Processive Interfacial Catalysis by Mammalian 85-Kilodalton Phospholipase A_2 Enzymes on Product-Containing Vesicles: Application to the Determination of Substrate Preferences†

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Received January 13, 1993; Revised Manuscript Received March 25, 1993

ABSTRACT: Substrate specificities of the human and rat kidney 85-kDa phospholipase A_2 enzymes (hmw-PLA_2) have been determined under conditions in which hydrolysis of substrate vesicles occurs without the desorption of enzyme from the interface (scooting mode catalysis). The rat kidney enzyme binds to vesicles of 1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (OPPC), which contain the substrate 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine (SAPC) and 10 mol % arachidonic acid (20:4) and 1-stearoyl-*sn*-glycero-3-phosphocholine (S-lyso-PC) as the hydrolysis reaction products, with a second-order rate constant $k_{on} \cong 2 \times 10^7$ M^{-1} s^{-1}. Upper limits of $k_{off} \leq 3 \times 10^{-4}$ s^{-1} and $K_D \leq 15$ pM for the dissociation rate and equilibrium constants, respectively, are estimated from the vesicle binding measurements. The initial rates of hydrolysis of either radiolabeled 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphoserine (^{3}H-SAPS), -phosphoethanolamine (^{3}H-SAPE), -phosphoinositol (^{14}C-SAPI), or -phosphate (^{3}H-SAPA) and either ^{3}H-SAPC or ^{14}C-SAPC, which were incorporated into product-containing OPPC vesicles, were simultaneously measured with dual isotope radiometric assays. The plasmenylcholine 1-*O*-(*Z*-hexadec-1′-enyl)-2-arachidonyl-*sn*-glycero-3-phosphocholine (^{3}H-PlasAPC) was also tested. Relative substrate specificity constants (k_{cat}/K_M* values) were determined from the concentrations and initial rates of hydrolysis of the labeled substrates; the rank order of the values is SAPC ≅ SAPI ≅ PlasAPC > SAPE > SAPA ≅ SAPS. The maximal difference in specificity constants is 3.5-fold, indicating that the hmw-PLA2 does not significantly discrimate between phospholipids with different polar head groups. The diglyceride 1-stearoyl-2-arachidonyl-*sn*-glycerol is not a substrate for the human hmw-PLA_2. Two mixtures of 1-stearoyl-2-acyl-*sn*-glycero-3-phosphocholine, which have different *sn*-2 acyl chains, were prepared and compared to SAPC as substrates. One mixture contained naturally-occurring unsaturated fatty acyl chains and the other contained a mixture of 20:4, all of its partially hydrogenated analogues (20:3, 20:2, and 20:1), and arachidic acid (20:0). The order of preference for the human hmw-PLA_2 is *sn*-2-20:4 > *sn*-2-α-linolenoyl > *sn*-2-linoleoyl > *sn*-2-oleoyl ≥ *sn*-2-palmitoleoyl. The preference order of the 20-carbon acyl chains is 20:4 > 20:3 > 20:2 > 20:1 > 20:0, and there is a preference for positional isomers with double bonds closest to the *sn*-2 ester. In contrast, the human non-pancreatic-secreted 14-kDa phospholipase A_2 does not discriminate significantly between the 20-carbon substrates.

An 85-kDa phospholipase A_2 (hmw-PLA_2)[1] has recently been purified from the cytosol of rat and mouse macrophages (Leslie et al., 1988; Wijkander & Sundler, 1989, 1991), the human monocytic cell line U937 (Clark et al., 1990; Diez & Mong, 1990; Kramer et al., 1991; Rehfeldt et al., 1991), human, bovine, and rabbit platelets (Kim et al., 1991a,b; Takayama et al., 1991), and rat kidney (Gronich et al., 1990). This enzyme may be responsible for initiating the production of eicosanoids in activated cells by catalyzing the lipolysis of membrane phospholipids to yield free arachidonic acid. The hmw-PLA_2 preferentially hydrolyzes *sn*-2-arachidonyl phospholipids; in contrast, the 14-kDa secreted phospholipases A_2 do not discriminate between saturated and unsaturated fatty acyl chains at the *sn*-2 position of phospholipids (Diez et al., 1992; Ghomashchi et al., 1991). Intracellular calcium levels play a role in the regulation of the hmw-PLA_2 enzyme by altering the intracellular location of the enzyme. At low calcium levels (<0.1 μM), the enzyme is found in the cytosolic fraction of cells, and it translocates to the membrane fraction in the presence of about 0.5–1 μM calcium (Clark et al., 1991; Diez & Mong, 1990; Krause et al., 1991; Channon & Leslie, 1990; Yoshihara & Watanabe, 1990). Translocation in cell-

