAP-DMK) can be nearly quantitatively acylated by acid anhydrides in the presence of catalysts. The resulting acyl DHAP-DMK can be hydrolyzed to acyl DHAP with high yield. Using this method, we synthesized a number of acyl analogs of acyl DHAP and determined their biological activities as substrates for two different enzymes.

Starting from these synthetic acyl DHAPs, we also describe methods for the preparation of the racemic and *sn*-3 isomers of different lysophosphatidic acids (LPAs; 1-acyl-glycero-3-phosphate). In recent years, LPA has been shown to be not only the metabolic precursor of all nonether glycerolipids (1) but also a novel lipid mediator with a wide range of biological effects (12).

The methods described here will be useful for the synthesis of different acyl analogs of acyl DHAP and LPA and will facilitate further study into the biological roles of these important lipids.

MATERIALS AND METHODS

DHAP-DMK di(cyclohexylammonium) salt, different acid anhydrides, fatty acids, 4-pyrrolidinopyridine (4-PrPy), dicyclohexylcarbodiimide (DCC), $NaCNBH_3$, porcine pancreatic phospholipase A_2 (1,000 U/mg protein), and anhydrous pyridine were obtained from Sigma-Aldrich (St. Louis, MO). Unisil (activated silicic acid) was from Clarkson Chemical Co. (Williamsport, PA), and AG 50W-X4 (H^+ form) resin was from Bio-Rad (Richmond, CA). Dry, alcohol-free chloroform was prepared by

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Abbreviations: DCC, dicyclohexylcarbodiimide; DHAP, dihydroxyacetone phosphate; DHAP-DMK, dimethyl ketal of dihydroxyacetone phosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid; PMR, proton magnetic resonance; 4-PrPy, 4-pyrrolidinopyridine.

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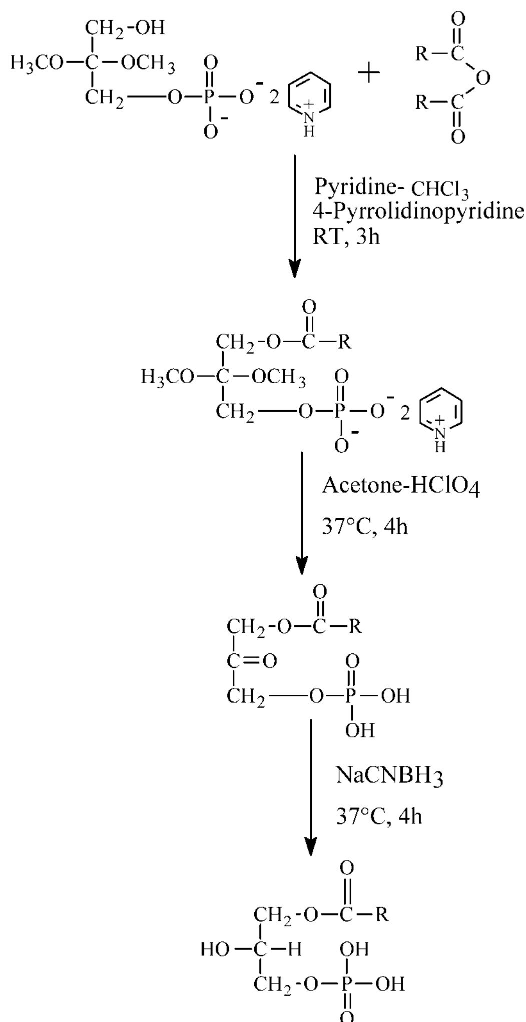
freshly distilling analytical-grade $CHCl_3$ over P_2O_5. Fura2-AM was from Molecular Probes (Eugene, OR). All other chemicals and supplies were as described previously (13). The total lipid phosphorus was assayed according to Ames (14). Proton magnetic resonance (PMR) spectra were taken on a 360 MHz spectrophotometer (model WM 360; Brucker Co.). Ligand-induced release of intracellular Ca^{2+} was measured by well-established procedures (12, 15).

