Membrane Lipids Have Multiple Effects on Interfacial Catalysis by a Phosphatidic Acid-Preferring Phospholipase A1 from Bovine Testis†

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ABSTRACT: We previously purified a cytosolic phospholipase A1 that could catalyze the preferential hydrolysis of phosphatidic acid in mixed-micelle assays. Here we studied the enzyme's interactions with unilamellar lipid membranes and examined effects of the lipids on enzyme binding, stability, and catalysis. A major finding was that membrane lipids could influence the stability, activity, and specificity of the enzyme under conditions where enzyme binding to the membranes was likely to be saturated. Thus, the enzyme was unstable at 37 °C in the absence of membranes but bound to membranes that contained anionic phosphoglycerides and could be stabilized by these membranes in the presence of albumin. The overall activity of the bound enzyme toward membrane phosphoglycerides, assayed in the presence of albumin, increased when phosphatidylethanolamine was substituted for phosphatidylcholine. Furthermore, the enzyme's catalytic preference for phosphatidic acid increased when cholesterol and diacylglycerol were included in the membranes, *sn*-1-stearoyl-2-arachidonoylphosphatidylethanolamine was substituted for *sn*-1-palmitoyl-2-oleoylphosphatidylethanolamine, and the concentration of phosphatidic acid was increased from 0 to 10 mol % of the total membrane phosphoglycerides. Finally, changes in the relative contents of phosphatidylcholine and phosphatidylserine in the membranes influenced the enzyme's catalytic preference for different molecular species of phosphatidic acid. These results provide the first available information about the enzyme's ability to interact with membranes and identify conditions that yield high enzyme activity toward membrane-associated phosphatidic acid.

Bovine testis contains a 97.6 kDa phospholipase A1 (PLA1)[1] that appears to be a homotetramer when it is highly purified (*1*−*3*). The PLA1 is of special interest because assays with mixed micelles have provided evidence that it catalyzes the hydrolysis of PA in preference to that of other phosphoglycerides (*1, 2*). In addition, the levels of both its activity and its mRNA are much higher in mature testis than in newborn testis (*1, 3*), and two different protein kinases can catalyze reactions that phosphorylate it with significant stoichiometry (Han et al., unpublished results). These observations raise the possibility that the enzyme may catalyze the preferential hydrolysis of membrane-associated PA in vivo and perhaps influence phosphatidic acid-dependent mechanisms that contribute to spermatogenesis. However, much more information about the enzyme is needed. For example, one immediate concern is that the results of the mixed micelle assays may have little relevance to the enzyme's action in intact cells. The mixed micelles were transient aggregates of unclear molecular structure that contained single diacylphosphoglycerides + at least 80 mol % neutral detergent, Triton X-100. In contrast, cell membranes consist of relatively stable lipid bilayers that contain complex mixtures of many diacylphosphoglycerides, sphingolipids, neutral lipids, and associated integral and peripheral membrane proteins (*4, 5*). Therefore, the mixed micelle assays could not have informed about potential effects of structural features of membranes, such as those that might have involved acyl chain interactions within lipid bilayers or phosphoglyceride headgroup interactions at the interface between membranes and the cytosol.

In view of this concern, the need to investigate the enzyme's ability to interact with membranes and membrane-associated substrates was clear. However, the membranes of cells are heterogeneous, and it is not yet known which membrane or membranes interact with the PLA1 in vivo. Therefore, we decided to use model membranes whose physical properties resembled those of cell membranes and whose lipid compositions could be varied to increase the chance of identifying specific, membrane-dependent effects.

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[1] Abbreviations: BSA, essentially fatty acid-free bovine serum albumin; PLA1, phospholipase A1; 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 20:4, arachidonic acid; 16:0-18:1, *sn*-1-palmitoyl-2-oleoyl; 18:0-20:4, *sn*-1-stearoyl-2-arachidonoyl; AOPA, *sn*-1-alkyl-2-oleoyl; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine (PA, PC, PE, PI, and PS refer to 16:0-18:1 molecular species unless specified otherwise); LysoPA, *sn*-1-hydroxyl-2-18:1 glycerophosphate; DAG, *sn*-1,2-diacylglycerol; chol, cholesterol; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Thesit, dodecylpoly(ethylene glycol ether)$_n$ where the average n is 9; K_D^{app}, apparent dissociation constant.

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We used an extrusion procedure to prepare 100 ± 20 nm diameter, unilamellar lipid membranes from several different mixtures of phosphoglycerides and neutral lipids; examined their physical properties by a laser light scattering method; and incubated them with purified preparations of the enzyme. Then we used an immunochemical method to identify lipid effects on the enzyme's ability to bind to the membranes, mixed-micelle-dependent enzyme activity assays to identify effects of the membranes on the enzyme's stability, and assays with membrane-associated substrates to analyze effects of the membrane lipids on the enzyme's ability to catalyze the hydrolysis of PA and other phosphoglycerides. The results of the incubation studies provide a first look at the enzyme's interactions with membranes and reveal a degree of complexity that would not have been anticipated on the basis of the results of our earlier experiments with mixed micelles.

MATERIALS AND METHODS

Materials. The PLA1 was partially purified from bovine testis through the Superdex 200 size-exclusion step, as described (*2*), and then stored at −70 °C in buffer containing 50 mM MOPS, pH 7.2, 100 mM KCl, 1 mM EGTA, and 0.1 mM Thesit. When assayed with mixed micelles that contained Triton X-100 (see below), the enzyme that had been purified from bovine testis could catalyze the hydrolysis of about (9.4−12.5) × 10^3 mol of PA min^{-1} (mol of enzyme)$^{-1}$. For incubation studies with membranes (see below) the enzyme and Thesit were diluted at least 100-fold, so the Thesit was always present at a concentration that was well below its critical micelle concentration (0.1 mM). Polyclonal antibody against the peptide TKRRL-REIEERLHGLKASS, which corresponded to the putative coiled-coil forming region (amino acids Thr 589−Ser 607), encoded by the enzyme's cDNA (*3*), was from Research Genetics Inc. BSA and most other reagents were from Sigma except where indicated. Immobilon-P PVDF membranes were from Millipore Corp. The enhanced chemiluminescence kit, Hyperfilm, and horseradish peroxidase-coupled anti-rabbit IgG antibody were from Amersham Life Science. [9,-10-^{3}H]16:0, [9,10-^{3}H]18:1, and 16:0-[1- ^{14}C]18:1-PC (25 mCi/mol) were from DuPont NEN. [^{3}H]16:0-18:1-PC was synthesized as described (*6*). The corresponding molecular species of labeled PA, PE, and PS were prepared by incubating portions of the labeled PC with *Streptomyces* sp. phospholipase D (*2*). [^{3}H]16:0-18:1-PI was synthesized from [^{3}H]16:0-18:1-PA (*7*) by use of a yeast membrane preparation kindly provided by Dr. George M. Carman (Department of Food Science, Rutgers University). Nonradioactive phosphoglycerides were from Avanti Polar Lipids (Alabaster, Alabama); and 18:0-20:4-PE was purified from bovine heart PE (Avanti) by reverse-phase high performance liquid chromatography. Organic solvents were reagent-grade or better from J. T. Baker. Plastic-backed silica gel 60 thin-layer chromatography plates were from E. M. Merck.

