On the Relation between a Stearoyl-Specific Transacylase from Bovine Testis Membranes and a Copurifying Acyltransferase†

David Hollenback and John A. Glomset*

Howard Hughes Medical Institute, Departments of Medicine and Biochemistry, and Regional Primate Research Center, University of Washington, Seattle, Washington 98195-7370

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ABSTRACT: Bovine testis membranes contain a coenzyme A-dependent transacylase that can catalyze the preferential transfer of stearoyl groups from phosphoglycerides to *sn*-2-acyl molecular species of lysophosphatidic acid and lysophosphatidylinositol [Itabe et al., (1992) *J. Biol. Chem. 267*, 15319−15325]. We have now purified this enzyme 1000-fold and shown that it copurifies with an acyltransferase. The purified transacylase can use phosphatidic acid, phosphatidylinositol, or phosphatidylinositol-4-phosphate as an acyl donor and catalyzes the transfer of stearoyl groups in preference to palmitoyl groups or oleoyl groups. In contrast, the purified acyltransferase uses acyl-coenzyme A as an acyl donor and shows no such preference for stearoyl group transfer. Furthermore, phosphatidylinositol-4,5-bisphosphate inhibits the two enzymes to different extents and by different mechanisms. Nevertheless, the enzymes are similar in several respects: they use the same acyl acceptors and, when assayed together, compete for the acyl acceptor, *sn*-2-oleoyl lysophosphatidic acid; they lose activity in parallel unless stabilized by the addition of an anionic phosphoglyceride or stearoyl-coenzyme A; and they show similar sensitivities to heat and pH. One way to explain these results is to postulate that the transacylase reaction occurs in two successive steps: a stearoyl-specific first step in which a stearoyl group is transferred from an *sn*-1-stearoyl-2-acyl phosphoglyceride to coenzyme A, and a relatively non-acyl-chain-specific second step in which a stearoyl group is transferred from stearoyl-coenzyme A to an *sn*-2-acyl lysophosphoglyceride. According to this line of reasoning, the transacylase assay that we have used measures the net effect of both steps, whereas the acyltransferase assay measures only the effect of the second step.

The membranes of animal cells contain enzymes that can catalyze the transfer of fatty acyl groups from donor to acceptor lipids (reviewed in refs *1*−*5*). These *transacylases* are of several types. For instance, some require unesterified coenzyme A (CoA)[1] as a cofactor; others do not. Some preferentially catalyze the transfer of 20:4 groups or 18:0 groups (*6*−*14*); others show little acyl group specificity. How these *transacylases* relate to membrane-associated *acyltransferases*, which catalyze the transfer of fatty acyl groups from acyl-CoA to lipid acceptors, is not clear. Furthermore, it's unclear how transacylase reactions influence membrane functions, though evidence is accumulating that 18:0-specific transacylases and 20:4-specific transacylases play important roles in the biosynthesis of special phosphoglyceride molecular species that contain esterified 18:0 in the *sn*-1 position and/or esterified 20:4 in the *sn*-2 position (*2, 5, 15*).

Much more information about the different transacylases will be needed if their functional significance is to be fully understood. The transacylases will have to be solubilized, purified, and characterized so that their properties can be compared. Unfortunately, most attempts to solubilize membrane-associated transacylases with detergents have caused an immediate and complete loss of enzyme activity. Therefore, it has been necessary to use unsolubilized membrane preparations as a source of enzyme activity, and it has been difficult to characterize the properties of individual transacylases with certainty. This problem will have to be solved if progress in the field is to continue.

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* To whom correspondence should be addressed: Howard Hughes Medical Institute, Box 357370, University of Washington, Seattle WA 98195-7370. Tel: 206-685-2503. Fax: 206-543-0858. E-mail: jglomset@u.washington.edu.

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[1] Abbreviations: acyl-CoA, fatty acyl ester of coenzyme A; CoA, unesterified coenzyme A; DG, *sn*-1,2-diacylglycerol; DTPA, diethyenetriaminepentaacetic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HPLC, high performance liquid chromatography; K_M^{app}, apparent K_M; lysoPA, lysophosphatidic acid; lysoPC, lysophosphatidylcholine; lysoPE, lysophosphatidylethanolamine; lysoPI, lysophosphatidylinositol; lysoPS, lysophosphatidylserine; *sn*-1-OPGE, *sn*-1-oleyl-3-phosphoglyceryl ether; *sn*-2-OPGE, *sn*-2-oleyl-3-phosphoglyceryl ether; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIP, phosphatidylinositol-4-monophosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; PS, phosphatidylserine; V_{max}^{app}, apparent V_{max}; vol, volume. Phosphoglyceride molecular species are designated as follows: *sn*-1-16:0, *sn*-1-palmitoyl; *sn*-1-18:0, *sn*-1-stearoyl; *sn*-1-18:1, *sn*-1-oleoyl; *sn*-2-18:1, *sn*-2-oleoyl; *sn*-2-20:4, *sn*-2-arachidonoyl; 14:0-20:4, *sn*-1-myristoyl-2-arachidonoyl; 16:0-18:1, *sn*-1-palmitoyl-2-oleoyl; 16:0-18:2, *sn*-1-palmitoyl-2-linoleoyl; 16:0-20:4, *sn*-1-palmitoyl-2-arachidonoyl; 16:0-22:6, *sn*-1-palmitoyl-2-docosahexenoyl; 18:0-18:1, *sn*-1-stearoyl-2-oleoyl; 18:0-18:2, *sn*-1-stearoyl-2-linoleoyl; 18:0-20:4, *sn*-1-stearoyl-2-arachidonoyl; 18:1-18:1, *sn*-1,2-dioleoyl.

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Fortunately, a testis membrane-associated transacylase was recently found to be stable in the presence of the neutral detergent, Triton X-100. Preliminary attempts to characterize the Triton X-100-solubilized transacylase showed that it could catalyze the preferential transfer of 18:0 groups from donor phosphoglycerides to *sn*-2-acyl molecular species of lysoPA or lysoPI by a CoA-dependent mechanism (*13*). This raised the possibility that the enzyme might be identical or closely related to 18:0-specific transacylases previously implicated in the biosynthesis of 18:0-20:4 PI, the major molecular species of PI in mammalian cells (*16*, *17*; see also ref *15* for review). The initial aim of the present study was to purify the transacylase, characterize its properties in more definitive terms, and examine its mechanism of action. However, early during the course of the study, we discovered that the transacylase copurified with an acyltransferase through five chromatographic steps. Therefore, we expanded the study to include an examination of the properties of this enzyme as well.

MATERIALS AND METHODS

Materials. [γ-^{32}P]ATP, [^{14}C]18:0, [^{14}C]18:1-[^{14}C]18:1 PC, 18:0-[^{14}C]20:4 PI, and [^{14}C]12:0-CoA were from NEN Life Science. 18:1-18:1 Phosphatidyl[^{14}C]ethanolamine and 18:1-18:1 phosphatidyl[^{14}C]serine were from Amersham. *N*-[2′-(1′,3′-Propanediol)]-*cis*-9-octadecenimide (a monoolein analog with an imide-linked fatty acid) was a gift from Dr. Henry Higgs (Salk Institute, San Diego, California). Bovine brain PIP_2, bovine brain PIP, reduced Triton X-100, and recombinant *Escherichia coli* DG kinase were from Calbiochem. Acyl-CoA ligase was from Boehringer Mannheim. 1-Monoolein and 2-monoolein were from Serdery. DG was from Sigma. All other lipids were from Avanti Polar Lipids. Mature bovine testes were from Pel Freeze. Q Sepharose, octyl-Sepharose, and Superdex 200 were from Pharmacia. Dextran Sulfate 500 was coupled to epoxy-activated Sepharose (both from Pharmacia) according to the manufacturer's instructions. Matrex Red A was from Amicon. SDS−PAGE reagents and Ultrafree-15 concentrator (Biomax filter, 10 kDa molecular weight cutoff) were from Millipore. 2,6-di-*tert*-Butyl-4-methylphenol, 1-methyl-3-nitro-1-nitrosoguanidine, anhydrous chloroform, dicyclohexylcarbodiimide, and 1,2-dichloroethane were from Aldrich. 4-Pyrrolidinopyridine was from Aldrich and was recrystallized in pentane before use. All other organic solvents were American Chemical Society grade or better from J. T. Baker. All other chemicals were of reagent grade from Sigma.

Preparation of ^{32}P-Labeled lysoPA and lysoPA Analogs. ^{32}P-Labeled *sn*-2-18:1 lysoPA (ca. 200 000 cpm/nmol) was prepared by a modified method of Walsh and Bell (*18*) using recombinant *E. coli* DG kinase to phosphorylate 2-18:1 glycerol with [γ-^{32}P]ATP. A typical (1.0 mL) reaction mixture contained 4 mM 2-monoolein; 2.8 mM 18:1-18:1 PC; 16.3 mM Triton X-100; 0.075 mg/mL of DG Kinase; 4.05 mM [γ-^{32}P]ATP; 12.5 mM $MgCl_2$; 50 mM imidazole, pH 6.6; 50 mM $LiCl_2$; 1 mM EGTA; 2 mM DTT; and 23 μM DTPA. The ^{32}P-labeled lysoPA was purified essentially as described (*13*). ^{32}P-Labeled *sn*-1-18:1 lysoPA, 1-OPGE, 2-OPGE, and *N*-[2′-(3′-[^{32}P]phospho-1′-propanol)]-*cis*-9-octadecenimide were prepared in a similar manner, substituting the appropriate precursor (1-18:1 glycerol, 1-oleyl glyceryl ether, 2-oleyl glyceryl ether, or *N*-[2′-(1′,3′-propanediol)]-*cis*-9-octadecenimide) for 2-monoolein in the reaction mixture.

Preparation of [^{14}C]18:0-18:1 PC. This synthesis was performed as described (*19*) with several modifications (*20*, *21*). Briefly, 32.3 μmol of *sn*-2-18:1 lysoPC and 64.6 μmol of [^{14}C]18:0 (11.5 cpm/pmol) were dried under a stream of Argon in a 3 mL amber reactivial (Pierce). The sample was dried further overnight *in vacuo* at a pressure of <50 mTorr, then suspended in 1 mL anhydrous chloroform. A Teflon-coated stir bar was added followed by 185 μL 0.5 M 4-pyrrolidinopyridine and 65 mL 0.5 M dicyclohexylcarbodiimide, both dissolved in anhydrous chloroform. The vessel was capped with a mini-inert valve (Pierce), flushed with argon and stirred for 6 h at room temperature, with three more additions of dicyclohexylcarbodiimide (6.5 μmol/addition). This treatment caused a nearly complete conversion of lysoPC to PC. After the incubation, the ^{14}C-labeled PC was purified on a Bakerbond diol column (Baker) as described (*13*) and repurified if necessary. The ^{14}C-labeled PC was greater than 95% pure as determined by TLC using two different solvent systems. Digestion of the purified, ^{14}C-labeled PC with phospholipase A_2 (*Naja naja naja*, Sigma) showed that 92% of the [^{14}C]18:0 was esterified at the *sn*-1 position.