RESULTS

Synthesis of palmitoyl DHAP-DMK

Method A: palmitic anhydride as the acylating agent. DHAP-DMK was acylated directly by palmitic anhydride to synthesize palmitoyl DHAP-DMK (**Scheme 1**). The cyclohexylammonium salt of DHAP-DMK was converted to the pyridinium salt by passing its aqueous solution (10 mg or 20 μmol in 1.0 ml) through a column (4 cm × 0.7 cm) of the pyridinium form of the AG 50W cation-exchange resin. Pyridinium salt of AG 50W-X4 (100–200 mesh) was prepared by treating the H^+ form of the resin with 50% aqueous



Scheme 1. Chemical synthesis of acyl dihydroxyacetone phosphate (DHAP) and lysophosphatidic acid (LPA). RT, room temperature.

pyridine. After eluting the pyridinium salt of DHAP-DMK from the column with water (total volume, 3.0 ml), it was lyophilized in a Speed-Vac concentrator and stored over P_2O_5. The dry glassy mass was dissolved in 0.3 ml of anhydrous pyridine to which a solution of 40 mg (80 μmol) of palmitic anhydride and 15 mg (100 μmol) of 4-PrPy in 0.3 ml of anhydrous $CHCl_3$ was added. The mixture was then stirred magnetically for 3 h at room temperature. When at the end of the reaction the products were examined by TLC ($CHCl_3$/methanol/acetic acid/water, 100:40:12:4), two main phospholipid-positive (16) spots having relative mobility (R_f) values of 0.60 and 0.85, along with an occasional minor one (R_f = 0.65), were seen to be present on the chromatogram. The compound having the R_f of 0.60 was identified as the acyl DHAP-DMK (see below), and the two faster moving phospholipids were tentatively identified as the monopalmitoyl and dipalmitoyl mixed anhydrides with the phosphoric group of the same compound (9). The pyridine and chloroform from the reaction mixture were removed by blowing a stream of dry N_2, and the residue was dissolved in 3.0 ml of $CHCl_3$/methanol (2:1). To the solution, 0.75 ml of 0.5 M HCl was added, mixed, and centrifuged at low speed (600 *g*, 10 min) to separate the phases. The upper aqueous phase containing most of the catalyst (4-PrPy) was removed and the lower phase containing the lipids was washed with the acidic theoretical upper phase ($CHCl_3$/methanol/0.01 M HCl, 3:48:47) to remove the rest of the 4-PrPy. The solvents from the lower phase were evaporated off by blowing a stream of N_2, and the residue was transferred onto a 0.5 g Unisil column (0.5 cm inner diameter) with $CHCl_3$. The column was first eluted with 15 ml of chloroform (fraction 1), which eluted the excess palmitic acid, and the phospholipid was then eluted using 15 ml of 30% methanol in chloroform (fraction 2). TLC analysis, performed as described above, of fraction 2 showed the presence of only a single compound (R_f = 0.60), which demonstrated positive reactions with molybdenum blue spray for phospholipids (16) and primuline spray for lipids (17) and was identified as the palmitoyl DHAP-DMK by NMR analysis (see below). The amount of phospholipid present in this fraction, as determined by phosphate analysis, was 15.6 μmol or 78% of the DHAP-DMK used for the reaction. PMR analysis of this phospholipid in $CDCl_3$, using tetramethylsilane as the internal standard, showed all of the peaks expected for palmitoyl DHAP-DMK: δ 0.75 [t, 3H for the terminal (C-16) CH_3 of the palmitoyl group], 1.15 (s, sharp 24H associated with C-4 to C-15 of the palmitoyl group), 1.46 (t, 2H for C-3 of the palmitoyl moiety), 2.21 (t, 2H at C-2 of the palmitoyl group), 3.15 (s, 6H of the 2-methoxy groups of the ketal), 3.45 (s, 2H at the carbon atom of DHAP esterified to the palmitoyl group), and 3.85 and 3.95 (each as a singlet for the 2H attached to the carbon atom of DHAP esterified to the phosphoric acid).