† Supported by a grant and a Research Career Development Award from the National Institutes of Health (HL36235 and GM562) and a grant from Sterling Winthrop. M.H.G. is a fellow of the Alfred P. Sloan Foundation (1991–1993), and A.M.H. and S.S. are the recipients of postdoctoral fellowships from the American Cancer Society and the Swiss National Science Foundation, respectively.

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; GC, gas chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; hmw-PLA_2, 85-kDa phospholipase A_2; K_M*, Michaelis constant for the enzyme bound to the interface; nps-PLA_2, non-pancreatic-secreted phospholipase A_2; OPPC, 1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine; PAPC, 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine; PlasAPC, 1-*O*-(*Z*-hexadec-1′-enyl)-2-arachidonyl-*sn*-glycero-3-phosphocholine; Plas-lyso-PC, 1-*O*-(*Z*-hexadec-1′-enyl)-*sn*-glycero-3-phosphocholine; SAG, 1-stearoyl-2-arachidonyl-*sn*-glycerol; SAPA, SAPC, SAPE, SAPI, and SAPS, 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphate, -3-phosphocholine, -3-phosphoethanolamine, -3-phosphoinositol, and -3-phosphoserine); S-lyso-PC, 1-stearoyl-*sn*-glycero-3-phosphocholine; SX_{20}PC, 1-stearoyl-2-(arachidonyl, its partially hydrogenated forms, and arachidyl)-*sn*-glycero-3-phosphocholine; SX_{nat}PC, 1-stearoyl-2-(palmitoleoyl, oleoyl, linoleoyl, α-linolenoyl, *cis*-11-eicosenoyl, *cis*-11,14-eicosadienoyl, *cis*-8,11,14-eicosatrienoyl, and arachidonyl)-*sn*-glycero-3-phosphocholine; TLC, thin layer chromatography; 20:4, arachidonic acid. All labeled phospholipids contain either ^{2}H, ^{3}H, or ^{14}C in their *sn*-2 acyl chains. All fatty acids are designated by specifying the number and location of their double bonds, and *cis* designates that all of the double bonds have the *cis* stereochemistry.

0006-2960/93/0432-5949$04.00/0

free systems can be induced by submillimolar concentrations of calcium (Clark et al., 1991; Ghomashchi et al., 1992; Wijkander & Sundler, 1992). It is likely that calcium does not serve as an active-site catalytic cofactor (Ghomashchi et al., 1992, Wijkander & Sundler, 1992), unlike the 14-kDa secreted phospholipases A_2, which require calcium for the chemical steps of the hydrolytic reaction (Scott et al., 1990).

The amino acid sequence of the hmw-PLA_2 from human U937 cells shows no regions of homology with the 14-kDa enzymes (Clark et al., 1991; Sharp et al., 1991). The N-terminal region of the 85-kDa phospholipase A_2 contains a sequence of approximately 50 amino acids that is apparently responsible for binding of the enzyme to membranes. This sequence motif is also found in other enzymes that bind to phospholipid membranes in a calcium-dependent manner [for example, protein kinase C-γ (Coussens et al., 1986), synaptotagmin (Perin et al., 1990), and certain forms of phosphatidylinositol-specific phospholipase C (Baker, 1989)]. There is evidence that the hmw-PLA_2 is hormonally regulated (Gronich et al., 1988) and that this regulation involves posttranslational modification of the enzyme by phosphorylation (Lin et al., 1992).