Preparation of Unilamellar Lipid Vesicles. Various mixtures of lipids in chloroform were successively dried under argon, suspended in 1-mL aliquots of MOPS−KCl buffer (50 mM MOPS, pH 7.2; 100 mM KCl; 1 mM EGTA, and 1 mM $MgCl_2$), and converted into unilamellar membranes by extrusion through 0.1-μm polycarbonate filters, as described (*8*). Lipid recoveries after extrusion were about 90%, as determined by measurements of radioactivity in phosphoglyceride-tagged membranes. Membrane diameters for each preparation were measured on a Brookhaven Instruments Corp. 90Plus particle size analyzer and, unless mentioned otherwise, found to be 100 ± 20 nm. The membranes were stable for at least 1 week after extrusion but were typically prepared and used on the same day. The relative distributions of lipids in membranes were always expressed as mole/mole ratios.

Assays of PLA1 Binding to Membranes. Enzyme-binding studies were done with unilamellar membranes that had been produced by suspension of lipid mixtures in MOPS−KCl buffer containing 170 mM sucrose, followed by extrusion, essentially as described (*9*). The composition of the lipid membranes varied, but each membrane contained trace amounts of ^{14}C-labeled 16:0-18:1-PC and, unless mentioned otherwise, 1 mol of chol/mol of total phosphoglyceride. After the extrusion procedure, aliquots of the membrane suspensions were diluted more than 5-fold with MOPS−KCl buffer, and mixtures of the diluted suspensions were prepared that contained 4−7 different concentrations of membranes and PLA1 (4.8 nM) in a total volume of 160 μL. The mixtures were incubated for 5 min at room temperature in polycarbonate centrifuge tubes (Beckman Instruments, Inc.) and then centrifuged at room temperature for 16 min at 160000*g* in a TLA 100.1 rotor of a Beckman Optima TLX Ultracentrifuge. The contents of each centrifuge tube were separated into a top fraction (140 μL) and a bottom fraction (20 μL), and the distribution of the PLA1 in the fractions (measured as described below) was compared with that of the membrane-associated, ^{14}C-labeled PC.

The distribution of PLA1 in the top and bottom fractions from each centrifuge tube was usually measured by an immunochemical method. PLA1 standards and material from each fraction were applied separately to the same Immobilon-P membrane with a Bio-Dot apparatus (Bio-Rad) according to the manufacturer's instructions. Then, antibody to the putative coiled-coil-forming region of the PLA1 was added, followed by horseradish peroxidase-coupled anti-rabbit IgG antibody; the antibody complex was visualized by enhanced chemiluminescence, and the chemiluminescent response was quantitated by analysis on a Bio-Rad model GS-700 imaging densitometer. Analysis of the PLA1 standards and the material from the top fraction obtained after centrifugation was straightforward. Appropriate concentrations of the PLA1 standards and a 70-μL aliquot of each top fraction, diluted with MOPS−KCl to a final volume of 200 μL, could be applied directly to an Immobilon-P membrane. But material from bottom fractions could not be applied directly to Immobilon-P membranes because the lipids of the unilamellar membranes in the bottom fractions interfered with the transfer of the PLA1. Removal of the lipids could be achieved by the following Triton X-114 phase-extraction approach. Each 20 μL bottom fraction was mixed with 10 μL of 10% Triton X-114 (Sigma) and 50 μL of MOPS−KCl buffer. The mixture was then vortexed, incubated for 5 min on ice, vortexed again, and warmed for 5 min at 37 °C to induce the formation of a Triton-rich phase (*10*). This phase was separated from the aqueous phase by centrifugation for 6 min at 14 000*g* at room temperature. Finally, a 40 μL aliquot of the upper aqueous phase was withdrawn, MOPS−KCl buffer was added to the aliquot to

bring its total volume to 200 μL, and the diluted sample was applied to an Immobilon-P membrane.

After the signals from the top and bottom fractions were quantitated by densitometry, they were converted into molar concentrations on the basis of comparisons with the signals from the PLA1 standards (which had been calibrated on the basis of comparisons with a purified recombinant form of the enzyme). About 90% of the enzyme protein was typically recovered. The percentage of membrane-bound versus unbound PLA1 was determined on the basis of comparisons with the amounts of [^{14}C]PC-labeled membranes that had been recovered in the top and bottom fractions. Apparent dissociation constants (K_D^{app}) were then determined on the basis of total membrane phosphoglyceride concentrations and expressed in millimolar, as described (*8*). Standard errors of fit were calculated with the GraFit program from Erithacus software (*11*).

Assays of PLA1 Stability. We examined the effects of membrane lipids on the enzyme's stability by incubating the enzyme in siliconized polypropylene tubes (Island Scientific, WA) in the absence or presence of membranes and then determining the enzyme's specific activity on the basis of mixed-micelle assays of the enzyme's activity (*2*) and immunochemical measurements of enzyme mass (see above). Determinations of enzyme specific activity were straightforward when the incubations were done in the absence of membranes, but a different approach had to be used when incubations were done in the presence of membranes because the phosphoglycerides of the membranes influenced the mixed micelle assays. In brief, aliquots (10 μL) were removed from the incubations and mixed with 2 μL of cold, 10% Triton X-114 in new polypropylene tubes; the mixtures were warmed for 5 min at 37 °C to separate a Triton-rich phase from an aqueous phase; and aliquots of the latter phase (8 μL) were removed and used for measurements of enzyme mass or assays of enzyme activity. The latter were done by incubating the aliquots in siliconized glass tubes in the presence of mixed micelles (100 μL) that contained a mixture of 44.75 mol % Triton X-100 + 44.75 mol % Triton X-114 + 0.5 mol % [^{3}H]16:0-18:1-PA + 10 mol % AOPA (16 mM total micellar lipid) and measuring the amounts of [^{3}H]-16:0 that were generated as described below. Control experiments showed that more than 95% of the enzyme activity was recovered after this procedure.

Membrane-Based Assays of Enzyme Activity. All assays were performed in siliconized glass tubes. The membranes used were usually composed of various mixtures of lipids that included either [^{3}H]16:0-18:1-PA or another, similarly labeled phosphoglyceride. Reaction mixtures (100 μL total volume) contained the membranes (1 mM total phosphoglyceride phosphorus) + MOPS−KCl buffer + PLA1 (0.03−1.59 nM) ± BSA (30 μM). Incubations were done for various periods of time at 37 °C. To measure apparent initial reaction rates, which depended not only on the enzyme's activity but also on its ability to hop from substrate-depleted membranes to fresh, substrate-containing membranes, enzyme-membrane mixtures were usually incubated for 15 min and five aliquots were taken from the mixtures at different time points. The radioactive 16:0 in each aliquot was analyzed as described below, and the data were graphed. Graphs from enzyme incubations with membranes (1 mM phosphoglycerides) + BSA were found to be linear, and the slopes of the lines were determined with Origin 5.0 from Microcal (Northhampton, MA). Finally, the error of each slope was calculated with the same program.