Preparation of Other Enzyme Substrates. *sn*-2-Acyl lysophosphoglycerides were prepared by digestion of the corresponding diacyl phosphoglycerides with neutral lipase (*13*). Molecular species of unlabeled or ^{14}C-labeled PA were prepared from the corresponding molecular species of PC by digestion with phospholipase D (*22*). [^{14}C]18:0-CoA was prepared essentially as described (*23*).

Enzyme Assays. All enzyme assays were performed in 12 × 75 mm borosilicate glass test tubes at 37 °C for 3−10 min. Product formation was linear with time and protein. Column fractions were assayed in singlets, all other samples in duplicate. ^{32}P-labeled PA products from the enzyme assays were prepared for C_{18} reverse phase HPLC as described (*13*). Any additional changes in either assay are described in the figure legends or text. mol % values were calculated as described (*24*). The computer program GraFit (Erithacus Software) used multiple nonlinear regression to determine Michaelis−Menton kinetic constants and inhibitor constants.

Transacylase Assay. Fatty acyl transfer was monitored as described for the CoA-dependent transacylation reaction (*13*) with the following modifications. In most experiments, assay mixtures (50 μL) consisted of 10 μL of enzyme, buffer A (40 mM Tris, pH 7.5; 2 mM DTT; 0.4 mM EDTA, and 640 μM Na acetate), 80 μM CoA, and mixed micelles containing 91 mol % Triton X-100, 8 mol % bovine liver PI, and 0.75 mol % ^{32}P-labeled *sn*-2-18:1 lysoPA; the total concentration of micellar components (the total concentration of detergent plus lipids in micelles) was 15 mM. The activity followed surface dilution kinetics at Triton X-100 concentrations of ≥10 mM (*24*, *25*).

Because the acyl chains of lysophosphoglycerides migrate readily between the *sn*-1 and *sn*-2 positions, in some cases, ^{32}P-labeled 2-OPGE or *N*-[2′-(3′-phospho-1′-propanol)]-*cis*-9-octadecenimide was used as the acyl acceptor instead of ^{32}P-labeled *sn*-2-18:1 lysoPA with qualitatively similar results (data not shown). Other changes in the assay are noted in

the text and figure legends. For cruder fractions prior to purification over Q Sepharose, the assay mixture was similar to the above except that it contained 0.5 mM CoA, 4 mM Na acetate, 25 mM NaF, and 0.1 mM $NaVO_4$. No ^{32}P-labeled PA was formed if PI, CoA or enzyme were omitted from the assay (data not shown), and CoA was routinely omitted to determine background counts.

Acyltransferase Assay. In most experiments, assay mixtures (50 μL) consisted of 10 μL enzyme, buffer A, and mixed micelles containing 99 mol % Triton X-100, 0.47 mol % 18:0-CoA, and 1 mol % ^{32}P-labeled *sn*-2-acyl lysoPA; the total concentration of micellar components was 15 mM. In some cases, ^{32}P-labeled 2-OPGE or *N*-[2′-(3′-phospho-1′-propanol)]-*cis*-9-octadecenimide was used as the acyl acceptor instead of ^{32}P-labeled *sn*-2-acyl lysoPA with qualitatively similar results (data not shown). The activity followed surface dilution kinetics with respect to *sn*-2-acyl lysoPA at Triton X-100 concentrations ≥ 15 mM (*24*). However, similar activities were observed when the total concentration of 18:0-CoA in the incubation mixture was held at 7.5 μM while the concentration of Triton X-100 was varied from 10 to 25 mM. [^{32}P]PA products were subsequently analyzed as described for the CoA-dependent transacylation reaction. Changes in the assay are noted in the text and figure legends. No ^{32}P-labeled PA was formed if 18:0-CoA or enzyme were omitted from the assay.

Competition Assays. To evaluate the abilities of the transacylase and acyltransferase to discriminate among different substrates, incubation experiments were done with mixtures of labeled and unlabeled acyl donors or acceptors. Labeled acyl donors or acceptors were used at concentrations that approximated their K_M^{app} values; unlabeled acyl donors or acceptors were used at a range of concentrations that extended from below to above their K_M^{app} values.

General Procedures Used in Protein Purification. All steps were performed at 0−4 °C. The pH of the buffer solutions was adjusted at room temperature prior to refrigeration. During chromatography on dextran sulfate-Sepharose, octyl-Sepharose, Matrex Red A, and Superdex 200, absorbance at 280 nm and, where applicable, conductivity of the column effluent were measured using the inline absorbance monitor and conductivity meter in the BioLogic chromatography system (BioRad, Hercules, CA). Fractions containing at least 50% of the peak activity from these columns were pooled. The concentration of reduced Triton X-100 in the octyl-Sepharose effluent was determined as described (*26*). Active material collected from liquid chromatography was flash-frozen and stored at −70 °C.

Membrane Preparation, Extraction, and Chromatography on Q-Sepharose. Testes were stored at −70 °C, and the membranes were prepared as described (*25*), except that they were not washed with salt. Transacylase and acyltransferase activities were extracted by mixing six membrane pellets with 11.4 mM Triton X-100 in buffer B (20 mM Tris, pH 7.5; 1 mM DTT; 1 mM EDTA; 20% w/v glycerol; 1 mM phenylmethylsulfonyl fluoride; 1 mM benzamidine; 0.5 mM leupeptin, and 1 mg/mL aprotinin) to make a final vol of 600 mL. The solution was stirred for 1 h and then centrifuged for 1 h at 100000*g* in a Ti45 rotor (Beckman) to remove insoluble material. Q Sepharose (600 mL) was washed with 3 bed volumes of buffer B containing 8.2 mM Triton X-100, resuspended in 600 mL of the same buffer, and allowed to settle by gravity overnight; then excess buffer was removed by aspiration. The clear, auburn supernatant and the Q Sepharose slurry were mixed and then gently stirred for 1 h. The mixture was poured into a 3 L sintered glass funnel, allowed to drain, and then washed with 1 bed vol of buffer B containing 8.2 mM Triton X-100; neither activity bound to this resin, and the flow-through material from the two washes was pooled.

Chromatography on Dextran Sulfate-Sepharose. The NaCl concentration of the active material from the Q Sepharose chromatography was adjusted to 100 mM using a 5 M NaCl stock. Then the material was loaded onto a Pharmacia XK50 column packed with 150 mL of dextran sulfate-Sepharose and pre-equilibrated with 5−10 bed vol of buffer B containing 8.2 mM Triton X-100 and 100 mM NaCl. The column was washed with 150 mL of buffer B containing 100 mM NaCl and mixed micelles of 98 mol % reduced Triton X-100 and 2 mol % egg PA (the total concentration of micellar components was 1.5 mM), followed by 225 mL of buffer B containing the same concentration and composition of mixed micelles but 0.25 M NaCl. Enzyme activities were eluted with a 450 mL gradient of 0.25 to 1.3 M NaCl in the same buffer. The flow rate was 150 mL/h, and 12.5 mL fractions were collected.

Chromatography on Octyl-Sepharose. Octyl-Sepharose (78 mL) was washed with 5 bed vol of buffer C (20 mM Tris, pH 7.5; 1 mM DTT; 1 mM EDTA; 20% w/v glycerol; and 0.5 mM leupeptin) and then resuspended as a 46% v/v slurry in the same buffer. To this was sequentially added 24.1 mL buffer C containing 81.5 mM reduced Triton X-100, 14 mL buffer C containing mixed micelles of 80 mol % reduced Triton X-100 and 20 mol % egg PA (the total concentration of micellar components was 102 mM), and 195 mL buffer C containing 1.2 M NaCl. Each addition was followed by a 1 h incubation with gentle mixing at 4 °C. The slurry was poured into a 2.6 cm diameter column and washed with 5−10 bed vol of buffer C containing 0.6 M NaCl and mixed micelles of 90 mol % reduced Triton X-100 and 10 mol % egg PA (the total concentration of micellar components was 1.6 mM) until the A_{280} returned to its base line value. The concentration of egg PA in the enzyme-containing effluent from the dextran sulfate-Sepharose chromatography was adjusted to 10 mol % by adding buffer C containing 0.6 M NaCl and mixed micelles of 78 mol % reduced Triton X-100 and 22 mol % egg PA (the total concentration of micellar components was 1.8 mM). Then the mixture was loaded onto the octyl-Sepharose column and washed with 90 mL of buffer C containing 0.6 M NaCl and mixed micelles of 90 mol % reduced Triton X-100 and 10 mol % egg PA (the total concentration of micellar components was 1.6 mM). The enzyme activities were eluted in 290 mL of a 1.6 to 22.6 mM gradient of mixed micelles of the same composition in the same buffer. The flow rate was 105 mL/h, and 10 mL fractions were collected.

Chromatography on Matrex Red A. Matrex Red A (15 mL) was poured into a 2.5 cm diameter column and washed with 5−10 bed vol of buffer C containing 0.1 M NaCl and mixed micelles of 90 mol % reduced Triton X-100 and 10 mol % 16:0-18:1 PA (the total concentration of micellar components was 1.6 mM). The NaCl concentration in active material from octyl-Sepharose chromatography was adjusted

Table 1: Purification of Bovine Testis Membrane Transacylase and Acyltransferase Activities[a]

step	protein (mg)	activity (nmol/min)	yield (%)	activity (nmol/min/mg)	enrichment (fold)	relative specific activities[b]
			Transacylase Activity			
4000*g* supernatant	7534	6700	100	0.889	1	
100000*g* pellet	2352	2817	42	1.20	1.3	
Triton X-100 extract	1478	2626	39	1.78	2.0	1.14
Q-Sepharose	319	1449	22	4.54	5.1	0.90
dextran sulfate-Sepharose	41.0	759	11	18.5	20.8	nd[c]
octyl-Sepharose	6.26	775	12	124	139	1.17
Matrex Red A	0.528	246	4	466	524	0.95
Superdex 200	0.204	210	3	1030	1158	1.46
			Acyltransferase Activity			
4000*g* supernatant	7534	4062	100	0.539	1	
100000*g* pellet	2352	3080	76	1.31	2.4	
Triton X-100 extract	1478	2310	57	1.56	2.9	
Q-Sepharose	319	1603	39	5.02	9.3	
dextran sulfate-Sepharose	41.0	nd	nd	nd	nd	
octyl-Sepharose	6.26	661	16	106	196	
Matrex Red A	0.528	260	6	492	913	
Superdex 200	0.204	143	4	704	1306	

[a] Results are representative of three experiments. [b] Relative specific activities = transacylase specific activity/acyltransferase specific activity. [c] nd, not determined.

to 0.1 M by adding buffer C, and the material was loaded onto the resin. The column was washed with 15 mL buffer C containing 0.1 M NaCl and mixed micelles of 90 mol % reduced Triton X-100 and 10 mol % 16:0-18:1 PA (the total concentration of micellar components was 1.6 mM). Active material was sequentially eluted with 1.6 mL of a 0.1 to 0.2 M NaCl gradient in buffer C containing the same concentration and composition of mixed micelles followed by 30 mL of a 0.2 to 1.5 M NaCl gradient in the same buffer. The flow rate was 0.8 mL/min, and 1.2 mL fractions were collected.