Previous studies from this laboratory show that the rat kidney hmw-PLA_2 binds more tightly to phosphatidylcholine vesicles that contain reaction products (1:1 mixture of 20:4 and lysophospholipid) than to vesicles that do not contain products (Ghomashchi et al., 1992). This product-dependent binding of the enzyme to the interface may explain why for certain experimental conditions the progress of the reaction stops before all of the available substrate is hydrolyzed (Ghomashchi et al., 1992; Leslie, 1991; Diez et al., 1992).

This paper describes further studies of the action of the rat kidney and human U937 cell recombinant hmw-PLA_2 enzymes on product-containing vesicles. It is shown that the enzyme binds tightly to such vesicles and undergoes processive interfacial catalysis; phospholipid hydrolysis occurs without desorption of the enzyme from the vesicle into the aqueous phase. This behavior, called *scooting*, has been documented for the action of the 14-kDa phospholipases A_2 on anionic phospholipid vesicles (Berg et al., 1991; Gelb et al., 1991; Jain et al., 1991, 1986). On the basis of theoretical considerations, the scooting mode analysis of interfacial catalysis by phospholipases A_2 is the only reliable way to determine the intrinsic substrate preferences (relative k_{cat}/K_M^* values) for the action of the enzymes on phospholipid substrate aggregates (Berg et al., 1991; Gelb et al., 1992; Ghomashchi et al., 1991). In this study, scooting mode analysis has been applied to the human and rat hmw-PLA_2 enzymes to obtain information about the relative k_{cat}/K_M^* values of phospholipid substrates that differ in the structure of their polar head groups and their *sn*-1 and *sn*-2 fatty acyl chains. The *sn*-2 acyl chain specificity of the hmw-PLA_2 is compared to that observed with the nps-PLA_2.

MATERIALS AND METHODS

Materials. The phospholipids OPPC, SAPA, SAPC, SAPE, SAPS, S-lyso-PC, and 1,2-dioleoyl-*sn*-glycerol are from Avanti Polar Lipids. The lysophospholipid Plas-lyso-PC was a gift from Dr. Richard Gross. The fatty acids 20:4, *cis*-9-16:1, 17:0, *cis*-9-18:1, *cis*-9,12-18:2, *cis*-9,12,15-18:3, and fatty acid methyl ester GC standards (GLC-63A) are from NuChek Prep. ^{2}H-20:4 ([5,6,8,9,11,12,14,15-$^2H_8$]eicosatetraenoic acid) is from Oxford Biochemicals. Phospholipase D from cabbage was purchased from Boehringer Mannheim. Phospholipase C (*Clostridium welchii*), *cis*-11-20:1, *cis*-11,14-20:2, and *cis*-8,11,14-20:3 are from Sigma. ^{3}H-20:4 [5,6,8,9,11,12,14,15-$^3H_8$(N)] (200 Ci/mmol) is from American Radiolabeled Chemicals, Inc., and ^{3}H-SAPC (200 Ci/mmol) is from Amersham. ^{14}C-SAPC (1-^{14}C of the *sn*-2 chain, 50 mCi/mmol) and ^{14}C-SAPI (1-^{14}C of the *sn*-2 chain, 50 mCi/mmol) are from New England Nuclear. The radiolabeled phospholipids ^{3}H-SAPC, ^{3}H-PlasAPC, ^{3}H-SAPA, ^{3}H-SAPE, and ^{3}H-SAPS and the diglyceride ^{3}H-SAG were synthesized either enzymatically or chemically and purified by conventional techniques. Descriptions of the syntheses and isolation procedures that were used are given as supplementary material.