Method B: using a palmitic anhydride-generating system. The acylation reaction described above can also be carried out by generating palmitic anhydride from palmitic acid in the reaction mixture using DCC as the condensing agent (18), as described below. Twenty micromoles of lyophilized

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pyridinium salt of DHAP-DMK was dissolved in 0.3 ml of anhydrous pyridine to which a mixture of 80 μmol of palmitic acid, 80 μmol of DCC, and 100 μmol of 4-PrPy dissolved in 0.3 ml of dry $CHCl_3$ was added. The resulting mixture was stirred at room temperature overnight (~15 h). TLC analysis of this reaction mixture showed the presence of the same two phospholipids described above, having R_f values of 0.60 (major) and 0.85 (minor). The solvents from the reaction mixture were removed by blowing dry N_2, and the residue was dissolved in $CHCl_3$/methanol (2:1), acid-washed, and subjected to Unisil chromatography as described above. The product, palmitoyl DHAP-DMK, was eluted out in fraction 2. From phosphate analysis, the yield of the product was calculated to be 72% of the DHAP-DMK used.

Deketalization

The acyl DHAP-DMK, synthesized as described above, was deketalized to the free ketone by treatment with $HClO_4$ in acetone (Scheme 1). The palmitoyl DHAP-DMK, eluted from the Unisil column, was converted to the free acid form by dissolving it in $CHCl_3$/methanol and washing with aqueous HCl as described above. After removing $CHCl_3$ and methanol, it was dissolved in a mixture of 3.0 ml of acetone and 15 μl of 70% $HClO_4$ and incubated at 37°C. The time course for this deketalization reaction was monitored by TLC analysis, as described above, in which the parent compound (R_f = 0.60) separated well from the product, palmitoyl DHAP (R_f = 0.35). After the deketalization was found to be complete (~4 h), 60 μl of 3.0 M sodium acetate was added to the reaction mixture to neutralize the $HClO_4$, and the acetone was removed from the mixture by blowing a stream of N_2. The residue was suspended in 4.5 ml of $CHCl_3$/methanol (1:2) and acidified by adding 10 μl of 6.0 M HCl. The insoluble materials were removed by centrifugation, and 2.7 ml of 0.05 M HCl and 1.5 ml of $CHCl_3$ were added to the extract. After mixing and low-speed centrifugation (600 *g*, 10 min), the upper aqueous phase was removed and the solvents were evaporated off from the lower phase by blowing N_2. The residue was dissolved in a small volume of $CHCl_3$ and subjected to Unisil chromatography as described above. The product, palmitoyl DHAP, that eluted in fraction 2 (30% methanol in $CHCl_3$) was identified by cochromatography with authentic palmitoyl DHAP in different TLC systems (5) and also by NMR analysis (see below). Phosphate analysis showed that the yield of palmitoyl DHAP was 80% of the palmitoyl DHAP-DMK used. The PMR spectrum taken as described previously (5) showed all of the peaks expected for palmitoyl DHAP: δ 0.76 (t, 3H for the terminal CH_3 of the palmitoyl group), 1.18 (s, sharp, 24H associated with C-4 to C-15 of the palmitoyl moiety), 1.51 (t, 2H for C-3 of the palmitoyl group), 2.29 (t, 2H for C-2), 4.5 (d, 2H for the CH_2 group attached to the phosphate), and 4.84 (s, 2H for the CH_2 of DHAP adjacent to the palmitoyl moiety).