Analysis of radioactive 16:0 that was generated during incubations was done as follows. Reactions were terminated by the addition of 25 μL of $CHCl_3$ (containing 5 mg/mL unlabeled 16:0 + 1 mg/mL unlabeled phosphoglyceride mixture) followed by 900 μL of 1:1 $CHCl_3$:methanol + 1% formic acid + 0.2% butylated hydroxytoluene. After thorough mixing, two phases were generated by the addition of 650 μL of 9:1 water/methanol. The mixture was vortexed and centrifuged briefly, and the upper phase was removed by suction. The lower phase was evaporated under vacuum and dissolved in 50 μL of $CHCl_3$, and 25 μL of the solution was spotted onto a plastic-backed silica thin-layer chromatography plate and developed for 15 min in 65:35:1 hexane/ethyl acetate/glacial acetic acid. Bands of radioactive product fatty acids were visualized with iodine vapor, cut out, and quantitated by scintillation counting in Ecolume (ICN).

Other Methods. Protein concentrations were determined by the Bradford method with BSA as standard at 595 nm (*12*). Phosphoglyceride concentrations were determined after digestion with perchloric acid by measurement of orthophosphate (*13*).

RESULTS

Effects of Lipids on the Enzyme's Ability To Bind to Membranes. Soluble enzymes that catalyze reactions involving membrane lipids must bind to the surfaces of membranes to access their substrates (*5*). To identify membrane components that might influence the ability of the bovine testis PLA1 to bind to unilamellar lipid membranes, we incubated the enzyme with sucrose-loaded vesicles and then centrifuged the vesicles and used an immunochemical method to measure the amounts of bound and free enzyme protein (Materials and Methods). The results of experiments with membranes that were composed of simple mixtures of 16:0−18:1 molecular species of phosphoglycerides + chol are shown in Figure 1. Calculated values of K_D^{app} for these and other experiments (expressed in terms of molar concentrations of total membrane phosphoglycerides) are presented in Table 1. Note that the various membranes were all prepared by extrusion through polycarbonate filters and had diameters of 100 ± 20 nm, as determined by laser light scattering (Materials and Methods).

The combined data support the following conclusions. (1) The enzyme had a relatively high affinity for membranes that contained PA + chol or AOPA (a nonhydrolyzable analogue of PA) + chol, a somewhat lower affinity for membranes that contained PS + chol, and very little affinity for membranes that contained PC + chol or PC + PE + chol. (2) Partial replacement of PA or PS with PC or PE was associated with decreased enzyme binding, with PC having the greatest effect. (3) Enzyme binding was essentially unchanged when chol or chol + DAG were included in membranes that contained a mixture of PC + PE + PS + PA or when 18:0-20:4-PE was substituted for 16:0-18:1-PE.

It was not surprising to find that the enzyme had a much higher affinity for membranes that contained acidic phosphoglycerides than for membranes that did not contain these phosphoglycerides because we had shown previously that it

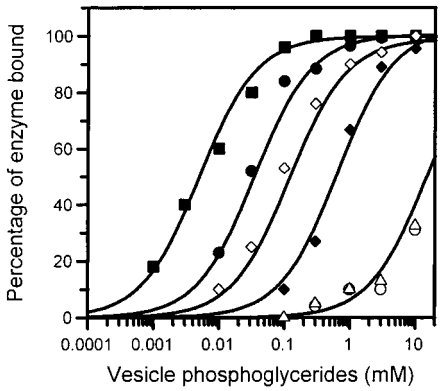

FIGURE 1: Effects of lipid composition on the ability of the PLA1 to bind to unilamellar membranes. Mixtures that contained PLA1 (4.8 nM) and separate sets of sucrose-loaded membranes at the indicated concentrations were incubated for 5 min at room temperature in MOPS−KCl buffer (total volume = 160 μL). The lipid compositions of the separate sets of membranes were as follows: (■) PA + chol (1:1), (●), PS + chol (1:1), (◇) PE + PS + chol (1:1:2), (◆) PC + PS + chol (1:1:2), (△) PC + PE + chol (1:1:2), and (○) PC + chol (1:1). After the incubations, the membranes in each incubation mixture were pelleted by centrifugation and the enzyme protein in the supernatant and pellet fractions was determined by an immunochemical method, as described under Materials and Methods. Results shown are mean values calculated on the basis of independent, duplicate incubations. Similar results were obtained in a second set of experiments.

could not bind to micelles that contained Triton X-114 unless they also contained an acidic phosphoglyceride (AOPA) (*2*). Furthermore, the replacement of PC with PE in acidic membranes has been reported to increase the affinity of several enzymes and proteins for the membranes (see, for example, ref *8*). However, it was of special interest that enzyme binding to membranes that contained PC + PE + PS + PA was unaffected when chol or chol + DAG was included in the membranes or when 18:0-20:4-PE was substituted for 16:0-18:1-PE, because experiments to be described later in this report showed that these changes significantly increased the enzyme's catalytic preference for PA.

Studies of the Enzyme's Stability. The enzyme that was used in the present study was purified and stored in buffer containing the neutral detergent Thesit to prevent it from losing activity (*2*). But both the enzyme and the Thesit had to be diluted manyfold before incubation of the enzyme with membranes, to prevent the Thesit from disrupting the membrane lipid bilayers (Materials and Methods). To examine the stability of the diluted enzyme preparations, we incubated aliquots of the preparations for 1 h at 37 °C in the absence of membranes and used mixed-micelle assays and immunochemical analyses to monitor the enzyme's specific activity during the course of the incubation. The results of these experiments revealed that the enzyme's specific activity decreased by about 75% during the incubation (Figure 2A), while the concentration of enzyme protein in the incubation mixture remained constant (data not shown). These results demonstrate that the purified enzyme is unstable at 37 °C.

To determine whether unilamellar lipid membranes could influence the enzyme's stability, we incubated the enzyme for 1 h at 37 °C in the presence of unlabeled membranes that contained PA, AOPA, or lysoPA in addition to PC + PE + PS + chol + DAG (1 mM total phosphoglycerides) and monitored the enzyme's specific activity during the incubations. Note that we removed lipids from aliquots of the incubation mixtures before measuring the enzyme's specific activity (Materials and Methods). The results of the experiments revealed that the membranes that contained AOPA could stabilize the enzyme effectively, whereas those that contained PA or lysoPA could not (Figure 2B). The mechanism of the stabilizing effect of the AOPA-containing membranes remains to be determined, but enzyme binding to AOPA on the surfaces of the membranes was probably involved. The concentrations of membranes used appeared high enough to saturate for enzyme binding (Table 1), and control experiments with membranes that contained only AOPA + chol showed that these membranes also could stabilize the enzyme (data not shown).