The hmw-PLA_2 from rat kidney was purified to a specific activity of 85 microunits/μg as described previously (Ghomashchi et al., 1992). The human hmw-PLA_2 was obtained from baculovirus-infected Sf9 cells and purified to near homogeneity (>80%, as determined by denaturing gel electrophoresis with a specific activity of 11 microunits/μg) (I. Street, P. Weech, and B. Kennedy, Merck Frosst Canada, unpublished results). The fraction of total enzyme that is phosphorylated is typically 0.6, and the specific activity of the phosphorylated enzyme is $\leq$2-fold greater than that of the nonphosphorylated enzyme. One microunit of enzyme activity is defined as the amount of enzyme that produces 1 pmol of ^{14}C-20:4/min from ^{14}C-SAPC at 37 °C using the assay described by Gronich et al. (1990). The human nps-PLA_2 was obtained as a gift from Dr. J. Browning (Biogen Inc., Cambridge, MA). It was purified to homogeneity as described previously [200-300 μmol/(min·mg) on radiolabeled *Escherichia coli* membranes] (Bomalaski et al., 1991).

Hydrogenation of 20:4 with Hydrazine. Unlabeled 20:4 was partially and fully hydrogenated by reacting 45 mg of 20:4 in 12 mL of absolute ethanol with 1.2 mL of hydrazine monohydrate (99%, Baker) at 50 °C under an atmosphere of oxygen (Sebedio & Ackman, 1982). Four equal volumes of the reaction mixture were removed at times t = 30, 60, 90, and 120 min and transferred to separate tubes, which contained 15 mL of 5% NaCl/1 M HCl. The resulting mixtures were extracted with three 6-mL portions of hexane. The extracts for each sample were combined and taken to dryness *in vacuo*. The residues were dissolved in ~0.2 mL of petroleum ether/diethyl ether/acetic acid (70:30:1 by volume) and purified by passage over a silica G column (0.5 × 5 cm) equilibrated with the same solvent. The purified fatty acids of each time point were taken to dryness with a stream of argon and dissolved in 0.5 mL of hexane. The fatty acids contained in a 5-μL aliquot of each sample time point were converted to the corresponding methyl ester derivatives as described below. The relative amounts of the fatty acid species were quantified by GC analysis of their methyl ester derivatives as described below. This information allowed the formulation of a mixture of 20:0, the 14 partial reduction products of 20:4, and 20:4 in which the 16 fatty acids were present in approximately equal amounts.

Synthesis of $SX_{20}PC$. An approximately equimolar mixture of 20-carbon fatty acids (previous paragraph) was esterified at the *sn*-2 position of S-lyso-PC. The reaction was carried out under an atmosphere of argon in a 4-mL screw-cap vial that was fitted with a Teflon septum and stir bar. The vial contained 3.4 mg of the mixture of fatty acids, 7 mg of S-lyso-PC, 30 mM *N*-hydroxybenzotriazole, 30 mM 4-pyrrolidinopyridine, and 17 mM 1,3-dicyclohexylcarbodiimide in 0.675 mL of dimethylformamide. The solution was stirred for 1 h at 70 °C, and a second addition of 1,3-dicyclohexylcarbodiimide was made to give a final concentration of 34 mM 1,3-dicyclohexylcarbodiimide. The reaction was stirred for 12 h

and cooled to room temperature, and the dimethylformamide was removed *in vacuo*. After removal of the dimethylformamide, the contents of the vial were dissolved in a small amount of $CHCl_3/CH_3OH/H_2O$ (25:10:1 by volume) and applied to a silica G column (1.5 × 15 cm) equilibrated with the same solvent. The phospholipids $SX_{20}PC$ eluted after 60–70 mL of solvent had passed through the column. Column fractions which contained $SX_{20}PC$ were identified by TLC and pooled, and the solvent was removed *in vacuo*. The remaining residue was lyophilized twice from benzene and dissolved in a small amount of $CHCl_3$. A 1- × 15-cm column of CM-52 cellulose (Whatman) equilibrated with 100% $CHCl_3$ was prepared as described previously (Kates, 1986). After the sample was applied, the column was washed with 25–30 mL of $CHCl_3$. The phospholipid was eluted with 8% CH_3OH in $CHCl_3$ (by volume). Column fractions containing the product were pooled and the elution solvent was removed *in vacuo*. The residue (2.1 mg) was lyophilized twice from benzene and dissolved in $CHCl_3$ to give a final concentration of 10 mg/mL. The concentration of phospholipid in the solution was estimated by phosphate analysis using a modification of the procedure of Bartlett as described by Christie (1982). The overall yield of the purified material was ~24%.