Synthesis and biological activity of different acyl DHAPs

The synthesis of hexanoyl (6:0), octanoyl (8:0), decanoyl (10:0), lauroyl (12:0), myristoyl (14:0), oleoyl (18:1, n-9, *cis*), elaidoyl (18:1, n-9, *trans*), and linoleoyl (18:2, n-6) DHAPs was carried out as described for palmitoyl DHAP, except that the intermediate acylated ketals were not purified by silicic acid chromatography. After acylation, 4-PrPy was removed from the reaction mixture by solvent partition under acidic conditions as described above, and the lipids present in the lower phase were treated with acetone/$HClO_4$ after removal of the solvents. The longer chain (>C10) acyl derivatives were purified as described above for palmitoyl DHAP. For the shorter chains (<C12), because of their partial solubility in the methanol-water upper phase, the solvent partition step after deketalization was omitted and the extracts were used directly for Unisil chromatography. All of the acyl DHAPs, except the hexanoyl DHAP, were completely eluted in fraction 2 (30% methanol in $CHCl_3$). For the hexanoyl DHAP, only a part of it was eluted in fraction 2, and a more polar solvent (60% methanol in $CHCl_3$) was found to be necessary for its complete elution from the column. On TLC analysis, all of the purified products gave single phospholipid-positive spots, the R_f values of which are given in **Table 1**. The amount of each purified acyl DHAP was determined by phosphate analysis, and the overall yield of each one, based on the starting amount of DHAP-DMK used, is also shown in Table 1.

The acyl DHAPs synthesized were tested as substrates for two peroxisomal enzymes, acyl DHAP:NADPH reductase and alkyl DHAP synthase (1). The enzymatic assays were performed as described previously (19, 20), and purified guinea pig liver peroxisomes (21) were used as the source of the enzymes. The specific activities of the enzymes, determined using each of the synthesized acyl DHAPs as the substrate, are shown in **Fig. 1A, B**.

Synthesis of LPA

Palmitoyl DHAP was reduced with sodium cyanoborohydride ($NaCNBH_3$) to make the corresponding LPA (Scheme 1). Five micromoles (2 mg) of the synthetic palmitoyl DHAP in a screw-top test tube was dissolved in 3.0 ml of methanol, and 1.0 ml of 0.2 M $NaCNBH_3$ was added, followed by the addition of 0.1 ml of glacial acetic acid. The tube was capped, and the mixture was incubated at 37°C for 4 h. The reaction was stopped by adding 4.0 ml of chloroform and 3.6 ml of 0.2 M HCl to the mixture.

TABLE 1. Migration properties on TLC plates and yields of different acyl DHAPs

Acyl DHAP Chain Length	Relative Mobility	Yield
		%
6:0	0.22	36.8
8:0	0.26	48.9
10:0	0.29	56.4
12:0	0.32	63.6
14:0	0.34	56.4
16:0	0.35	60.0
18:1 *cis*	0.40	74.6
18:1 *trans*	0.40	72.0
18:2	0.40	50.0

DHAP, dihydroxyacetone phosphate. Yield calculations are based upon the dimethyl ketal of DHAP used.

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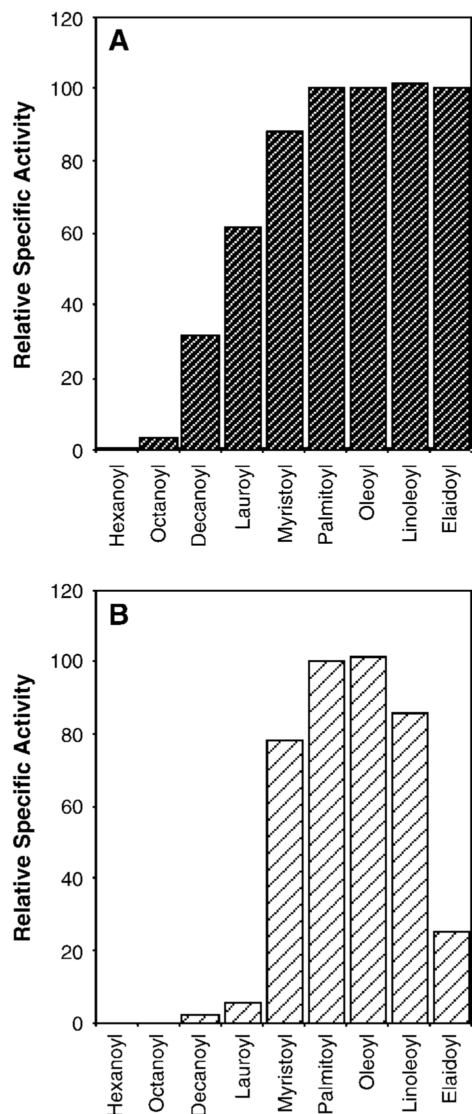