To examine the possibility that the membranes that contained PA or lysoPA may have failed to stabilize the enzyme because of a lysoPA-dependent effect, we incubated the enzyme with lysoPA-containing membranes in the presence or absence of BSA, which can bind lysoPA (*14*). BSA stabilized the enzyme during incubations with lysoPA-containing membranes at 37 °C but had no effect during incubations without such membranes (Figure 2C). Furthermore, BSA had no effect on the enzyme's specific activity in control experiments when it was added to mixtures of PLA1 + lysoPA-containing membranes *after* they had been incubated for 60 min at 37 °C (data not shown). These results

Table 1: Effects of Lipids on Enzyme Binding to Unilamellar Membranes

lipid composition of membranes (mol/mol)	K_D^{app} [a] (mM)
PC + chol (1:1)	>10
PS + chol (1:1)	0.035 ± 0.005
PA + chol (1:1)	0.005 ± 0.001
AOPA + chol (1:1)	0.003 ± 0.001
PC + PE + chol (1:1:2)	>10
PC + PS + chol (1:1:2)	0.65 ± 0.10
PE + PS + chol (1:1:2)	0.12 ± 0.02
PC + PA + chol (9:1:10)	1.5 ± 0.1
PC + PE + PA + chol (4.5:4.5:1:10)	1.0 ± 0.1
PC + PS + PA + chol (4.5:4.5:1:10)	0.2 ± 0.004
PE + PS + PA + chol (4.5:4.5:1:10)	0.1 ± 0.006
PC + PE + PS + PA + chol (2.25:4.5:2.25:1:10)	0.3 ± 0.008
PC + PE + PS + PA (1:4.5:3.5:1)[b]	0.07 ± 0.008
PC + PE + PS + PA + chol (1:4.5:3.5:1:10)	0.05 ± 0.007
PC + PE + PS + PA + chol + DAG (1:4.5:3.5:1:10:1)	0.035 ± 0.004
PC + PE + PS + 18:1-18:1-PA + chol + DAG (1:4.5:3.5:1:10:1)	0.04 ± 0.004
PC + 18:0-20:4PE + PS + PA + chol + DAG (1:4.5:3.5:1:10:1)	0.06 ± 0.008
PC + PE + PS + chol + DAG (1:4.5:3.5:10:1)	0.08 ± 0.006
PC + PE + PS + PA + PI + chol (1:1:1:1:1:5)	0.16 ± 0.02

[a] K_D^{app}, expressed in terms of millimolar membrane phosphoglycerides, were measured for membranes containing various mixtures of 16:0-18:1-phosphoglycerides ± chol ± DAG. But in one case 18:1-18:1-PA was substituted for 16:0-18:1-PA and in another 18:0-20:4-PE was substituted for 16:0-18:1 PE. Incubations were done for 5 min at room temperature with mixtures that contained PLA1 (4.75 nM) and sucrose-loaded membranes (0.001−5 mM phosphoglycerides) in MOPS−KCl buffer (total volume = 160 μL). Bound and unbound enzymes, separated by centrifugation, were measured by an immunochemical method. Then binding curves were generated and the standard error of the fit was determined as described under Materials and Methods. Similar results were obtained in at least one additional experiment.[b] Note that no chol was included in these membranes.

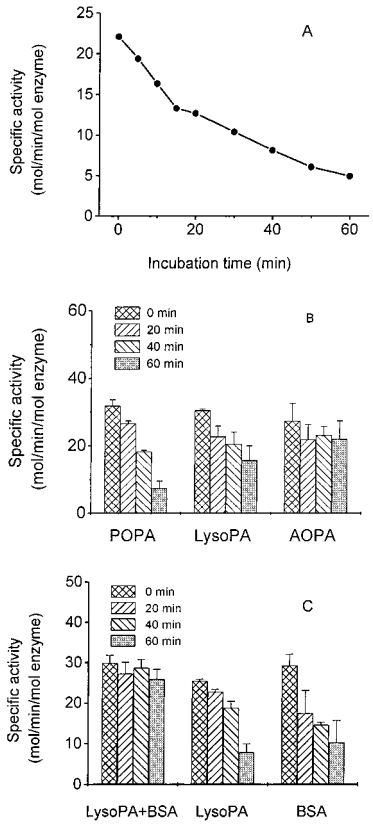

FIGURE 2: Membranes and BSA influence the enzyme's stability at 37 °C. (A) PLA1 (30.9 nM) was incubated at 37 °C in MOPS−KCl buffer in the absence of membranes (total volume = 300 μL). At the indicated times, aliquots (5 μL) of the incubation mixture were removed, and enzyme mass and activity were measured by an immunochemical method and mixed-micelle assays, respectively (Materials and Methods). Data represent means of duplicate, calculated enzyme specific activities. Similar results were obtained in four additional experiments. (B) Mixtures of PLA1 (30.9 nM) and membranes (1 mM phosphoglycerides) that contained unlabeled PA, lysoPA, or AOPA + PC + PE + PS + chol + DAG (1:1.5:4.5:2:10:1) were incubated at 37 °C in MOPS−KCl buffer (total volume = 100 μL). At the indicated times aliquots (6 μL) of the incubation mixtures were removed and mixed with 5 μL of cold 5% Triton X-114, the mixtures were warmed to separate a Triton phase from an aqueous phase, and the latter (8 μL) was collected and assayed for enzyme mass and activity as in panel A. Data represent means of quadruplicate determinations of enzyme specific activity ± SD. Similar results were obtained in another set of experiments. (C) PLA1 (30.9 nM) was incubated in MOPS−KCl buffer at 37 °C ± BSA (30 μM) ± membranes (1 mM phosphoglycerides) that contained lysoPA + PC + PE + PS + chol + DAG (1:1.5:4.5:2:10:1) in a total volume of 100 μL. At the indicated times aliquots (6 μL) were removed and assayed for enzyme mass and activity as described in panel B. Data represent means of quadruplicate determinations of enzyme specific activity ± SD. Similar results were obtained in a second set of experiments.

indicate that lysoPA-containing membranes have a destabilizing effect on the enzyme that can be prevented but not reversed by BSA. In addition, they show that the Triton X-114 phase-separation procedure used to remove membrane lipids from incubation mixtures and the Triton X-100 used in subseqent mixed micelle assays also cannot reverse the effects of the lysoPA-containing membranes.

Assays of the Enzyme's Catalytic Activity toward Vesicle-Associated Substrates Reveal Rapid Stalling of Hydrolysis Reactions. To investigate the enzyme's ability to catalyze the hydrolysis of membrane-associated phosphoglycerides,

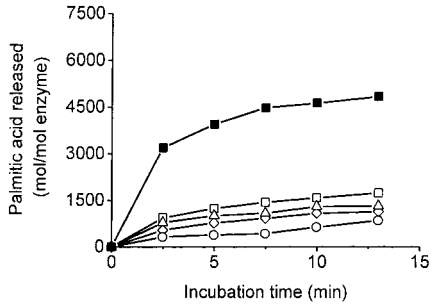

FIGURE 3: Phosphoglyceride hydrolysis stalls quickly during enzyme incubations with membrane-associated substrates. Results are shown of parallel enzyme assays using five different types of selectively labeled, radioactive membranes. All assays (total volume = 1 mL) contained PLA1 (1.24 nM) and membranes (0.5 mM phosphoglycerides) that were prepared from a mixture of the following lipids: PC + PE + PS + PI + PA + chol (1:1:1:1:1:5). But the membranes in the assays were labeled selectively with one of the following [^{3}H]16:0-18:1-labeled phosphoglycerides: (■) PA, (□) PE, (△) PS, (◇) PI, or (○) PC, open circles. Results shown are from a single experiment; a second, independent experiment gave similar results.

we initially incubated the enzyme with membranes that contained various mixtures of phosphoglycerides + chol in the absence of BSA. However, hydrolysis of the phosphoglycerides stalled after a few minutes of incubation in each case. For example, in the experiment shown in Figure 3 we investigated the enzyme's catalytic preference for 16:0-18:1 molecular species of PA, PC, PE, PI, and PS by incubating the enzyme with separate sets of selectively tagged membranes that contained equimolar quantities of all five phosphoglycerides. The results of the experiments provided evidence that the enzyme could catalyze the hydrolysis of PA in preference to that of each of the other four phosphoglycerides, but it was difficult to evaluate this preference in quantitative terms because the hydrolysis of each of the five phosphoglycerides quickly stalled.