Synthesis of $SX_{nat}PC$. A mixture of 1.5 mg each of *cis*-9-16:1, *cis*-9-18:1, *cis*-9,12-18:2, and *cis*-9,12,15-18:3, 1.6 mg each of *cis*-11-20:1, *cis*-11,14-20:2, *cis*-8,11,14-20:3, and 20:4, and 24 mg of S-lyso-PC was dissolved in 1.9 mL of dimethylformamide and placed in a 4-mL screw-cap vial, which contained a stir bar and was fitted with a Teflon septum. The coupling reaction was carried out as described for $SX_{20}PC$. *N*-Hydroxylbenzotriazole, 4-pyrrolidinopyridine, and 1,3-dicylohexylcarbodiimide were added in 0.93 mL of dimethylformamide to give final concentrations of 30, 30, and 17 mM, respectively, in a reaction volume of 2.83 mL. After removal of the dimethylformamide at the end of the reaction, the phospholipid products were purified as described above for the purification of $SX_{20}PC$. The yield of purified material was ~36%.

Preparation of Sonicated Substrate Phospholipid Vesicles. Aqueous suspensions of phospholipid vesicles for use as substrates were typically prepared at concentrations 5–10-fold greater than that required in the assays. The required amounts of stock solutions of phospholipids in $CHCl_3$, S-lyso-PC in $CHCl_3/CH_3OH$ (1:1 by volume), fatty acids in ethanol, and commercially obtained radiolabeled phospholipids in toluene/ethanol (1:1 by volume) were transferred to a 1- × 7.5-cm glass test tube, and the solvents were removed with a stream of argon and then *in vacuo* for 30 min. The residue was resuspended in 0.1 mL of H_2O and frozen, and the water was removed by lyophilization; this step ensured that all of the solvents had been removed. Sufficient 10 mM HEPES, pH 8.0, and 0.1 mM EDTA was added to the lipid film to give the desired final concentration of phospholipids, and the suspension was thoroughly mixed using a vortex mixer. The dispersion of lipids was frozen and sonicated for 1 min at room temperature using a bath sonicator (Lab Supplies, Model G11 2SPIT) (Jain & Gelb, 1991). Aqueous stock solutions of substrates were used on the day of the experiment and were not stored for later use.

Enzymatic Reactions. Reaction mixtures that were not analyzed by GC contained 10 mM HEPES, pH 8.0, 0.6 mM $CaCl_2$, and 0.1 mM EDTA (buffer A) in a total volume of 0.05 mL in polypropylene tubes at room temperature unless otherwise noted. The compositions and concentrations of the substrate vesicle components are given in the figure and table legends. Reactions were initiated by the addition of enzyme and quenched at the times given in the text by the addition of 0.37 mL of $CHCl_3/CH_3OH$/concentrated HCl (200:100:1 by volume). The phospholipids and fatty acids were extracted and the labeled fatty acids were quantified as described previously (Ghomashchi et al., 1992).

The products of reactions with vesicles that contained both ^{14}C-SAPC and ^{3}H-SAG as the labeled substrates were extracted with $CHCl_3/CH_3OH$ as previously described (Ghomashchi et al., 1992). Unlabeled 20:4 and 1,2-dioleoyl-*sn*-glycerol (5 μg each) were added to the samples before removal of the $CHCl_3$ layer. The $CHCl_3$ layer was evaporated with a stream of nitrogen, and the residue was dissolved in 0.05 mL of hexane/diethyl ether/acetic acid (80:20:2 by volume). The samples were applied to individual lanes of a silica G TLC plate (Merck), and the plate was developed with the same solvent. The hydrolysis products ^{14}C-20:4 and ^{3}H-20:4 ($R_f \sim 0.2$) and the substrates ^{14}C-SAPC ($R_f \sim 0$) and ^{3}H-SAG ($R_f \sim 0.1$) were visualized with I_2 vapor. The areas of the plate containing ^{14}C-20:4 and ^{3}H-20:4 were removed, and the fatty acids were eluted by shaking the silica gel with 0.2 mL of CH_3OH for 5 min. Scintillation fluid was added, and the samples were counted using a Beckman Model LS 1801 scintillation counter with channel settings that permitted 34% and 66% counting efficiency of ^{3}H and ^{14}C, respectively. The spillover of ^{3}H cpm into the ^{14}C channel was <1%, and spillover of the ^{14}C cpm into the ^{3}H channel was 8%.