Fig. 1. Activities of guinea pig liver peroxisomal acyl DHAP: NADPH reductase and alkyl DHAP synthase toward synthetic acyl DHAPs of different acyl chain lengths. A: Acyl DHAP reductase activity toward different acyl DHAPs was determined by incubating the acyl DHAPs (200 μM) with *S*-[4-^{3}H]NADPH (70 μM, 10,000 dpm/nmol) and peroxisomes (0.5–1.0 μg of protein) in phosphate buffer (pH 7.5) and determining the amount of 1-acyl[2-^{3}H]*sn*-glycero-3-phosphate formed, as described previously (19). The data presented are averages of two determinations that varied within ±3% of the average. B: Alkyl DHAP synthase activity was assayed by incubating the acyl DHAPs (200 μM) with [1-^{3}H]hexadecanol (170 μM, 30,000 dpm/nmol) and peroxisomes (20–40 μg of protein) in Tris buffer (pH 8.0) and measuring the amount of 1-*O*-[1′-^{3}H]hexadecyl DHAP formed using a solvent partition method, as described previously (20). The data presented are averages of two determinations that varied within ±5% of the average.

After vortexing and centrifuging at 1,000 *g* for 10 min, the upper layer was aspirated off and the lower chloroform layer was washed once with 6.5 ml of chloroform/methanol/0.01 M HCl (3:48:47). The solvents from the washed lower layer were removed by blowing a stream of N_2. TLC of the product using chloroform/methanol/5% aqueous $NaHSO_3$ as the developing solvent showed the presence of a single primuline- and molybdenum blue-positive spot of R_f = 0.35, the same as authentic LPA, indicating complete reduction of palmitoyl DHAP. In this bisulfite-containing TLC solvent system, acyl DHAP migrates more slowly (R_f = 0.20) than LPA (22).

The synthesized LPA was purified by Unisil column chromatography as described above. LPA was mainly eluted out in the 30% methanol-70% chloroform fraction (fraction 2). Organic phosphate analysis of this purified LPA indicated that the yield was 78% of the palmitoyl DHAP used. On TLC analysis using different solvent systems, as described above, the synthesized LPA always had the same migration rate as authentic LPA. Proton NMR analysis showed all of the peaks expected for 1-palmitoyl glycero-3-phosphate: δ 0.90 [t, 3H for the terminal CH_3 (i.e., C-16 of the palmitoyl moiety)], 1.27 (s, sharp, 24H associated with C-4 to C-15 of the palmitoyl group), 1.60 (s, broad, 2H of the C-3 of the palmitoyl group), 2.25 (t, 2H at C-2 of the palmitoyl group), 3.75–3.78 (m, broad, 1H at C-2 of the glycerol moiety) 3.80 (s, broad, 1H of the OH group at C-2), 4.08–4.13 (d, 2H of the C-1 of the glycerol moiety), and 7.80 (s, broad, 2H of the phosphate group).

1-Oleoyl-*rac*-glycero-3-phosphate and 1-elaidoyl-*rac*-glycero-3-phosphate also were synthesized by the $NaCNBH_3$ reduction of the ketone group of the corresponding synthetic acyl DHAPs using the same method described above for the palmitoyl LPA. The yields were between 75% and 80% of the starting compounds.

Biological activity of the synthetic LPAs

The identity of the synthetic LPAs was also verified by measuring their biological activity in activating specific cell surface LPA receptors. Both *R*- and *S*- (*sn*-3 and *sn*-1) isomers of LPA were shown to be equally effective at activating these G-protein-coupled LPA receptors (12). We tested their effect in an established assay of Ca^{2+} mobilization in human A431 cells. Three synthesized LPAs (oleoyl, palmitoyl, and elaidoyl as their racemic mixture) and one *sn*-3 (or *R*-) isomer of LPA obtained from Sigma Co. were used for this purpose. At a saturating receptor concentration (1 μM), all four compounds were equally potent (**Fig. 2**). However, at a nonsaturating dose (500 nM), we observed a significant reduction in the ability of 1-palmitoyl and 1-elaidoyl LPA to elicit Ca^{2+} mobilization compared with oleoyl LPA, either synthesized as described here or in a commercially available form (Fig. 2). These results confirm that the 1-oleoyl analog is the most potent biological form of LPA (12).