Chromatography on Superdex 200. A column of Superdex 200 (XK26/60, Pharmacia) was pre-equilibrated with 5 bed vol of a solution containing 20 mM Tris, pH 7.5; 1 mM DTT; 1 mM EDTA; 0.5 mM leupeptin; 0.5 M NaCl; and mixed micelles of 95 mol % reduced Triton X-100 and 5 mol % 16:0-18:1 PA (the total concentration of micellar components was 1.5 mM). Active material from Matrex Red A chromatography was concentrated with a Millipore Concentrator according to the manufacturer's instructions and loaded onto the resin. The column was developed in the same buffer; the flow rate was 0.5 mL/min and 1.2 mL fractions were collected.

Stability Studies. Dextran sulfate-Sepharose (0.5 mL in Pierce EconoColumns, 0.75 cm diameter) was pre-equilibrated with 5 mL of buffer B containing 0.05 M NaCl and 1.63 mM reduced Triton X-100. The NaCl concentration of the active material from Q Sepharose Chromatography was adjusted to 0.05 M from a 5 M stock and 5 mL of sample were loaded onto the resin. The column was washed with 2.5 mL of buffer B containing 0.3 M NaCl and 1.63 mM reduced Triton X-100 and the active material was eluted with 1.2 mL of buffer B containing 1.3 M NaCl and 1.63 mM reduced Triton X-100. To measure the stability of this material, the eluate was immediately mixed with an equal volume of buffer B containing 1.63 mM reduced Triton X-100 and then assayed for activity over time. To measure the effects of potential stabilizers, the eluate was immediately mixed with an equal volume of buffer B containing mixed micelles of 99 mol % reduced Triton X-100 and 1 mol % phosphoglyceride or acyl-CoA (the total concentration of micellar components was 1.5 mM in both experiments), then assayed for activity immediately and after a 20 h incubation on ice.

Attempts to Isolate an Acyl-CoA Intermediate. Aliquots of pooled fractions from the Superdex 200 chromatography were incubated in buffer A, 80 μM CoA and mixed micelles of 97−99 mol % Triton X-100 and 1 mol % [^{14}C]18:0-18:1 PA (14.7 cpm/pmol) with or without 1.5 mol % *sn*-2-18:1 lysoPA (the total concentration of micellar components was 15 mM). After a 3 min incubation at 37 °C the reaction was quenched with 200 μL of ice-cold acetone. Then the samples were incubated on ice for 15 min and centrifuged for 15 min at 16000*g*. The supernatant was removed and dried *in vacuo*; the sample was suspended in 50 μL of methanol, spotted on to a silica gel 60 TLC plate (Merck), and developed in isobutanol/acetic acid/water = 8/3/3.

Other Methods. Protein concentrations were assayed using the bicinchoninic acid method (Pierce); before quantification, interfering substances were removed with trichloroacetic acid precipitation (*25*). SDS−PAGE on 12% acrylamide slab gels was performed by the method of Laemmli (*27*). Protein bands were identified by silver staining (*28*). Thin layer chromatography of phospholipids and phospholipid quantification were as described (*13*).

RESULTS

Partial Purification of Transacylase and Acyltransferase Activities; Requirement for an Added Lipid Stabilizer. When Triton X-100-solubilized material from bovine testis membranes was treated with Q Sepharose (Materials and Methods), 80% of the transacylase activity remained soluble, based on the CoA-dependent transfer of acyl groups from bovine liver PI to ^{32}P-labeled *sn*-2-18:1 lysoPA (Table 1). Furthermore, about the same percentage of the acyltransferase activity in the Triton X-100-solubilized material also remained soluble, based on the transfer of 18:0 groups from 18:0-CoA to ^{32}P-labeled *sn*-2-18:1 lysoPA (Table 1). On the other hand, both of the soluble activities subsequently

adsorbed to a column of dextran sulfate-Sepharose and coeluted when a linear gradient of 0.25 to 1.3 M NaCl was applied (Figure 1A).

The coeluted activities decayed rapidly at the same rate (Figure 2A) unless they were stabilized by the addition of PA, PIP, PIP_2, or 18:0-CoA (Figure 2, panels B and C, and data not shown). Importantly, the half maximal concentration of 16:0-18:1 PA required for stabilization of the transacylase and acyltransferase activities was approximately 0.3 mol % in each case (Figure 2B). Moreover, the half maximal concentrations of 18:0-CoA required for stabilization of the activities under comparable conditions were similar: transacylase, 0.25 mol %, and acyltransferase, 0.2 mol % (Figure 2C).

On the basis of these results, we included a minimum of 2 mol % egg PA or 16:0-18:1 PA in the chromatography buffers used for the dextran sulfate-Sepharose step in the enzyme purification and in all subsequent steps as well. Furthermore, because pooled fractions from the dextran sulfate column were next chromatographed on octyl-Sepharose, pre-equilibration of the octyl-Sepharose with buffer containing egg PA was essential (Materials and Methods). The transacylase- and acyltransferase activities adsorbed to the pre-equilibrated octyl-Sepharose column and coeluted on a 1.6 to 22.6 mM linear gradient of mixed micelles containing 90 mol % reduced Triton X-100 and 10 mol % egg PA (Figure 1B).

When the activities recovered from this column were subsequently chromatographed on Matrex Red A, they coeluted on a linear gradient of 0.2 to 1.5 M NaCl (Figure 1C). Finally, when the activities recovered from the Matrex Red A column were passed through a Superdex 200 column, they again coeluted (Figure 1D). Thus, the enzyme activities behaved similarly during five successive chromatography steps, and each one was purified approximately 1000-fold (see also Table 1).

The calculated *apparent* molecular mass of each enzyme was 250 kDa, based on the enzymes' elution volume from the Superdex 200 column in the presence of reduced Triton X-100 and 16:0-18:1 PA. But this value is very likely to be an overestimate. When we chromatographed material from a Matrex Red A column on Superdex 200 in the presence of Triton X-305 and 16:0-18:1 PA, the enzyme activities again coeluted but had an apparent molecular mass of 467 kDa (not shown). This indicates that the presence of bound Triton influenced the calculated molecular masses in these experiments. The difference between the calculated values obtained remains to be explained, but probably reflects physical differences in the two types of Triton: Triton X-305 has a larger monomer size than reduced Triton X-100 does (average molecular mass 1526 versus 629) but a smaller aggregation number (4.2 versus 100−150; ref *29*). It was not possible to determine the molecular masses of the enzymes by SDS−PAGE because the pooled active fractions from a Superdex 200 experiment showed several major and minor silver-staining bands that ranged from 20 to 100 kDa (data not shown).

Acyl Donor and Acceptor Specificities of the Partially Purified Transacylase. Several sets of experiments were done to examine the acyl donor specificity of the partially purified transacylase from the Superdex 200 column step. One set examined the enzyme's ability to use different *classes*

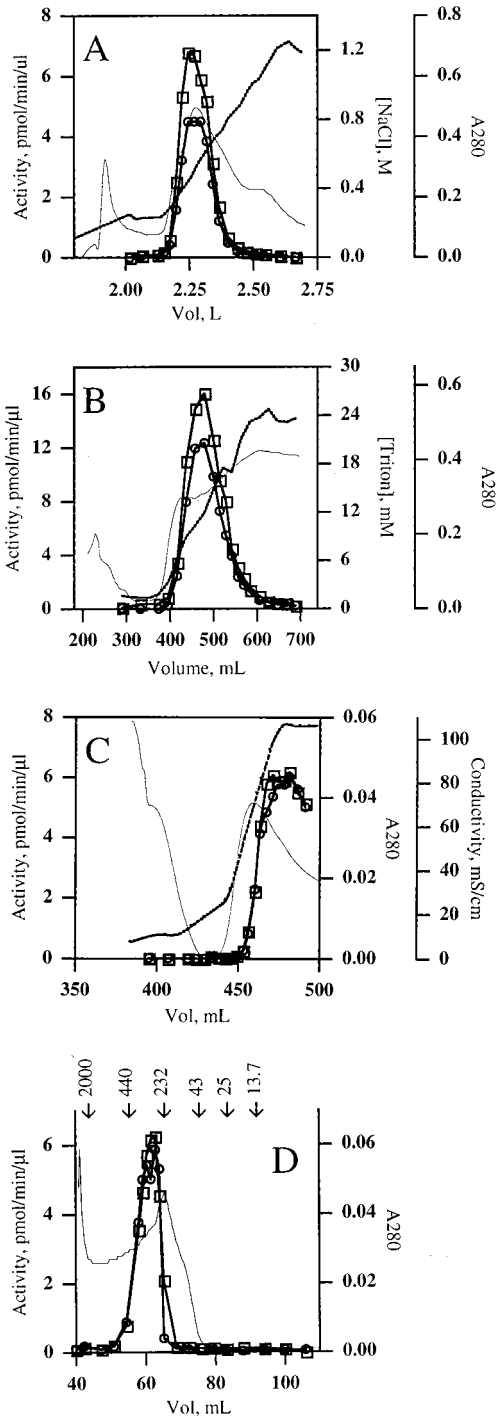

FIGURE 1: Copurification of transacylase and acyltransferase activities. Shown are elution profiles from successive chromatographic experiments on dextran sulfate-Sepharose (A), octyl-Sepharose (B), Matrex Red A (C), and Superdex 200 (D), done as described in Material and Methods. Transacylase activity (circles) and acyltransferase activity (squares), measured as described in Materials and Methods, are graphed in relation to the elution of absorbing material at A_{280} nm (thin lines), conductivity or NaCl concentration (heavy lines, panels A and C), and/or reduced Triton X-100 concentration (heavy lines, panel B). Similar results were obtained in several purifications through dextran sulfate-Sepharose and octyl-Sepharose ($n = 5$), matrex Red A ($n = 2$), and Superdex 200 ($n = 5$). Elution of size exclusion chromatography standards (Pharmacia) is shown in panel D. SDS−PAGE analysis of fractions eluting from the Superdex 200 column (excluded volume = 40 mL and included volume = 120 mL) showed that the majority of protein eluted between 58 and 70 mL and that the chromatography caused some resolution of the silver-staining bands, yet none of the bands appeared to track with the eluted activities.