Enzymatic Reactions for GC Analysis. Reaction mixtures contained 10 mM HEPES, pH 8.0, 0.1 mM EDTA, and 0.6 mM $CaCl_2$ (buffer A), 30 μM total phospholipid, and 300 microunits human hmw-PLA_2 in a total volume of 10 mL at room temperature. The specific compositions of the substrate vesicles are given in the legend of Figure 3. Reactions were initiated by the addition of enzyme and quenched at the times given in the figure and table legends by the addition of 0.033 mL of concentrated HCl. Background levels of fatty acids were measured for similar reaction mixtures that did not contain enzyme. The fatty acid 17:0 (2.5 μg) was added after the quench as an internal standard. Total phospholipids and fatty acids were extracted into $CHCl_3$ by the addition of 10 mL of $CHCl_3/CH_3OH$ (2:1 by volume) followed by a second extraction with 5 mL of the same solvent. The combined extracts were evaporated with a stream of nitrogen, and the residue was dissolved in ~0.2 mL of petroleum ether/diethyl ether/acetic acid (70:30:1 by volume) and applied to a silica G column (0.5 × 5 cm) equilibrated with the same solvent. Unoxidized free fatty acids elute completely from the column with 4 mL of solvent; oxidized fatty acids are retained on the column. The unoxidized fatty acids were collected in a screw-cap vial, and the solvent was evaporated with a stream of nitrogen. The residue was resuspended in 1 mL of hexane, and 0.2 mL was removed for radiometric analysis by scintillation counting. The remaining 0.8 mL was mixed with 0.5 mL of 5% HCl in CH_3OH (by weight) and heated overnight at 50 °C to form the methyl ester derivatives of the fatty acids. The reaction mixture was cooled to room temperature and neutralized by the addition of 2 mL of 5% $NaHCO_3$ (by weight), and the hexane layer was transferred to a 1.5-mL screw-cap vial. The aqueous layer was extracted with a second portion of hexane, and the combined extracts were evaporated with a stream of argon. The residue was dissolved in 0.05 mL of toluene and stored under argon at −80 °C. The amounts of the individual substrate phospholipid molecular species in the reactions were measured by extracting the phospholipids from reaction mixtures which did not contain enzyme, as

described above. The acyl chains of the phospholipids were directly converted to fatty acid methyl esters as described above. Flame-ionization GC analysis (see below) gave the mass fraction of the individual fatty acids esterified at the *sn*-2 position of the substrate phospholipids.

Analysis of Fatty Acid Methyl Esters by GC. Fatty acid methyl esters were quantified either by flame-ionization detection GC or by quantitative combined GC/mass spectrometry. Analyses by flame-ionization detection were performed on a Hewlett-Packard 5840 gas chromatograph equipped with a splitless injector and an integrator–controller terminal. A Perkin-Elmer CPS2 capillary column (50 m × 0.25 mm), which separates all of the methyl ester derivatives of the fatty acids tested except *cis*-11-20:1 from *cis*-5,11-20:2 and 20:4 from ^{2}H-20:4, was used for GC analyses by flame-ionization detection. The initial settings of the instrument were injector 275 °C, detector 275 °C, and column oven 80 °C. Splitless sample injections of 0.2–2.0 μL were made, and 2 min later the column oven temperature was increased from 80 to 160 °C at a rate of 20 °C/min and then to 180 °C at a rate of 5 °C/min.