We investigated the basis of the stalling effect by incubating the enzyme for 20 min with membranes that contained [^{3}H]16:0-18:1-PA + a mixture of unlabeled PC + PE + PS + chol + DAG and then (after the hydrolysis of PA had stalled) adding either fresh, labeled membranes or fresh enzyme to the reaction mixture and continuing the incubation (Figure 4A). Measurements of the amounts of radioactive products that were formed during the two-step incubation experiments showed that (1) the hydrolysis of PA stalled during the first step of the incubation though only about 14% of the available PA had been hydrolyzed, (2) the addition of fresh membranes during the second step of the incubation failed to relieve the stalling effect, and (3) only the addition of fresh enzyme to the stalled reaction mixtures caused the hydrolysis of PA to resume. This suggested that the hydrolysis of PA had stalled during the first step of the incubation because the enzyme had lost activity and/or had bound so tightly to a subset of membranes that it could access only those substrates that were located there.

To examine the possibility that products of the hydrolysis of PA might have contributed to the stalling phenomenon, we tested the effects of BSA on the enzyme's ability to catalyze the hydrolysis of membrane-associated PA in three parallel incubation experiments (Figure 4B). In the first of these experiments, we incubated the enzyme for 20 min with [^{3}H]16:0-18:1-PA-labeled membranes ± BSA and measured

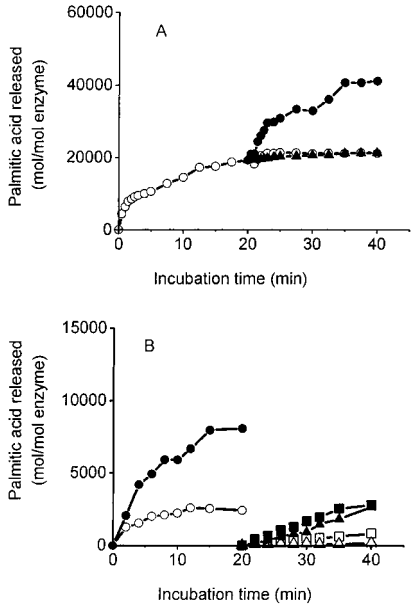

FIGURE 4: Kinetic analysis of the stalling effect. (A) Effects of the addition of fresh membranes or fresh enzyme after stalling of the PLA1-dependent hydrolysis of PA. Assay mixtures (total volume = 1.6 mL) containing PLA1 (0.477 nM) and PA-labeled membranes (1 mM phosphoglycerides) that consisted of PC + PE + PS + PA + chol + DAG (1.5:4.5:2:1:10:1) were incubated for 20 min at 37 °C. Then the incubation was continued in the presence or absence of fresh, added PA-labeled membranes (1 mM phosphoglycerides) or enzyme (0.477 nM). At the times indicated, aliquots of the reaction mixtures were removed and their contents of unesterified [^{3}H]16:0 were determined as described under Materials and Methods. Results shown are from a single experiment: (○) original incubation mixture, (●) incubation mixture after the addition of fresh enzyme, and (▲) incubation mixture after the addition of fresh membranes. Similar results were obtained in a second experiment. (B) Effects of BSA on the PLA1-dependent hydrolysis of membrane-associated PA. Results of three parallel sets of assays are shown. In one set of assays, whose results are shown toward the left of the figure, PLA1 (1.59 nM) was incubated for 20 min at 37 °C with PA-labeled membranes (1 mM phosphoglycerides) that contained PC + PE + PS + PA + chol + DAG (1.5:4.5:2:1:10:1) in the presence (●) or absence (○) of BSA (30 μM). In the second set of assays, whose results are shown toward the right, PLA1 (1.59 nM) was incubated for the same time and temperature with *unlabeled* membranes (1 mM phosphoglycerides) that contained PC + PE + PS + PA + chol + DAG (1.5:4.5:2:1:10:1); then fresh, PA-labeled membranes of the corresponding composition and concentration were added to the incubation mixture (final assay volume = 1.2 mL) and the incubation was continued in the presence (■) or absence (□) of BSA (30 μM). In the third set of assays, whose results also are shown toward the right, PLA1 (1.59 nM) was incubated for the same time and temperature with unlabeled membranes (1 mM phosphoglycerides) that contained PC + PE + PS + lysoPA + 16:0 + chol + DAG (1.5:4.5:2:1:1:10:1). Then fresh membranes (1 mM phosphoglycerides) that contained PC + PE + PS + [^{3}H]16:0-18:1-PA + chol + DAG (1.5:4.5:2:1:10:1) were added and the incubation (final volume = 1.2 mL) was continued in the presence (▲) or absence (△) of 30 μM BSA. Results are from single sets of experiments; similar results were obtained in two additional three-set experiments.

the amounts of [^{3}H]16:0 that were generated as a function of time. The results of the experiments (shown toward the left in the figure) indicated that the hydrolysis of PA quickly stalled in the absence of BSA but in the presence of BSA initially proceeded with linear kinetics and stalled only when the amount of [^{3}H]16:0 that had accumulated was 3-fold greater than that observed in the control. In the second experiment, we preincubated the enzyme for 20 min with *unlabeled* membranes in the absence of BSA to induce stalling, then added [^{3}H]16:0-18:1-PA-containing membranes ± BSA to the reaction mixture, continued the incubation for another 20 min, and made similar measurements. In this experiment (shown toward the right of Figure 4B), BSA again increased the amount of [^{3}H]16:0-18:1-PA that was hydrolyzed. However, the apparent initial rate of hydrolysis of [^{3}H]16:0-18:1-PA in the presence of BSA was about 6-fold lower than the corresponding rate of hydrolysis of [^{3}H]16:0-18:1-PA in the first experiment, i.e., considerably lower than could have been accounted for by the mixing of preincubated, unlabeled membranes with [^{3}H]16:0-18:1-PA-containing membranes. In the third experiment (also shown toward the right in Figure 4B), we preincubated the enzyme with unlabeled membranes that contained lysoPA instead of PA before incubating it with [^{3}H]16:0-18:1-PA-containing membranes ± BSA. In this case, the amount of [^{3}H]16:0-18:1-PA that was hydrolyzed in the presence of BSA was similar to that observed in the presence of BSA in the second experiment, while almost no [^{3}H]16:0-18:1-PA was hydrolyzed in the absence of BSA.