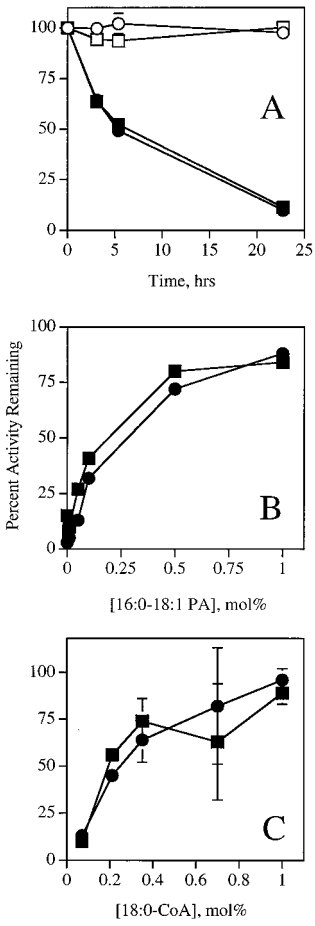

FIGURE 2: Decay of transacylase and acyltransferase activities after elution from dextran sulfate-Sepharose or prevention of decay by added PA or 18:0-CoA. (A) Enzyme-containing fractions, pooled after chromatography on Q-Sepharose or after successive chromatographies on Q-Sepharose and dextran sulfate-Sepharose (Materials and Methods) were assayed immediately or after being incubated for various periods of time at 4 °C. Transacylase (circles) and acyltransferase (squares) activities were measured as described in Materials and Methods; pooled material from Q Sepharose chromatography (open symbols); pooled material from dextran sulfate-Sepharose chromatography (filled symbols). Two separate experiments done in duplicate gave similar results. (B) Stabilization by added 16:0-18:1 PA of the transacylase and acyltransferase activities after chromatography on dextran sulfate-Sepharose. Enzyme-containing fractions were pooled, mixed with the indicated concentrations of 16:0-18:1 PA, and assayed immediately or after being incubated for 20 h at 4 °C. Symbols are the same as in panel A. A second experiment using egg PA as the stabilizer gave qualitatively similar results. (C) Stabilization by added 18:0-CoA of the transacylase and acyltransferase activities after chromatography on dextran sulfate-Sepharose. Enzyme-containing fractions were pooled and mixed with the indicated concentrations of 18:0-CoA, then treated as in B. Symbols are the same as in panel A. The results are the average ± standard deviation of two separate experiments done in duplicate.

of phosphoglycerides as acyl donors in the presence or absence of CoA. The results revealed that the enzyme could catalyze the CoA-dependent transfer of acyl groups from 18:0-20:4 PA, bovine brain PIP, or bovine liver PI to ^{32}P-labeled *sn*-2-18:1 lysoPA, but could not catalyze the corresponding CoA-independent reactions (Table 2 and data not shown). Furthermore, the enzyme could not catalyze the CoA-dependent or CoA-independent transfer of acyl groups from 18:0-18:1 PS, 18:0-20:4 PE, 18:0-20:4 PC or *sn*-1-18:0 lysoPA to ^{32}P-labeled *sn*-2-18:1 lysoPA (Table 2 and data not shown). The major difference between these results and the results reported previously for the unfractionated Triton X-100 extract of bovine testis membranes (*13*) is that the partially purified transacylase was unable to use *sn*-1-18:0-containing molecular species of PS, PE, and lysoPA as acyl donors.

Table 2: Phosphoglyceride Acyl Donors in the Transacylase Assay[a]

phosphoglyceride acyl donor	V_{max}^{app} [b] (pmol/min/μg protein)	K_M^{app} (mol %)	V_{max}^{app}/K_M^{app}	*n*
18:0-20:4 PA	67 ± 1	0.096 ± 0.006	700 ± 40	3
bovine brain PIP	350 ± 50	0.71 ± 0.07	493 ± 5	2
bovine liver PI	340 ± 30	0.9 ± 0.1	380 ± 50	5
18:0-18:1 PS	na[c]	na		
18:0-20:4 PC	na	na		
18:0-20:4 PE	na	na		
sn-1-18:0 lysoPA	na	na		

[a] The activities of different classes of acyl donors were determined in separate assay containing aliquots from a Superdex 200 column, buffer A, 80 μM CoA, and mixed micelles of 87−99 mol % Triton X-100, 1 mol % ^{32}P-labeled *sn*-2-18:1 lysoPA and variable concentrations of phosphoglyceride acyl donor. [b] V_{max}^{app} values and K_M^{app} values shown are from separate experiments that used the same Superdex 200 pool as the enzyme source. The error was calculated by nonlinear regression of duplicate data points in this analysis. Similar relative V_{max}^{app}/K_M^{app} values for the different classes of phosphoglycerides were observed for the indicated number of determinations (*n*) from at least two separate purifications. [c] na, not active.

A second set of experiments examined the possibility that the above-mentioned enzyme reactions with PA, PIP, and PI might have been catalyzed by a single transacylase. The results of acyl donor competition assays (Materials and Methods) revealed that unlabeled 18:0-18:1 PA, bovine brain PIP, or bovine liver PI could separately compete with [^{14}C]-18:0-18:1 PA in transacylase assays that used unlabeled *sn*-2-acyl lysoPI as an acceptor; the order of effectiveness was 18:0-18:1 PA > bovine brain PIP > bovine liver PI (Figure 3A). Furthermore, the results of separate heat-inactivation experiments showed that the ability of the transacylase to catalyze the CoA-dependent transfer of acyl groups from 0.7 mol % 18:0-20:4 PA, 2 mol % bovine brain PIP, or 8 mol % bovine liver PI to 0.75 mol % ^{32}P-labeled *sn*-2-18:1 lysoPA declined in parallel when the enzyme was treated for various periods of time at 45 or 50 °C ($t_{1/2}$ value for inactivation at 45 °C = 13 min, Figure 7 and data not shown). The combined results of these experiments provided strong evidence that only a single transacylase was present.

A third set of experiments tested the enzyme's specificity for acyl group transfer. The transacylase was separately incubated with different *sn*-1-acyl-2-18:1 molecular species of PA in the presence of CoA and ^{32}P-labeled *sn*-2-18:1 lysoPA; and V_{max}^{app}/K_M^{app} was determined for each reaction. The results indicated that V_{max}^{app}/K_M^{app} was 12-fold greater for the reaction with 18:0-18:1 PA than it was for the reaction with 18:1-18:1 PA and that no reaction occurred with 16:0-18:1 PA (Figure 4A; Table 3). In other experiments, the enzyme was incubated with *equimolar mixtures* of unlabeled 18:0-18:1 PA + 16:0-18:1 PA or 18:0-18:1 PA + 18:1-18:1 PA in the presence of CoA and ^{32}P-labeled *sn*-2-18:1 lysoPA, and the molecular species composition of the ^{32}P-labeled PA that was formed was analyzed by reverse phase HPLC. The results of these experiments showed that only 18:0 was transferred (Figure 5, panels A and B). This

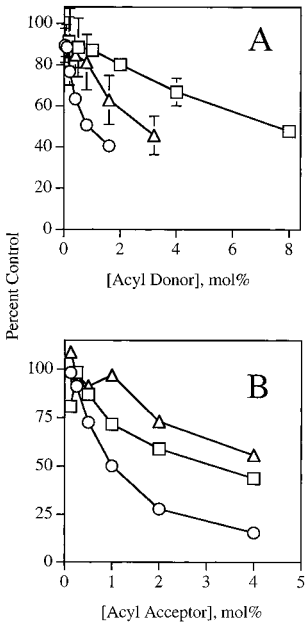

FIGURE 3: Competition among classes of transacylase substrate phosphoglycerides and lysophosphoglycerides. (A) Competition among classes of phosphoglyceride acyl donors was determined in transacylase assays (8 min, 37 °C) containing aliquots of pooled material from a Superdex 200 column; buffer A; 80 μM CoA; and mixed micelles of 91−99 mol % Triton X-100, 0.3 mol % [^{14}C]-18:0-18:1 PA, 0.5 mol % *sn*-2-acyl lysoPI and the indicated concentrations of unlabeled 18:0-18:1 PA (circles), bovine brain PIP (triangles), or bovine liver PI (squares). Amounts of radioactive 18:0-18:1 PI formed during the incubations were determined by thin layer chromatography (*13*). The results are the average ± standard deviation from two separate experiments done in duplicate. (B) Competition among classes of lysophosphoglyceride acyl acceptors was determined in transacylase assays (8 min, 37 °C) containing aliquots of pooled material from a Superdex 200 column; buffer A; 80 μM CoA; and mixed micelles of 95−99 mol % Triton X-100, 2 mol % bovine liver PI, 0.5 mol % of ^{32}P-labeled *sn*-2-18:1 lysoPA, and the indicated concentrations of unlabeled *sn*-2-18:1 lysoPA (circles), *sn*-1-18:1 lysoPA (squares), or *sn*-2-acyl lysoPI (triangles). Two separate experiments done in duplicate gave similar results.

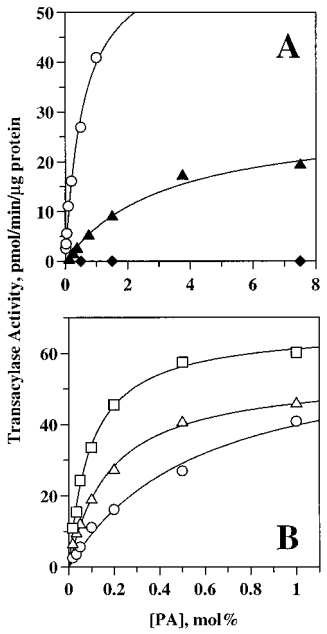

FIGURE 4: Phosphatidic acid molecular species preference of the transacylase. (A) Preference of the transacylase from a Superdex 200 column for different *sn*-1-acyl-2-18:1 molecular species of PA was determined by measuring the amount of labeled PA formed in separate incubation assays (8 min, 37 °C) containing buffer A; 80 μM CoA; and mixed micelles of 92−99 mol % Triton X-100, 0.75 mol % ^{32}P-labeled *sn*-2-18:1 lysoPA and the indicated concentrations of 18:0-18:1 PA, hollow circles; 18:1-18:1 PA, filled triangles; and 16:0-18:1 PA, filled diamonds. Two separate experiments done in duplicate gave similar results. (B) Preference of the transacylase from a Superdex 200 column for different *sn*-1-18:0-2-acyl molecular species of PA was determined by measuring the amount of labeled PA formed in separate incubation assays similar to those in panel A except with the indicated concentrations of 18:0-20:4 PA, hollow squares; 18:0-18:2 PA, hollow triangles; or 18:0-18:1 PA, hollow circles. For comparison, 18:0-18:1 PA is shown in both panels. Two separate experiments done in duplicate gave similar results.

latter observation in particular provided compelling evidence that the transacylase reaction was highly 18:0 specific.