The amounts of the 17:0 internal standard and all the hydrolysis products except 20:4 were determined by flame-ionization detection analysis. The signal response for the 17:0 internal standard, which was measured for each injected sample, permitted the calculation of the mass amounts of the methyl esters of the enzymatic reaction products in the entire sample. Known amounts of fatty acid methyl ester standards (NuChek Prep) were routinely injected to verify their relative retention times and the response of the detector; a response of 150 ± 15 integrator area units/ng was consistently measured. The response of the detector was verified by cutting and weighing the peaks of the chromatogram traces and comparing the weights of the peaks to the area units given by the detector.

The order of elution of the fatty acid methyl esters formed from the partial and full hydrogenation of 20:4 was determined by combined GC/mass spectrometry. GC separations were made with a Hewlett-Packard 5890 gas chromatograph equipped with a splitless injector, and mass spectra were obtained with a Kratos Profile HV3 mass spectrometer. The hydrogenation products of 20:4 were separated on a Perkin-Elmer CPS2 capillary column using the temperature settings described above. The ion currents for the parent ions of the methyl ester derivatives of 20:0 (326.32 amu), the four positional isomers of 20:1 (324.30 amu), the six positional isomers of 20:2 (322.29 amu), the four positional isomers of 20:3 (320.27 amu), and 20:4 (318.26 amu) were measured. For a given class of positional isomers, the assignments of the relative retention times in this work are based on those obtained by Sebedio and Ackman (1982) with the same column.

Analysis of the Amount of 20:4 Enzymatically Generated in Product-Containing Vesicles. Flame-ionization detection GC analysis cannot measure the amount of 20:4 that is enzymatically hydrolyzed from SAPC of substrate vesicles that initially contain 10 mol % 20:4. Two methods were used to measure the hydrolysis of SAPC. First, the amount of 20:4 hydrolyzed from SAPC was determined radiometrically. Substrate vesicles contained ^{3}H-SAPC (200 Ci/mmol) and the amount of the total label that was enzymatically converted to ^{3}H-20:4 was determined as described above; this amount represents the fraction of total SAPC that is hydrolyzed by the enzyme. The total mass of SAPC in the substrate vesicles was determined from the total mass of $SX_{20}PC$ in the reaction mixture (phosphorus analysis) and the mass fraction that was SAPC (flame-ionization detection GC).

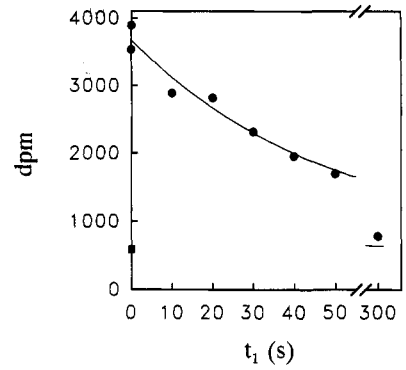

FIGURE 1: Binding of rat kidney hmw-PLA_2 to phospholipid vesicles. Binding of enzyme to unlabeled vesicles was initiated by the 9-fold dilution of a vesicle stock solution into 0.05 mL of buffer A at room temperature containing 3 microunits of enzyme. The final concentrations of the components of the vesicles were 6.3 μM OPPC, 0.33 μM S-lyso-PC, 0.33 μM 20:4, and 0.011 μM unlabeled SAPC. After incubation for the times t_1 given at the bottom of the figure, a stock solution of labeled vesicles was diluted 10-fold into the reaction mixture to give final concentrations of components in the labeled vesicles of 6 μM OPPC, 0.3 μM S-lyso-PC, 0.3 μM 20:4, and 0.01 μM ^{3}H-SAPC (200 Ci/mmol). The reactions were quenched after t_2 = 20 min and the amount of ^{3}H-20:4 was measured as described under Materials and Methods (circles). Maximum hydrolysis of labeled vesicles was measured by the simultaneous addition of unlabeled and labeled vesicles to the enzyme solution and the reactions were quenched after t_2 = 20 min and analyzed as above (circles, t_1 = 0 s). Background hydrolysis was measured with reaction mixtures that did not contain enzyme (square) and were quenched after t_2 = 20 min. The solid line is drawn for a first-order rate constant k_{obs} = 0.02 s^{-1}.