In view of the combined results of these experiments and the experiments that are shown in Figure 2B,C, it seems likely that one effect of the BSA on the stalling phenomenon was to prevent lysoPA from inactivating some of the enzyme irreversibly during the incubations. But BSA also may have had other effects, which remain to be investigated. For example, by removing negatively charged products of the enzyme reaction from the surfaces of substrate-depleted membranes, it may have influenced the ability of the active enzyme to hop from these membranes to fresh, substrate-containing membranes in the incubation mixture (*5*). Moreover, it may have relieved the reversible effects of product-dependent enzyme inhibition by a similar mechanism. Further work will be required to evaluate these possibilities.

Changes in the Composition of Membrane Phosphoglycerides Influence the Hydrolysis of the Phosphoglycerides. Having shown that BSA had to be included in incubation experiments to prevent the enzyme reaction from stalling, we examined the possibility that changes in the phosphoglyceride composition of membranes might influence the apparent initial rates of hydrolysis of the phosphoglycerides. We used membranes that contained PC + PA + chol (9:1:10), PC + PS + PA + chol (4.5:4.5:1:10), PC + PE + PA + chol (4.5:4.5:1:10), or PC + PE + PS + PA + chol (2.25:4.5:2.25:1:10) and measured the apparent initial rates of hydrolysis of each of the phosphoglycerides by selectively labeling subsets of the membranes with [^{3}H]16:0-18:1 molecular species of PA, PC, PE, or PS (Figure 5). The results of the experiments showed that (1) the enzyme catalyzed the hydrolysis of PA in preference to that of PC, PE, or PS; (2) the substitution of PE for PC in membranes caused a 3-fold increase in both the apparent initial rate of hydrolysis of PA and the apparent initial rate of total phosphoglyceride hydrolysis; and (3) the substitution of PS for PC in membranes had little apparent effect.

The effect of the substitution of PE for PC on the hydrolysis of PA warrants special comment. It probably did not depend on increased enzyme binding to the membranes, because the concentrations of membranes used in the experiments were high enough (1mM phosphoglycerides) to cause effective enzyme binding even when the membranes

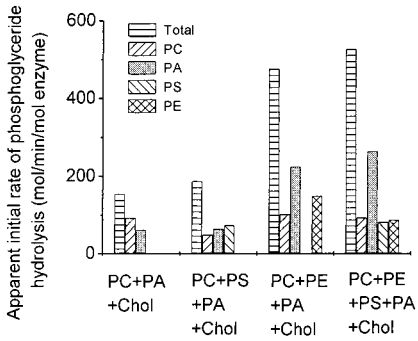

FIGURE 5: Changes in the composition of membrane phosphoglycerides affect the enzyme's activity. PLA1 (0.477 nM) was incubated for 15 min at 37 °C with BSA (30 μM) + separate sets of membranes (1 mM phosphoglycerides) in parallel assays (total volume = 0.5 mL). The membranes contained: PC + PA + chol (9:1:10), PC + PS + PA + chol (4.5:4.5:1:10), PC + PE + PA + chol (4.5:4.5:1:10), or PC + PE + PS + PA + chol (2.25:4.5:2.25:1:10). Furthermore, subsets of the membranes were labeled selectively with [^{3}H]16:0-18:1-PC, -PE, -PS, or -PA to allow the apparent initial rates of hydrolysis of each of the phosphoglycerides to be determined. Results shown are mean values from two independent incubations; similar results were obtained in an additional set of experiments.

contained no PE (see Table 1). It probably did not depend on competition for the enzyme's substrate-binding site because it was accompanied by an *increase* in the combined apparent initial rates of hydrolysis of PC + PE. And it probably did not depend on increased enzyme hopping because control experiments indicated that the substitution of PE for PC had no effect on enzyme hopping (data not shown). Therefore, the possibility has to be considered that the substitution of PE for PC may have increased the ability of membrane-bound PLA1 to access its substrates. Whether the substitution of PS for PC had a similar effect is unclear because control experiments showed that the presence of PS in membranes was associated with a reduction in enzyme hopping (data not shown).

Changes in the Content of Chol, DAG, and PE Fatty Acyl Groups Influence the Enzyme's Catalytic Preference for PA. To determine whether changes in the contents of chol, DAG, and PE fatty acyl groups in membranes could influence the enzyme's activity, we prepared sets of membranes from the following lipid mixtures: (1) a mixture that contained only 16:0-18:1 molecular species of PC + PE + PS + PA (1:4.5:3.5:1); (2) a mixture that contained the same phosphoglycerides + chol (1:4.5:3.5:1:10); (3) a similar mixture of phosphoglycerides + chol that also contained DAG (1:4.5:3.5:1:10:1); and (4) a similar mixture of phosphoglycerides + chol + DAG (1:4.5:3.5:1:10:1) that contained 18:0-20:4-PE instead of 16:0-18:1-PE. In addition, we labeled each set of membranes selectively with either [^{3}H]16:0-18:1-PC or [^{3}H]16:0-18:1-PA and did parallel incubation experiments with the labeled membranes to compare the enzyme's activity toward the two phosphoglyceride substrates. The results of these experiments demonstrated that chol, DAG, and 18:0-20:4-PE had cummulative effects on the enzyme's ability to catalyze the hydrolysis of PA and together increased the enzyme's activity toward PA by 8−10-fold (Table 2). In contrast, they seemed to have little or no effect on the enzyme's ability to catalyze the hydrolysis of PC (Table 2). Because chol, DAG, and 18:0-20:4-PE have little or no effect on the enzyme's ability to bind to membranes (Table 1), it is unlikely that they influence enzyme hopping. Therefore, as in the case of PE (see above), it is possible that they may have selectively increased the enzyme's ability to access PA in the membrane lipid bilayer.

The Enzyme Shows Sigmoidal Responses to Membrane-Associated 16:0-18:1-PA and 18:1-18:1-PA. Having identified conditions that yield high selective enzyme activity toward 16:0-18:1-PA, we examined the enzyme's ability to distinguish between 16:0-18:1-PA and 18:1-18:1-PA by incubating it with membranes that contained increasing amounts of [^{3}H]16:0-18:1-PA or [^{3}H]18:1-18:1-PA in addition to a mixture of unlabeled 16:0-18:1-PC + 18:0-20:4-PE + 16:0-18:1-PS + chol + DAG (2.5:4.5:2:10:1). The results of the experiments revealed that the apparent initial rates of hydrolysis of the [^{3}H]16:0-18:1-PA and [^{3}H]18:1-18:1-PA were similar sigmoidal functions of the relative concentrations of the two substrates in the membranes (Figure 6A). Several independent experiments yielded apparent Hill coefficients of 6−8 for each one, and the apparent initial rates of hydrolysis of the two substrates were maximal