A fourth set of experiments investigated the influence of PA *sn*-2-acyl groups on the 18:0 transfer reaction. *sn*-1-18:0−2-Acyl molecular species of PA that contained 18:1, 18:2, or 20:4 in the *sn*-2-position were used as 18:0 donors in reaction mixtures that contained CoA and the acyl chain acceptor, ^{32}P-labeled *sn*-2-18:1 lysoPA. Under these conditions, each molecular species of PA was an effective donor, though the reactions showed a 6-fold range in V_{max}^{app}/K_M^{app} values: 18:0-20:4 > 18:0-18:2 > 18:0-18:1 (Figure 4B, Table 3). These results were of interest in relation to the result obtained with *sn*-1-18:0 lysoPA, shown in Table 2. Taken together, they provided evidence that the presence of *sn*-2-acyl groups in phosphoglyceride acyl donors was absolutely required for the transacylation reaction and that different esterified acyl groups in this position affected the reaction differently.

We also tested the acyl acceptor preference of the partially purified transacylase in several sets of experiments. As in the case of the acyl donor experiments, one set of experiments investigated the enzyme's ability to use different *classes* of lysophosphoglycerides as acyl chain acceptors in separate assays. The results revealed that the enzyme could effectively catalyze the CoA-dependent transfer of acyl groups from bovine liver PI or 18:0-18:1 PA to *sn*-2-acyl lysoPI, *sn*-2-18:1 lysoPA, or *sn*-1-18:1 lysoPA and that it could also effectively catalyze the CoA-dependent transfer of acyl groups from bovine liver PI to 2-OPGE, the ether-linked analog of *sn*-2-18:1 lysoPA (Table 4; note that *sn*-2-acyl lysoPIP was not tested). However, no transfer of acyl groups to 1-OPGE or to the *sn*-2-18:1 molecular species of lysoPC, lysoPE, or lysoPS could be detected (Table 4 and data not shown).

A second set of experiments examined the possibility that a single transacylase might have catalyzed the above-mentioned reactions. Acyl acceptor competition assays were done in which increasing concentrations of unlabeled *sn*-2-acyl lysoPI, *sn*-2-18:1 lysoPA, or *sn*-1-18:1 lysoPA were added to incubation mixtures containing unlabeled bovine liver PI as an acyl donor, CoA, and ^{32}P-labeled *sn*-2-18:1 lysoPA as an acyl acceptor. The results of these assays showed that each of the unlabeled lysophosphoglycerides could inhibit the formation of labeled product, in the order of effectiveness: *sn*-2-18:1 lysoPA > *sn*-1-18:1 lysoPA > *sn*-2-acyl lysoPI (Figure 3B). Taken together with the results of the acyl donor competition assays shown in Figure 3A, this provided definitive evidence that the

Table 3: Preference of the Transacylase and Acyltransferase for Acyl Group Transfer[a]

	acyl donor	V_{max}^{app} [b] (pmol/min/μg of protein)	K_M^{app} (mol %)	V_{max}^{app}/K_M^{app}	*n*
transacylase assay[c]	16:0-18:1 PA	na[d]	na		
	18:1-18:1 PA	29 ± 3	3.2 ± 0.8	9 ± 2	3
	18:0-18:1 PA	63 ± 6	0.6 ± 0.1	110 ± 20	2
	18:0-18:2 PA	54 ± 2	0.18 ± 0.02	300 ± 30	2
	18:0-20:4 PA	67 ± 1	0.096 ± 0.006	700 ± 40	3
acyltransferase assay[e]	10:0-CoA	19.0 ± 3	0.05 ± 0.03	400 ± 200	2
	12:0-CoA	71 ± 3	0.08 ± 0.01	900 ± 100	2
	14:0-CoA	23.8 ± 0.8	0.051 ± 0.006	470 ± 60	2
	16:0-CoA	136 ± 7	0.09 ± 0.01	1500 ± 200	2
	18:0-CoA	208 ± 10	0.048 ± 0.008	4300 ± 800	4
	18:1-CoA	141 ± 20	0.19 ± 0.06	700 ± 300	2
	18:2-CoA	36 ± 3	0.16 ± 0.03	230 ± 80	2
	20:4-CoA	na	na		

[a] All values were determined using material from a Superdex 200 column. [b] V_{max}^{app} and K_M^{app} values are defined in Table 2. [c] The activities were determined in separate assays similar to those in Table 2. [d] na, not active. [e] The activities were determined in separate assays containing buffer A and mixed micelles of 93−99 mol % Triton X-100, 1 mol % of ^{32}P-labeled *sn*-2-18:1 lysoPA and variable concentrations of the indicated molecular species of acyl-CoA.

partially purified enzyme preparation contained only one transacylase.

Because acyl chains of lysophospholipids migrate readily between *sn*-1 and *sn*-2 positions (*30*), the ester-linked lysophosphoglycerides used in these assays were probably mixtures of *sn*-1-acyl- and *sn*-2-acyl molecular species. We therefore did a third set of experiments to evaluate the enzyme's preference for acyl group transfer to the *sn*-1- and *sn*-2- positions of lysoPA. We incubated the transacylase with a mixture of liver PI, CoA, and ^{32}P-labeled *sn*-1-18:1 lysoPA or ^{32}P-labeled *sn*-2-18:1 lysoPA, then analyzed the distribution of fatty acyl groups in the PA products that were formed as described in Materials and Methods. In each case, a 3:1 ratio of 18:0-18:1 PA to 18:1-18:0 PA was observed (data not shown). The combined evidence from these experiments and the above-mentioned experiments with the stable, lysoPA analogs, 2-OPGE and 1-OPGE, strongly suggested that the transacylase catalyzed the preferential transfer of 18:0 groups from phosphoglyceride acyl donors to the *sn*-1-position of *sn*-2-acyl lysophosphoglyceride acyl acceptors.

Acyl Donor and Acceptor Specificities of the Partially Purified Acyltransferase. Two sets of experiments were done to examine the acyl donor specificity of the partially purified acyltransferase from the Superdex 200 chromatography step. One set of experiments investigated the enzyme's ability to catalyze separate transfer reactions involving one of several different molecular species of acyl-CoA and ^{32}P-labeled *sn*-2-18:1 lysoPA. The results revealed that the enzyme could use each one of the tested molecular species of acyl-CoA as a substrate, though V_{max}^{app}/K_M^{app} for the reaction with 18:0-CoA was 3−9-fold higher than the corresponding ratios for the reactions with 12:0-CoA, 14:0-CoA, 16:0-CoA, or 18:1-CoA (Table 3).

The second set of experiments examined the enzyme's ability to catalyze acyl transfer reactions in competition assays. Unlabeled 18:0-CoA, 16:0-CoA, 18:1-CoA, or 12:0-CoA was separately incubated with [^{14}C]18:0-CoA and unlabeled *sn*-2-18:1 lysoPA, and the amount of labeled PA that was formed was determined. The results demonstrated that unlabeled 18:0-CoA, 16:0-CoA, and 18:1-CoA were equally effective inhibitors of the formation of [^{14}C]18:0 PA, though 12:0-CoA was less effective (Figure 6A). A follow-

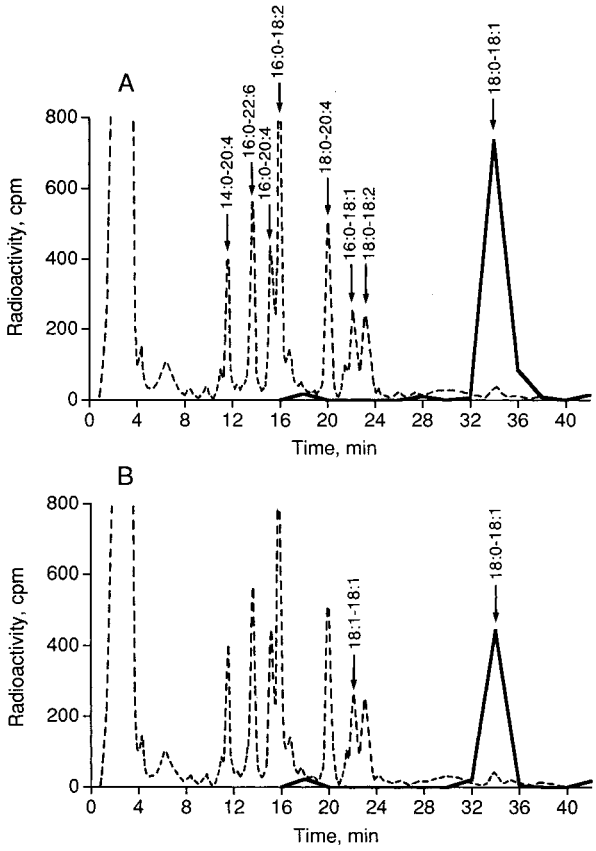

FIGURE 5: Competition among molecular species of acyl donors in transacylase assays. (A) Competition between 2 mol % 18:0-18:1 PA and 2 mol % 16:0-18:1 PA was determined in a transacylase assay (10 min, 37 °C) containing aliquots of pooled material from a Superdex 200 column, buffer A, 80 μM CoA, and mixed micelles of 95 mol % Triton X-100 and 0.75 mol % ^{32}P-labeled *sn*-2-18:1 lysoPA. The [^{32}P]PA that was formed was subsequently methylated and analyzed on reversed-phase HPLC as described in Materials and Methods. The results are the average of two HPLC experiments. (B) Competition between 2 mol % 18:0-18:1 PA and 2 mol % 18:1-18:1 PA was determined in a transacylase assay similar to that in panel A. The results are the average of two HPLC experiments. The arrows in the figure indicate the retention times of standards; dashed lines indicate the absorbance of carrier lipids at 206 nm; solid lines indicate radioactivity. Note that the 16:0-18:1 and 18:1-18:1 standards eluted as a single peak in this solvent system.