Preparation of 1-palmitoyl-*sn*-glycero-3-phosphate

The optically active natural *sn*-3 (*R*- or *L*-) isomer of the palmitoyl LPA was prepared from the corresponding racemic mixture by first chemically acylating its C-2 hydroxyl group with oleic acid to phosphatidic acid (PA) followed by hydrolysis by pancreatic phospholipase A_2 to natural LPA. Phospholipase A_2 hydrolyzes only the *sn*-3 isomer of phospholipids (23).

The free acid form of synthetic 1-palmitoyl-*rac*-glycero-3-phosphate (2.5 mg, 6.2 μmol) was dissolved in 0.5 ml of

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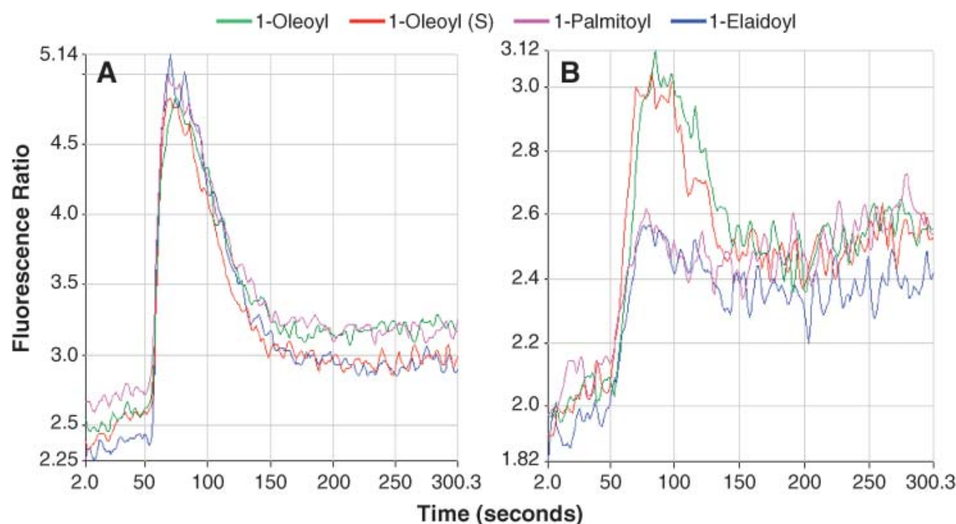

Fig. 2. LPA-induced Ca^{2+} mobilization in human A431 cells. Cells were preincubated with 2.5 μM Fura2-AM for 30 min at 37°C, washed, and then transferred to fluorimeter (Perkin-Elmer LS-50) cuvettes. LPAs in 0.1 M Tris (pH 7.5) were added to the cells, and changes in fluorescence were measured (excitation at 340 and 380 nm, emission at 510 nm) as described previously (15). Representative Ca^{2+} traces are shown in response to three synthesized racemic LPAs, 1-oleoyl (green), 1-palmitoyl (pink), and 1-elaidoyl (blue), and one authentic 1-oleoyl-*sn*-glycero-3-phosphate (red) at 1 μM (A) and 500 nM (B). Each experiment was repeated at least three times. (S), Sigma.