Table 2: Effects of chol, DAG, and 18:0-20:4-PE on the Apparent Initial Rates of Hydrolysis of Membrane-Associated PC or PA[a]

				apparent initial rate [mol min^{-1} (mol of enzyme)$^{-1}$]		increase (*x*-fold)	
membranes	chol	DAG	18:0-20:4-PE	PC	PA	PC	PA
				Experiment 1			
A	−	−	−	2.60 ± 0.55	10.9 ± 0.54	1	1
B	+	−	−	2.56 ± 0.42	26.6 ± 1.43	0.98 ± 0.37	2.45 ± 0.25
C	+	+	−	3.20 ± 0.87	62.3 ± 2.12	1.23 ± 0.59	5.73 ± 0.48
D	+	+	+	5.49 ± 5.9	89.2 ± 1.91	2.11 ± 1.16	8.20 ± 0.59
				Experiment 2			
A	−	−	−	2.82 ± 0.72	11.0 ± 0.79	1	1
B	+	−	−	2.51 ± 0.30	30.7 ± 1.77	0.89 ± 0.33	2.79 ± 0.36
C	+	+	−	3.39 ± 0.49	58.5 ± 1.24	1.20 ± 0.48	5.31 ± 0.49
D	+	+	+	4.67 ± 0.80	94.3 ± 0.94	1.66 ± 0.71	8.57 ± 0.70

[a] Parallel incubations were done for 15 min at 37 °C with mixtures that contained PLA1 (0.6 nM), BSA (30 μM), and separate sets of membranes (1 mM phosphoglycerides) in a total volume of 100 μL. A, PC + PE + PS + PA (1:4.5:3.5:1); B, PC + PE + PS + PA + chol (1:4.5:3.5:1:10); C, PC + PE + PS + PA + chol + DAG (1:4.5:3.5:1:10:1); D, PC + 18:0-20:4-PE + PS + PA + chol + DAG (1:4.5:3.5:1:10:1). In each experiment, the membranes were labeled with either [^{3}H]16:0-18:1-PA or [^{3}H]16:0-18:1-PC. Apparent initial rates of hydrolysis of PA or PC were determined for each membrane by graphing the data obtained from measurements of the amounts of labeled fatty acid released at five different time points during the course of a 15 min incubation (Materials and Methods). Slopes of the essentially linear graphs and standard errors of fit of the data from the experiments were calculated with Origin 5.0 from Microcal (Northampton, MA).

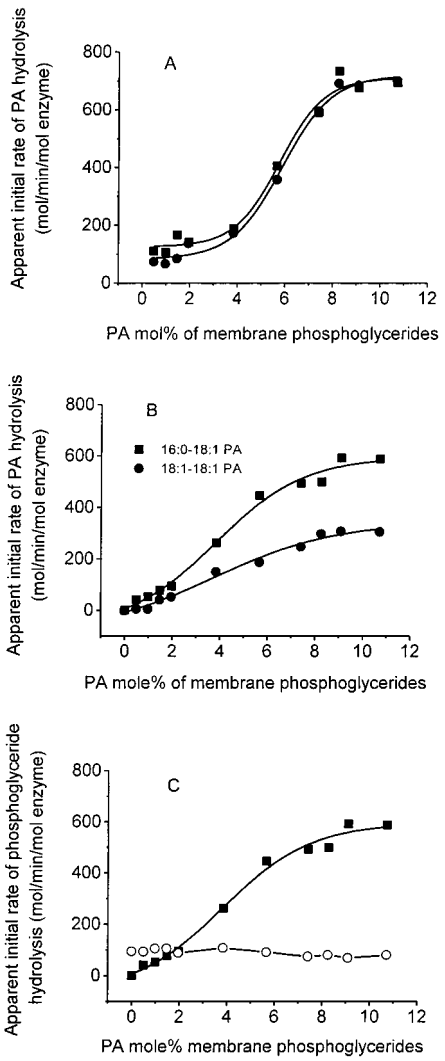

FIGURE 6: Relation between the content of PA in membranes and the rates of hydrolysis of PA and PC. (A) PLA1 (0.477 nM) was incubated for 15 min at 37 °C with BSA (30 μM) and membranes (1 mM total phosphoglycerides) that contained increasing amounts of [^{3}H]16:0-18:1-PA (■) or [^{3}H]18:1-18:1-PA (●) in addition to a fixed amount of PC + 18:0-20:4-PE + PS + chol + DAG (2.5:4.5:2:10:1). After the incubations (total volume = 0.1 mL), the amounts of released radioactive 16:0 or 18:1 were measured. Similar results were obtained in three additional experiments. (B) Incubations were done as in panel A but with membranes that contained increasing amounts of [^{3}H]16:0-18:1-PA (■) or [^{3}H]18:1-18:1-PA (●) in addition to a different mixture of PC + 18:0-20:4-PE + PS + chol + DAG (1:4.5:3.5:10:1). Similar results were obtained in three additional experiments. (C) Results from incubations (total volume = 1 mL) that were done in parallel with those shown in panel B are presented. PLA1 (0.477 nM) was incubated for 16 min with membranes (1 mM phosphoglycerides) that contained increasing amounts of unlabeled 16:0-18:1-PA in addition to a fixed amount of [3H]16:0-18:1-PC + PE + PS + chol + DAG (1:4.5:3.5:10:1). Then the amounts of radioactive 16:0 were measured (○) Similar results were obtained in a second set of experiments. Results from panel B related to incubations with membranes that contained increasing amounts of [^{3}H]16:0-18:1-PA in addition to unlabeled PC + PE + PS + chol + DAG (1:4.5:3.5:10:1) are shown for comparison (■).

when each substrate accounted for about a tenth of the total membrane phosphoglycerides.

In another set of experiments, we incubated the enzyme with membranes that contained increasing amounts of [^{3}H]-16:0-18:1-PA or [^{3}H]18:1-18:1-PA in addition to a mixture of unlabeled lipids that included a lower relative proportion of PC and a higher relative proportion of PS (16:0-18:1-PC + 18:0-20:4-PE + 16:0-18:1-PS + chol + DAG = 1:4.5:3.5:10:1). The results of these experiments revealed that the apparent initial rates of hydrolysis of the radioactive 16:0-18:1-PA and 18:1-18:1-PA again increased with sigmoidal kinetics and reached maximum values when each phosphoglyceride accounted for about a tenth of the total vesicle phosphoglycerides (Figure 6B). But four independent experiments yielded apparent Hill coefficients of 1.7−1.8, and the maximum apparent initial rate of hydrolysis of 16:0-18:1-PA was about 2-fold faster than that of 18:1-18:1-PA.

In a third set of experiments, we incubated the enzyme with membranes that contained increasing amounts of unlabeled 16:0-18:1-PA in addition to a fixed amount of [3H]-16:0-18:1-PC + unlabeled PE + PS + chol + DAG to examine the effect of the PA on the enzyme's ability to catalyze the hydrolysis of PC. The results of the experiments provided evidence that changes in the concentration of PA in the membranes affected the hydrolysis of PA selectively because they had little or no effect on the hydrolysis of PC (Figure 6C and data not shown). Finally, we incubated the enzyme with membranes that contained increasing amounts of AOPA in addition to fixed amounts of 16:0-18:1-PC + 18:0-20:4-PE + PS + [^{3}H]16:0-18:1-PA + chol + DAG (1:4.5:3.5: 0.2:10:1), but we could detect no AOPA-dependent effect on the apparent initial rate of hydrolysis of the [^{3}H]16:0-18:1-PA (data not shown).