Table 4: Lysophosphoglyceride Acyl Acceptors Used by the Transacylase and Acyltransferase[a]

	acyl acceptor	V_{max}^{app} [b] (pmol/min/μg of protein)	K_M^{app} (mol %)	V_{max}^{app}/K_M^{app}	*n*
transacylase assay[c]	*sn*-2-acyl lysoPI	720 ± 10	0.66 ± 0.03	1090 ± 50	2
	sn-2-18:1 lysoPA	1800 ± 200	0.4 ± 0.1	5000 ± 1000	6
	sn-1-18:1 lysoPA	1100 ± 600	0.41 ± 0.06	1200 ± 200	3
	sn-2-OPGE	230 ± 30	0.09 ± 0.03	3000 ± 1000	2
	sn-1-OPGE	na[d]	na		
acyltransferase assay[e]	*sn*-2-acyl lysoPI	8100 ± 300	3.8 ± 0.2	2100 ± 100	2
	sn-2-18:1 lysoPA	2000 ± 200	0.7 ± 0.1	2900 ± 500	4
	sn-1-18:1 lysoPA	590 ± 30	1.1 ± 0.1	540 ± 60	3
	sn-2-OPGE	200 ± 5	0.25 ± 0.02	800 ± 70	2
	sn-1-OPGE	na	na		

[a] All values were determined using material from a Superdex 200 column. [b] V_{max}^{app} and K_M^{app} values are defined in Table 2. [c] The activities were determined in separate assays containing buffer A, 80 μM CoA and mixed micelles of 94−98 mol % Triton X-100, 2 mol % [^{14}C]18:0-18:1 PA and variable concentrations of unlabeled *sn*-2-acyl lysoPI, or mixed micelles of 87−92 mol % Triton X-100, 8 mol % bovine liver PI and variable concentrations of ^{32}P-labeled lysophosphoglyceride acceptors. [d] na, not active. [e] The activities were determined in separate assays containing buffer A, and mixed micelles of 95−99 mol % Triton X-100, 0.5 mol % ^{14}C-labeled or unlabeled 18:0-CoA and variable concentrations of unlabeled or ^{32}P-labeled lysophosphoglyceride acceptors.

up competition assay, done with a mixture of unlabeled 18:0-CoA, [^{14}C]12:0-CoA, and unlabeled *sn*-2-18:1 lysoPA, extended these observations by showing that 18:0-CoA was a very effective inhibitor of the formation of [^{14}C]12:0 PA (Figure 6B). The combined results of these experiments indicated that the acyltransferase was relatively non-acyl-chain-specific. In addition, the results of the competition assays provided evidence that the partially purified enzyme preparation contained only one acyltransferase.

Other experiments examined the acyl acceptor specificity of the acyltransferase. One set of experiments primarily evaluated the enzyme's ability to use different *classes* or *subclasses* of lysophosphoglycerides as acyl chain acceptors. The enzyme was separately incubated with [^{14}C]18:0-CoA as a donor and one of several potential lysophosphoglyceride acyl acceptors. The results showed that *sn*-2-acyl lysoPI, *sn*-2-18:1 lysoPA, *sn*-1-18:1 lysoPA, and 2-OPGE were effective acceptors, though the values calculated for V_{max}^{app}/K_M^{app} in the different reactions varied within a several fold range (Table 4; note that *sn*-2-acyl lysoPIP was not tested). In contrast to these results, assays with 1-OPGE or the *sn*-2-18:1 molecular species of lysoPC, lysoPE, and lysoPS demonstrated that the enzyme was unable to catalyze the transfer of acyl groups to any of these compounds (Table 4 and data not shown).

A second set of experiments investigated the positional specificity of the acyltransferase. The enzyme was incubated with unlabeled 18:0-CoA and either ^{32}P-labeled *sn*-2-18:1 lysoPA or ^{32}P-labeled *sn*-1-18:1 lysoPA, and the distribution of fatty acyl chains in the labeled PA that was formed was analyzed as described in Materials and Methods. The results revealed that the ratio of ^{32}P-labeled 18:0-18:1 species to ^{32}P-labeled 18:1-18:0 species was 3:1 in both cases (data not shown). On the basis of these results and the results obtained in the above-mentioned studies with 2-OPGE and 1-OPGE, it seemed clear that the acyltransferase, like the transacylase, catalyzed the preferential transfer of acyl groups to the *sn*-1-position of lysophosphoglyceride acyl acceptors.

A third set of experiments tested the enzyme's ability to distinguish between different acyl chain acceptors in competition assays. The enzyme was incubated with unlabeled 18:0-CoA and ^{32}P-labeled *sn*-2-18:1 lysoPA in the presence of increasing concentrations of unlabeled *sn*-2-18:1 lysoPA, *sn*-1-18:1 lysoPA, or *sn*-2-acyl lysoPI. The results revealed that each of the three unlabeled lysophosphoglycerides inhibited the formation of radioactive PA, in the order of effectiveness: *sn*-2-18:1 lysoPA > *sn*-1-18:1 lysoPA > *sn*-2-acyl lysoPI (Figure 6C). The combined results of the competition studies with acyl donors (Figure 6, panels A and B) and acyl acceptors (Figure 6C) provided definitive evidence that only one acyltransferase was involved.

Sensitivities of the Partially Purified Transacylase and Acyltransferase to Heat and pH, and Competition of the Enzymes for the Same Acyl Acceptor. A comparison of the heat sensitivities of the transacylase and acyltransferase reactions showed that the enzymes displayed similar sensitivities, measured at 45 or 50 °C (Figure 7 and data not shown). In addition, the reactions showed similar sensitivities to pH (Figure 8). Furthermore, three different types of competition assays showed that the partially purified transacylase and acyltransferase could compete for the same acyl acceptor. (A) When the activities of both enzymes were measured in the same assay at saturating substrate concentrations, the amount of product formed was not the sum of the products formed when the enzymes were measured separately. In a typical experiment, the combined transacylase and acyltransferase activity was 3.55 pmol/min/μL when measured in the presence of 80 μM CoA and mixed micelles containing 8 mol % bovine liver PI, 0.47 mol % 18:0-CoA, 1 mol % ^{32}P-labeled *sn*-2-18:1 lysoPA, and 91 mol % Triton X-100. In contrast, the transacylase activity was 2.1 pmol/min/μL when measured alone in the absence of 18:0-CoA, and the acyltransferase activity was 3.55 pmol/min/μL when measured alone in the absence of bovine liver PI and CoA. (B) When the activity of each enzyme was measured in a second type of assay, containing 18:0-20:4 PA, CoA and ^{32}P-labeled sn-2-18:1 lysoPA, addition of increasing concentrations of 16:0-CoA to the incubation mixture increased the acyltransferase activity, measured by the formation of ^{32}P-labeled material that comigrated with 16:0-18:1 PA on reverse phase HPLC, but decreased the transacylase activity, measured by the formation of ^{32}P-labeled material that comigrated with 18:0-18:1 PA (Figure 9A). At 0.33 mol % 16:0-CoA, the acyltransferase activity was 97% of maximum, whereas the transacylase activity was 20% of maximum. (C) When the activity of the acyltransferase was tested in an assay containing [^{14}C]18:0-CoA and unlabeled *sn*-2-18:1 lysoPA, addition of saturating concentrations of *either* bovine

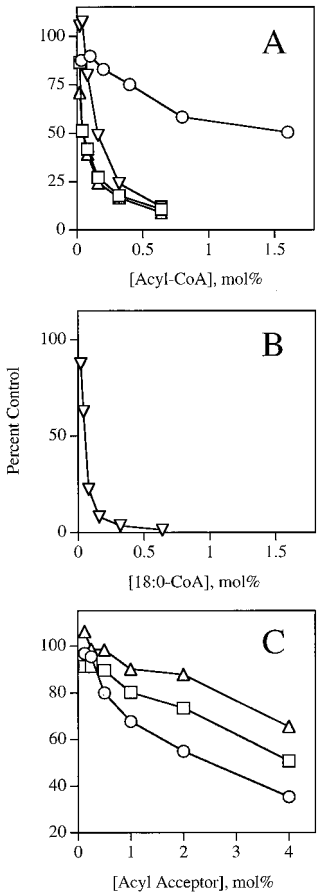

FIGURE 6: Competition among molecular species of acyl donors and acceptors in acyltransferase assays. (A) Competition among acyl-CoA molecular species was determined by incubating aliquots of pooled material from a Superdex 200 column for 6 min at 37 °C in buffer A in the presence of mixed micelles containing 99 mol % Triton X-100, 0.8 mol % *sn*-2-18:1 lysoPA, 0.02 mol % [^{14}C]18:0-CoA, and the indicated concentrations of unlabeled 12:0-CoA (circles), 16:0-CoA (squares), 18:0-CoA (inverted triangles), or 18:1-CoA (triangles). (B) Competition between the indicated concentrations of unlabeled 18:0-CoA and 0.033 mol % ^{14}C-labeled 12:0-CoA was determined with an assay comparable to that shown in panel A. (C) Competition among acyl acceptors was determined using assay conditions similar to those in panel A but in the presence of 0.47 mol % 18:0-CoA, 0.5 mol % ^{32}P-labeled *sn*-2-18:1 lysoPA, and the indicated concentrations of unlabeled *sn*-2-18:1 lysoPA (circles), *sn*-1-18:1 lysoPA (squares), or *sn*-2-acyl lysoPI (triangles). Two separate experiments done in duplicate gave similar results. The results for panels A−C are representative of two separate experiments done in duplicate.

liver PI (12 mol %) *or* CoA (80 μM) had only a slight inhibitory effect (see 0 mol % value for PI in Figure 9B and 0 μM value for CoA in Figure 9C). However, when the effects of increasing concentrations of bovine liver PI were tested in the *presence* of 80 μM CoA (Figure 9B) or when the effects of increasing concentrations of CoA were tested in the *presence* of 12 mol % bovine liver PI (Figure 9C), the acyltransferase activity decreased. Maximal inhibition (typically 50−60%) occurred in the presence of saturating concentrations of both bovine liver PI and CoA. The combined results of these experiments provided strong evidence that the activities of the partially purified transacylase and acyltransferase depended on the actions of a single enzyme protein or a tightly associated enzyme complex containing a shared protein subunit.

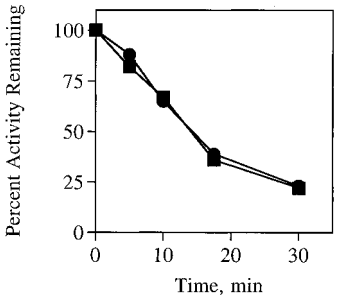

FIGURE 7: Heat sensitivities of the transacylase and acyltransferase. The stabilities of the transacylase and acyltransferase were determined after aliquots of pooled material from a Superdex 200 column were separately incubated for the indicated times at 45 °C and then immediately cooled on ice. Transacylase activity (circles) and acyltransferase activity (squares) were measured as described in Materials and Methods. Two separate experiments done in duplicate gave similar results.