anhydrous chloroform, and 13.6 mg (24.8 μmol) of oleic anhydride and 2.0 mg (13.4 μmol) of 4-PrPy were added. The mixture was magnetically stirred under N_2 at 35°C in a capped Reactivial (Pierce) for 3.5 h. The products were isolated by solvent extraction under acidic conditions as described above for the isolation of acyl DHAPs. TLC examination (chloroform-methanol-acetic acid-water, 25:10:3:1) demonstrated that the LPA (R_f = 0.35) was completely acylated to PA (R_f = 0.75) and also the formation of some phosphoanhydrides of PA (R_f values between 0.8 and 0.9). The latter compounds were hydrolyzed to PA by dissolving the products in 2.0 ml of acetone containing 20 μl of 6.0 M HCl and incubating the solution at 37°C for 15 min. The incubation was stopped by adding 50 μl of 3.0 M sodium acetate to the mixture. The acetone was removed from the mixture by blowing a stream of N_2, the residue was dissolved in 3.0 ml of chloroform-methanol (1:1), and 1.35 ml of 0.05 M HCl was added, mixed, and centrifuged at 1,000 *g* for 10 min. The upper aqueous layer was removed and the lower layer was washed once with 2.5 ml of Bligh and Dyer (24) upper layer (chloroform-methanol-water, 1:12:12). TLC examination of the washed lower layer showed a single molybdenum blue-positive spot having the same R_f as authentic PA. This crude preparation, contaminated with free oleic acid, was purified by silicic acid (Unisil) column chromatography as described above. The oleic acid was removed by eluting the column with chloroform, and the PA was then eluted out with 20% methanol in chloroform. Phosphate analysis showed the yield of PA to be 70% of the starting LPA.

A portion (1.0 mg) of the PA was enzymatically hydrolyzed by suspending it in 1.0 ml of 50 mM Tris buffer (pH 7.5) containing $CaCl_2$ (10 mM), sodium deoxycholate (1.0 mM), and 30 units of porcine pancreatic phospholipase A_2. The mixture was magnetically stirred under N_2 in a Reactivial at room temperature for 15 h. The phospholipids were isolated from the mixture by Bligh and Dyer (24) solvent extraction under acidic conditions as described above. TLC examination using the same chloroform-methanol-acetic acid-water solvent system showed the presence of two phosphate-positive lipids having the same R_f values of LPA (0.35) and PA (0.75). Phosphate analysis of these spots showed that 47% of the racemic PA was hydrolyzed to LPA. The LPA and PA were separated from each other as described below.

A solvent partition method (25) was used to separate the LPA formed after hydrolysis from the less polar unhydrolyzed PA. The remaining mixture obtained after phospholipase A_2 treatment was dispersed in 1.2 ml of citrate-phosphate (0.05–0.1 M, pH 4.4) buffer and dissolved in 4.5 ml of $CHCl_3$/methanol (1:2) to which 1.5 ml of water and 1.5 ml of additional $CHCl_3$ were added to make two phases (24). The upper phase containing the LPA was transferred to another tube, and the lower phase was reextracted with 5.0 ml of $CHCl_3$/methanol/water (1:12:12). The combined upper phase was acidified with 0.1 M HCl, and the free acid form of LPA was extracted back in chloroform as described previously (25). TLC examination of the extracted lower phase and the recovered LPA in chloroform showed a complete separation of PA (*sn*-1 isomer) from LPA (*sn*-3 isomer) by this solvent partition. Phosphate content analysis of these solutions showed the molar ratio of PA to LPA to be 52:48.

DISCUSSION

The two-step chemical synthesis of acyl DHAP and its reduction to LPA are summarized in Scheme 1. In the first

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step, the acylation of DHAP-DMK by acid anhydrides was complete in a short period of time (3–4 h), in contrast to the significantly longer time (2–3 days) previously reported to be necessary for similar acylation reactions (9–11). This rapid rate of acylation was probably attributable to our use of a pyridine/$CHCl_3$ mixture as the solvent, in which the reactants and the catalyst dissolved well, and to the use of a better catalyst, 4-PrPy (26), instead of the dimethylaminopyridine used by others (9–11). The reaction rate was slower when the acid anhydrides were generated in the reaction mixture from free fatty acids and DCC, so a longer reaction time was necessary. The yield of the acyl DHAP-DMK, however, was the same in both methods. This anhydride-generating system will be particularly useful when the anhydride form of a fatty acid is not commercially available. We synthesized both short-chain (octanoyl) and unsaturated (oleoyl) acyl DHAP with good overall yields (50–60%) using this acid anhydride-generating system.