It is noteworthy that our previous experiments using mixed micelles also showed that the enzyme catalyzed the hydrolysis of PA with sigmoidal kinetics (*1*, *2*). However, it had a distinct catalytic preference for 18:1-18:1-PA over 16:0-18:1-PA in those experiments and catalyzed the hydrolysis of 18:1-18:1-PA with sigmoidal kinetics even when it was incubated with mixed micelles that contained a small, fixed amount of this substrate in addition to increasing amounts of AOPA. The basis for these differences remains to be determined, but the difference in substrate preference may depend on the enzyme's ability to access substrates that are contained within stable, unilamellar membranes as opposed to transient aggregates of Triton X-100. Furthermore, the difference in reponse to AOPA may depend on the fact that the present experiments were done under conditions where enzyme binding to the membranes was probably saturated even in the absence of PA or AOPA, whereas the enzyme did not bind to mixed micelles of Triton unless they contained AOPA or another anionic phosphoglyceride and the sigmoidal effect of AOPA on catalysis correlated with an effect on enzyme binding (*2*).

DISCUSSION

The results of this study provide the first available evidence concerning the effects of membrane lipids on the activity of the bovine testis PLA1. They show that these lipids influence not only the enzyme's ability to bind to membranes (Table 1) but also its stability at 37 °C (Figure 2) and its ability to catalyze the hydrolysis of membrane-associated phosphoglycerides (Table 2, Figures 5 and 6). When the relative distribution of the lipids is adjusted to optimize the enzyme's catalytic preference for 16:0-18:1-PA, the membranes contain (1) a low relative amount of PC; (2) high relative amounts of PE, PS, and chol; (3) DAG; (4) 18:0-20:4-PE instead of

16:0-18:1-PE; and (5) 10 mol % PA per 100 mol % total phosphoglycerides. Under these conditions, the enzyme catalyzes the hydrolysis of PA at an apparent initial rate that is about 18-fold greater than the rate of hydrolysis of PC. However, many questions about the molecular basis and functional significance of these results remain to be answered.

The results must reflect both the structural characteristics of the enzyme and the properties of the membrane lipids. But all that is currently known about the enzyme's structure is that the sequence of its cDNA encodes both a small lipase consensus region and a putative coiled-coil-forming region and that serine 540 in the lipase consensus region is required for catalysis (*3*). It is not clear which of the enzyme's amino acids comprise its interfacial binding region, which amino acids form its substrate-binding region, or which amino acids directly effect catalysis. Furthermore, though the purified enzyme is oligomeric, it is not known whether the enzyme functions as an oligomer or whether interactions of the enzyme oligomer with membranes lead to its dissociation into active monomers or cause changes in its conformation. Therefore, much more structural work on the enzyme is needed.

On the other hand, a considerable amount of information is available about the properties of the membrane lipids. For example, studies of the interactions of other interfacial enzymes with membranes have provided evidence that PC can inhibit the binding of these enzymes to anionic phosphoglycerides, and PC has even been shown to inhibit the binding of calcium ions to anionic phosphoglycerides (ref *8* and references therein). Model experiments with NMR have suggested that inhibitory effects of PC, such as these, may depend on the ability of the phosphorylcholine headgroup of PC to interact with the headgroups of PA and other negatively charged phosphoglycerides (*15*, *16*). Furthermore, headgroup-dependent interactions might conceivably have contributed to the 300-fold difference, shown in Table 1, between the enzyme's K_D^{app} for membranes that contained PA + chol (1:1) and that for membranes that contained PC + PA + chol (9:1:10).

PE also could interfere with enzyme binding to membranes in our experiments, though its effect was weaker than that of PC (Table 1). Moreover, chol, DAG, and 18:0-20:4-PE could influence the enzyme's activity without apparently affecting enzyme binding (Tables 1 and 2, Figure 5). Extensive biophysical studies have provided evidence that all three of these lipids have a tendency to promote the formation of nonbilayer phases in membranes (*17*, *18*). Therefore, changes in the lamellar structure of the membranes (not detectable by analysis with the Brookhaven particle sizer) could potentially have caused the changes in enzyme activity that we observed. On the other hand, it remains possible that chol, DAG, and 18:0-20:4-PE might have influenced the enzyme's activity by selectively increasing its ability to access PA. A key point is that chol, DAG, and 18:0-20:4-PE promoted the hydrolysis of PA without significantly affecting the enzyme's ability to catalyze the hydrolysis of PC or bind to the membranes (Tables 2 and 1).

A related point is that increasing the concentration of PA in membranes had a sigmoidal effect on the enzyme's catalytic activity that seemed to reflect selective changes in the enzyme's ability to access PA within the membrane lipid bilayer rather than changes in the enzyme's overall ability to bind to membranes (Figure 6). chol and DAG may conceivably have contributed to the sigmoidal effect by acting as molecular spacers to relieve inhibitory effects of PC and Mg^{2+} on PA [Mg^{2+} has been shown to interact with the phosphate groups of PA molecules in bilayers and monolayers (*19*, *20*)]. On the other hand, 18:0-20:4-PE may have increased the *local* concentration of 16:0-18:1-PA in membranes through phase-separation effects (King et al., to be submitted).

With regard to the basis of the sigmoidal effect, at least two possibilities have to be considered. One is that the effect might depend on complex competitive interactions between PA and the other substrate phosphoglycerides in the membranes. Another is that the effect may reflect some kind of cooperativity that requires concomitant enzyme interactions with two or more molecules of membrane PA. It might be possible to distinguish between these alternatives by examining the effect of changes in the concentration of membrane PA on the hydrolysis of each of the other membrane phosphoglycerides. But all that is known at present is that increasing the concentration of PA had no detectable effect on the hydrolysis of PC (Figure 6C).

The above-described effects of membrane lipids are of potential interest because the PLA1 may conceivably function in vivo by attenuating 16:0-18:1-PA-dependent signals that are generated by a PC-specific phospholipase D reaction on the cytoplasmic surface of a cell plasma membrane. In support of this possibility, consider the following: (1) The best-studied cell plasma membrane, that of the human erythrocyte, is known to have an asymmetric, chol-rich lipid bilayer (*21*). (2) The cytoplasmic leaflet of this bilayer contains only about 20% of the total membrane PC and sphingomyelin but about 80% of the membrane ethanolamine-containing phosphoglycerides (PE and plasmenylethanolamine) and 100% of the total PS (*21*, *22*). (3) The PC of the cytoplasmic leaflet consists mainly of 16:0-18:1 and 16:0-18:2 molecular species, whereas the PE, plasmenylethanolamine, and PS consist mainly of 18:0-20:4 or related molecular species (*23*, *24*). (4) Agonists can activate a PC-specific phospholipase D reaction that appears to generate PA on the plasma membrane (reviewed in ref *25*). (5) The generation of PA in agonist-stimulated cells is followed in many cases by the accumulation of DAG (*26*, *27*). (6) As mentioned earlier, the results of the present study show that PLA1 has the highest catalytic preference for PA when the PA is present in model membranes that contain low amounts of DAG and 16:0-18:1-PC but high amounts of chol, 18:0-20:4-PE, and PS. Therefore, it can be argued that these membranes may represent a rough model of the cytoplasmic surface of an activated plasma membrane. Because the lipid compositions of the model membranes that we used were much simpler than those of native cell membranes and the model membranes contained no proteins, much more work with better models will clearly be required to evaluate this possibility.

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