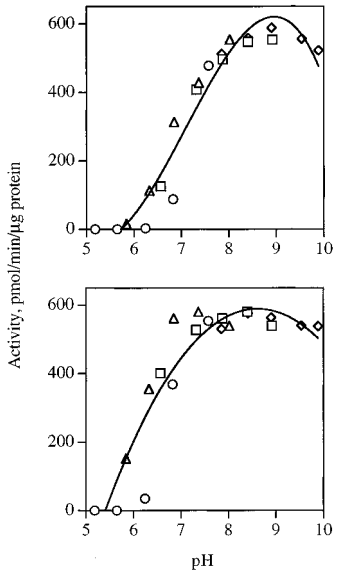

FIGURE 8: pH Optima of the transacylase and acyltransferase. (A) Effect of pH on the transacylase reaction was determined in separate assays (4 min, 37 °C) containing aliquots of pooled material from a Superdex 200 column. In addition, incubation mixtures contained: 2 mM DTT; 0.4 mM EDTA; 80 μM CoA; 0.64 mM Na acetate; mixed micelles of 90 mol % Triton X-100, 8 mol % bovine brain PI and 1 mol % ^{32}P-labeled *sn*-2-18:1 lysoPA; and 0.1 M of the indicated buffer (circles, 2-[*N*-morpholino]ethanesulfonic acid; triangles, imidazole; squares, Tris; diamonds, 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid). The line was drawn by fitting all the data points to a third order polynomial. Two separate experiments done in duplicate gave similar results. (B) Effect of pH on the acyltransferase reaction was determined in separate assays (4 min, 37 °C) containing aliquots of pooled material from a Superdex 200 column. In addition, incubation mixtures contained: 2 mM DTT; 0.4 mM EDTA; 0.64 mM Na acetate; mixed micelles of 98 mol % Triton X-100, 0.5 mol % 18:0-CoA, and 1 mol % ^{32}P-labeled *sn*-2-18:1 lysoPA; and 0.1 M of the indicated buffer as described in panel A. The line was drawn as in panel A. Two separate experiments done in duplicate gave similar results.

Inhibition of the Transacylase and Acyltransferase Reactions by PIP$_2$. Bovine brain PIP$_2$ has been shown to inhibit the 18:0-specific transacylase activity in Triton X-100 extracts of bovine testis membranes (*13*). When we tested its effects on the partially purified transacylase and acyltransferase activities, we found that it inhibited greater than 90% of the transacylase activity but only about 50% of the acyltransferase activity (Figure 10A). In addition, double-

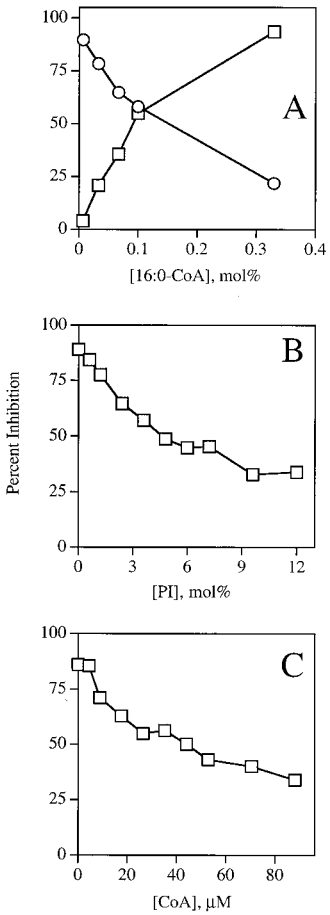

FIGURE 9: Competition of the transacylase and acyltransferase for the same acyl acceptor. (A) Competition of the transacylase and acyltransferase for 1.5 mol % ^{32}P-labeled *sn*-2-18:1 lysoPA was determined by incubating aliquots of pooled material from a Superdex 200 column in assays (8 min, 37 °C) containing buffer A, 82 μM CoA, and mixed micelles of 98 mol % Triton X-100, 0.75 mol % 18:0-20:4 PA and the indicated concentrations of unlabeled 16:0-CoA. Amounts of radioactive 18:0-18:1 PA (circles) and 16:0-18:1 PA (squares) formed during the incubations were determined by reverse phase HPLC (Materials and Methods). The results are from a single experiment done in duplicate. Two similar, separate experiments, one that used pooled material from a Superdex 200 column and one that used material from an octyl-Sepharose column, yielded similar results. (B) Competition of the transacylase and acyltransferase for 1 mol % unlabeled *sn*-2-18:1 lysoPA was determined by incubating aliquots of pooled material from a Superdex 200 column in assays (8 min, 37 °C) containing buffer A, 88 μM CoA, and mixed micelles of 87−99 mol % Triton X-100, 0.02 mol % [^{14}C]18:0-CoA, and the indicated concentrations of unlabeled bovine liver PI. The results are from a single experiment done in duplicate. Two similar, separate experiments, one that used pooled material from a Superdex 200 column and one that used material from an octyl-Sepharose column, yielded similar results. (C) Requirement for added CoA in the competition assay with unlabeled bovine liver PI and 0.02 mol % [^{14}C]18:0-CoA was determined in assays (8 min, 37 °C) with aliquots of the same pooled enzyme material and by a comparable assay as in panel B but in the presence of mixed micelles of 87 mol % Triton X-100 and 12 mol % (total concentration 1.8 mM) bovine liver PI and the indicated concentrations of CoA. The results are from a single experiment done in duplicate. Two similar, separate experiments, one that used pooled material from a Superdex 200 column and one that used material from an octyl-Sepharose column, yielded similar results.

reciprocal plots of PIP_2 inhibition versus bovine liver PI or 18:0-20:4 PA in transacylase assays (Figure 10, panels B and C) showed strictly competitive inhibition for each donor, with a K_i of 0.023 ± 0.018 mol % (n = 2) for PI and a K_i of 0.0349 ± 0.0028 mol % for 18:0-20:4 PA (n = 2). PIP_2 inhibition was also strictly competitive for CoA [K_i = 0.0180 ± 0.0076 (n = 2)] but was strictly uncompetitive for *sn*-2-18:1 lysoPA [K_i = 0.052 ± 0.048 (n = 2)]. In contrast, PIP_2 inhibition of the acyltransferase activity displayed what appeared to be a different mechanism of inhibition (Figure 10, panels D and E): replots of the slopes and y-axis intercepts, which were derived from double-reciprocal plots (*31*), versus PIP_2 concentration showed hyperbolic noncompetitive inhibition for 18:0-CoA and hyperbolic competitive inhibition for *sn*-2-18:1 lysoPA (Figure 10, insets to panels D and E). These results provided new information about the mechanism of PIP_2 inhibition of the transacylase reaction and showed that the acyltransferase reaction was inhibited by PIP_2 as well, probably by a different mechanism.

DISCUSSION

This report describes the partial purification of an 18:0-specific transacylase found in bovine testis membranes and its copurification with an acyltransferase. Substrate and product competition assays with mixed micelle systems provided strong evidence that the partially purified enzyme preparation contained only one transacylase and one acyltransferase, and this established the basis for detailed studies of the enzymes' properties. Studies of the partially purified transacylase showed that it had a more restricted acyl donor specificity than that described for the transacylase in unfractionated Triton X-100 extracts of bovine testis membranes (*13*). It used PA, PI, and PIP, but did not use PE, PS, or *sn*-1-18:0 lysoPA. However, the partially purified transacylase was similar to the unfractionated transacylase in that it catalyzed the preferential transfer of 18:0 groups, as demonstrated dramatically by substrate competition experiments. In addition, it showed the same acyl acceptor specificity for *sn*-2-acyl molecular species of lysoPI and lysoPA and was inhibited by PIP_2.

The acyltransferase in the partially purified enzyme preparation had not been identified previously. It used several different molecular species of acyl-CoA as acyl donors and, unlike the transacylase, did not discriminate between 18:0 groups and 16:0 groups or 18:1 groups. Nevertheless, it used the same acyl acceptors as the transacylase did and competed with the transacylase for the acyl acceptor, *sn*-2-18:1 lysoPA, when the two enzymes were assayed in the same incubation mixture. Furthermore, the acyltransferase reaction was inhibited by PIP_2, though to a lesser extent than the transacylation reaction was and by what appeared to be a different mechanism.

The facts that the transacylase and acyltransferase copurified (through five chromatographic steps); lost activity in parallel during the purification unless a lipid stabilizer was added; showed quantitatively equivalent responses to the lipid stabilizers, 16:0-18:1 PA and 18:0-CoA; displayed similar sensitivities to heat and pH; and competed for the same acyl group acceptor suggested that they might contain one or more common components. Definitive information about the relation between the two enzymes will probably have to await molecular cloning and expression studies. But for now it seems reasonable to postulate that the transacylase and acyltransferase assays that we used in this study actually measured two different aspects of the same reaction. Thus, the transacylase reaction may occur in two successive steps,