As reported previously (9), we also found that acid anhydrides acylated not only the hydroxyl group of DHAP but also the acidic hydroxyl groups of the phosphate, forming mixed acid anhydrides. Therefore, a large excess (4×) of acid anhydride was found to be necessary for the maximum yield of the desired product. These acyl phosphates are, of course, more unstable than the ester group and decomposed in acidic solution during purification.

The acyl DHAP-DMK was found to be fairly refractory to acid hydrolysis, such as treatment with methanolic HCl (5), so that during such a long hydrolytic process a part of the acyl ester was also hydrolyzed, resulting in a low yield of the product. We found, however, that treatment of the ketal by the strong perchloric acid in acetone resulted in the rapid formation of the ketolipid with little hydrolysis of the ester bond. This rapid deketalization was probably attributable to transketalization of the lipid ketal with acetone (forming 2,2′-dimethoxypropane), which was used as the solvent.

Using the present method (Scheme 1), we synthesized acyl DHAPs having different acyl chain lengths, including unsaturated ones. The relatively lower yield of the short- and medium-chain acyl DHAPs (Table 1) was attributable to their lower hydrophobicity compared with the long-chain DHAPs, so that there were more losses of these compounds during the purification process. Many of these compounds, such as the short and medium chains and the unsaturated acyl derivatives, have not been synthesized previously. These are probably "unnatural" acyl DHAPs, because it has been shown that only the saturated long-chain acyl CoAs can serve as substrates for the enzymatic acylation of DHAP (27, 28). However, these unnatural derivatives are also used as substrates by the two enzymes that metabolize acyl DHAP (Fig. 1). The specificities of the enzymes toward these acyl DHAPs are somewhat different. Whereas alkyl DHAP synthase uses only the long-chain saturated and unsaturated acyl DHAPs with an abrupt cutoff below C-14, acyl DHAP reductase, although preferring the long-chain acyl DHAPs, also uses the medium-chain acyl DHAPs in decreasing activities with the decreasing length of the acyl chain (Fig. 1). Interestingly, although elaidoyl DHAP was found to be a good substrate for alkyl DHAP reductase, unlike its *cis* isomer (oleoyl DHAP), we found that it is a poor substrate for alkyl DHAP synthase (Fig. 1).

These synthetic acyl DHAPs provided us substrates for a simple borohydride reduction method to prepare the corresponding LPAs. Previously, we used $NaBH_4$ to reduce acyl DHAPs to racemic LPA (25). However, because acyl DHAP is labile at high pH but $NaBH_4$ is not stable at pH $<$ 7.0, we had to use a buffer to keep the pH at ~8.0, which was sometimes difficult to maintain. The use of $CNBH_3^-$ at low pH, as demonstrated here, solved that problem. The reduction of acyl DHAPs was complete, and as shown here (Fig. 2), the synthesized LPAs were active in releasing intracellular Ca^{2+} via LPA-specific cell surface receptors. The relative activity of palmitoyl and oleoyl LPAs as ligands is the same as reported in the literature (12). It is interesting that although oleoyl LPA showed the highest activity in stimulating the LPA receptor, its *trans* isomer (elaidoyl) had lower activity, similar to the saturated palmitoyl LPA analog. We also describe here a method for resolving the optical isomers of LPA. In a separate experiment, we found that the resulting *sn*-3 LPA is fully active as a substrate for guinea pig liver microsomal LPA acyltransferase (data not shown).

The synthetic methods described here for the preparation of acyl DHAPs and LPAs are simple, and all of the starting chemicals are commercially available. The overall yields are high, higher than those obtained by previously described methods (3–5). Also, many acyl derivatives (e.g., unsaturated ones) that could not be synthesized by previous methods could be successfully prepared using the methods described here. Using the procedures described above, one could also synthesize PAs having two different acyl groups at C-1 and C-2 of the glycerol moiety. These methods should prove valuable not only for making different acyl DHAPs and LPAs as substrates and ligands for different enzymes and receptors but also for making analogs of these physiologically important intermediates, which may act as inhibitors or antagonists of different enzymes and receptors for these lipids.

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