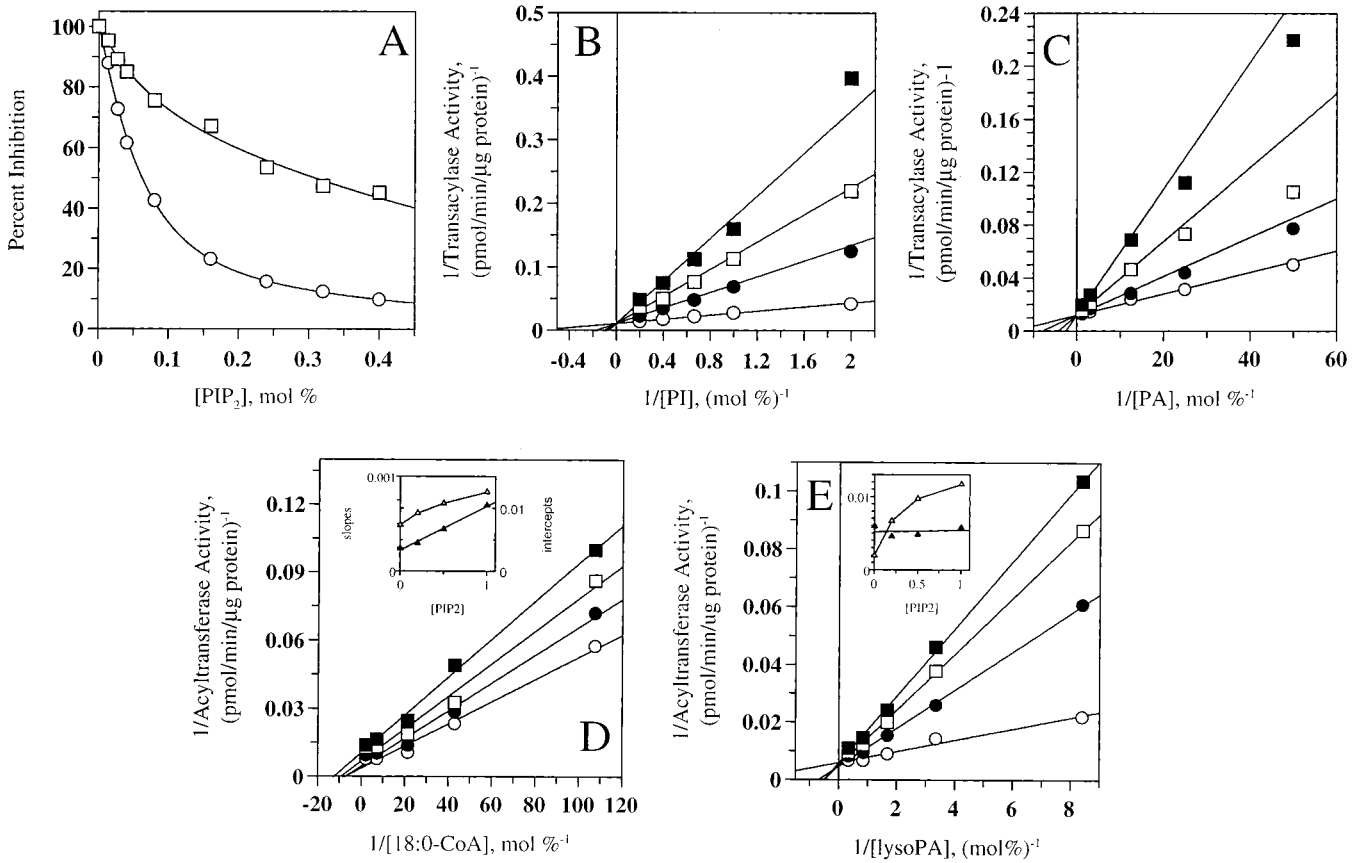

FIGURE 10: Inhibition of the transacylase and acyltransferase by PIP_2. (A) Effects of PIP_2 on the transacylase and acyltransferase reactions were determined by separately incubating aliquots of pooled material from a Superdex 200 column for 8 min at 37 °C in buffer A in the presence of mixed micelles of 95−99 mol % Triton X-100, 0.5 mol % ^{32}P-labeled *sn*-2-18:1 lysoPA, and the indicated concentrations of PIP_2. In addition, the transacylase assay (circles) contained 3.9 mol % bovine liver PI and 24 μM CoA, and the acyltransferase assay (squares) contained 0.07 mol % 18:0-CoA. (B) PIP_2 inhibition of the transacylase reaction at variable bovine liver PI concentrations was determined by separately incubating aliquots of pooled material from a Superdex 200 column in buffer A, 24 μM CoA and mixed micelles of 95−99 mol % Triton X-100, 0.5 mol % ^{32}P-labeled *sn*-2-18:1 lysoPA, the indicated concentrations of bovine liver PI, and one of four PIP_2 concentrations (open circles, no PIP_2; filled circles, 0.03 mol % PIP_2; open squares, 0.09 mol % PIP_2; filled squares, 0.18 mol % PIP_2). (C) PIP_2 inhibition of the transacylase reaction at variable 18:0-20:4 PA concentrations was determined by incubating aliquots of pooled material from a Superdex 200 column in assays similar to those in panel B but in the presence of the indicated concentrations of 18:0-20:4 PA. The symbols used are the same as in panel B. (D) PIP_2 inhibition of the acyltransferase reaction at variable 18:0-CoA concentrations was determined by separately incubating aliquots of pooled material from a Superdex 200 column in buffer A and mixed micelles of 98−99 mol % Triton X-100, 0.5 mol % ^{32}P-labeled *sn*-2-18:1 lysoPA, the indicated concentrations of 18:0-CoA, and one of four PIP_2 concentrations (open circles, no PIP_2; filled circles, 0.2 mol % PIP_2; open squares, 0.5 mol % PIP_2; filled squares, 1.0 mol % PIP_2). The insets show the slopes (hollow triangles) and intercepts (filled triangles) of the primary plots as a function of PIP_2. (E) PIP_2 inhibition of the acyltransferase reaction at variable *sn*-2-18:1 lysoPA concentrations was determined by separately incubating aliquots of pooled material from a Superdex 200 column using assay conditions similar to those in panel D but in the presence of 0.067 mol % 18:0-CoA and the indicated concentrations of ^{32}P-labeled *sn*-2-18:1 lysoPA. The symbols used for the panel and the inset are the same as in panel D. The results for panels A−E are from single experiments done in duplicate; similar results were obtained in experiments that used pooled material from an octyl-Sepharose column (A, B, D, and E) or a Superdex 200 column (C).

a first step in which an 18:0 group is transferred preferentially from PA, PI, or PIP to CoA, and a subsequent, relatively non-acyl-chain-specific step, in which an 18:0 group is transferred from 18:0-CoA to *sn*-2-acyl molecular species of lysoPA or lysoPI. If this two-step reaction model is correct, the transacylase assays that we used measured the net effects of both reactions whereas the acyltransferase assays that we used measured the effect of the second reaction only.

The possibility that a single, membrane-associated, CoA-dependent enzyme might catalyze a two-step transacylation reaction has been suggested previously (*9*, *32*, *33*). It has also been proposed that two separate, membrane-associated enzymes might catalyze sequential reactions that result in transacylation (*34*, *35*). In contrast, other investigators have postulated that a single, CoA-dependent enzyme that can bind phosphoglyceride acyl donors and lysophosphoglyceride acyl acceptors at a single site might act as a transacylase by catalyzing a single, reversible reaction (*36*−*39*).

Present information with regard to the 18:0-specific transacylation reaction seems most consistent with the first model involving a single-enzyme, two-step reaction or with a variant of this model involving two tightly associated enzyme subunits that catalyze sequential reactions. If the second model of two physically separated enzymes were applicable, one might expect 18:0-CoA to accumulate at least transiently during the reaction. But we have obtained no evidence for this so far. We failed to detect the formation of [^{14}C]18:0-CoA in transacylase assays done with CoA and the phosphoglyceride acyl donor, [^{14}C]18:0-18:1 PA, in the presence or absence of the acyl acceptor, *sn*-2-18:1 lysoPA (detection limit of [^{14}C]18:0-CoA = 14 pmol/assay; forma-

tion of PA product under similar assay conditions was typically >200 pmol/assay). Furthermore, addition of 70 μM unlabeled 18:0-CoA to the reaction mixture to serve as a trap had no effect on the accumulation of [^{14}C]18:0-CoA (>85% of the added radioactivity was recovered, all of which comigrated with PA on thin layer chromatography).

The third model involving a single-enzyme, single-reversible reaction also seems unlikely. To account for the differences in acyl chain specificity between the transacylase and the acyltransferase, one would have to postulate that the enzyme binds different diacylphosphoglycerides with similar affinities but transfers 18:0 groups at a higher rate than other fatty acyl groups. However, 16:0-18:1 PA was a weak inhibitor of the transacylase reaction (half maximal concentration = 6 mol %; data not shown), which is inconsistent with this mechanism. Furthermore, PIP_2 showed competitive inhibition kinetics toward the transacylase acyl donors, bovine liver PI and 18:0-18:1 PA, and is structurally similar to these phosphoglycerides, so it probably inhibited the transacylase reaction by interacting with the enzyme's phosphoglyceride acyl donor-binding site. But PIP_2 showed different inhibition kinetics toward *sn*-2-acyl lysoPA in the acyltransferase assays and may have inhibited the acyltransferase reaction by a different mechanism.

If the transacylase reaction does involve a 18:0-specific first step and a relatively non-acyl-chain-specific second step, as we have postulated, many important questions remain to be answered. For example, one set of questions concerns the catalytic properties of the enzyme. The properties that we have identified in this study all depend on the use of mixed micelle systems. Might association of the transacylase-acyltransferase with membranes affect these properties? Might proteins that control the delivery of substrates to the membrane-associated enzyme play important regulatory roles? Might proteins that control the removal of products from the membrane also be involved?

It may be possible to address some of these questions by experimentation with enzyme preparations that have been reconstituted into well-characterized, unilamellar vesicles. To identify factors that control the delivery of substrates to the reconstituted enzyme, assay systems might be devised that include (A) vesicles containing both the transacylase and potential phosphoglyceride acyl donors and (B) incubation medium containing CoA, acyl-CoA bound to an acyl-CoA-binding protein (*40*, *41*), and *sn*-2-acyl lysoPA or lysoPI bound to a lysophosphoglyceride-binding protein (*42*). To identify factors that control the removal of products from the reconstituted enzyme, an assay might be developed that includes (A) vesicles containing the transacylase and 18:0-18:1 PA; (B) medium containing CoA, *sn*-2-20:4 lysoPI and PI transfer protein (*43*); and (C) additional vesicles, which contain only PC, to act as acceptors of PI.

A related set of questions concerns the possible role of the transacylase-acyltransferase *in vivo*. What are the principal substrates and products of the transacylation reaction in intact cells? Do reactions that control the metabolic flow of these substrates and products determine the net effects of the enzyme's activity? How do the *sn*-1-18:0-2-acyl phosphoglycerides that are generated affect the structure and function of cell membranes?

One possibility that warrants attention is that the CoA-dependent, 18:0-specific transacylase may contribute to intracellular pathways that preferentially recycle 2-acylglycerol or *sn*-2-acyl lysoPA into *sn*-1-18:0-2-acyl molecular species of PI, PE, and PS. Evidence that an enzyme of this type in Swiss 3T3 cells may play a role in such pathways has been reported (*15*, *16*). Another possibility is that the CoA-dependent, 18:0-specific transacylase may play a role in acyl chain remodeling reactions involving PI. Evidence that a similar enzyme in rat liver plays such a role also has been reported (*17*). Though it has generally been assumed that acyltransferases rather than transacylases catalyze acyl chain remodeling reactions (*44*), remodeled molecular species accumulate preferentially in specific phosphoglyceride classes, as in the case of 18:0-20:4 PI. Because the molecular basis for this preference remains poorly understood, the possibility has to be considered that highly specific transacylases, such as the 18:0-specific transacylase, may be involved.

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