The extent of oxidation of 20:4 at the *sn*-2 position of commercially prepared ^{3}H-SAPC was measured by subjecting a known amount to methanolysis by heating the sample in 0.33 mL of 5% HCl in CH_3OH and 0.66 mL of hexane at 95 °C under argon for 2 h. The reaction mixture also contained a known amount of [1-^{14}C]-*cis*-4,7,10,13,16,19-22:6 and 0.05 mg of unlabeled 20:4. The mixture was cooled, and the HCl was neutralized by the addition of 1 mL of 5% $NaHCO_3$. The hexane layer was removed and applied to a silica G TLC plate, which was then developed with hexane/diethyl ether (85:15 by volume). The methyl ester derivative of 20:4 was visualized with I_2 vapor and the regions of the plate which contained either the nonoxidized ($R_f \sim 0.5$) or oxidized methyl esters ($R_f < 0.45$) were scraped off, eluted from the silica with CH_3OH, and quantified by scintillation counting. The [1-^{14}C]-*cis*-4,7,10,13,16,19-22:6 present in the reaction served as a control to measure any oxidation that occurred during the methanolysis reaction. The results of this analysis are that ~26% of the total ^{3}H-20:4 in the sample was oxidized and ~12% oxidation of the ^{14}C-22:6 occurred during the procedure. The fraction of oxidized material was subtracted from the total radioactivity in the enzymatic assays to give the total ^{3}H radiolabel that was nonoxidized ^{3}H-20:4 at the *sn*-2 position of ^{3}H-SAPC.

Combined GC/mass spectrometry was used as a second method to measure the amounts of nondeuterated 20:4 enzymatically produced from substrate vesicles that initially contained 10 mol % ^{2}H-20:4. Chromatographic separations of 20:4 were made with a J & W DB1 capillary column (30 m × 0.25 mm). The initial settings of the instrument were injector 300 °C, transfer line 290 °C, reentrant 290 °C, and column oven 100 °C. Splitless injections of ≤2 μL were made and the temperature was immediately increased to 310 °C at

a rate of 20 °C/min. The amount of 20:4 produced by the enzyme was measured by comparing the intensity of the parent ion signal for 20:4 methyl ester to the intensity of the parent ion signal for the internal standard 17:0 methyl ester (parent molecular ion mass = 284.27 amu). The ion intensities were calibrated by measuring the signal strengths of known amounts of 20:4 and 17:0 methyl esters.

RESULTS

Binding of hmw-PLA_2 to Product-Containing Vesicles. Previous studies have shown that the rat kidney hmw-PLA2 binds relatively weakly to phospholipid vesicles, which contain only phosphatidylcholine, and that tight binding of the enzyme to these vesicles is promoted by the addition of small amounts of enzymatic reaction products (typically 10 mol % of a 1:1 mixture of S-lyso-PC and 20:4) (Ghomashchi et al., 1992). The experiments established that the residency time of the enzyme on the product-containing vesicles is longer than it is on vesicles that do not contain products, but they did not address whether the enzyme is reversibly or irreversibly bound to the vesicles with products over the time course of the reaction.

To determine whether binding of the enzyme to product-containing vesicles is reversible, the following experiment was carried out. Binding reactions were initiated by mixing the rat kidney hmw-PLA_2 with unlabeled vesicles (OPPC/10% products/SAPC), and the reactions proceeded for increasing times t_1, which are given at the bottom of Figure 1. A second addition of similar vesicles, which contained ^{3}H-SAPC, was made at the end of t_1, and the reactions were quenched 20 min later. The assay is shown in eq 1. The amount of hydrolysis of ^{3}H-SAPC is a measure of the amount of enzyme present in the reaction after t_1 that can bind to labeled vesicles and hydrolyze ^{3}H-SAPC.

$$\begin{array}{l} \text{vesicle} + \text{hmw-PLA}_2 \xrightarrow[t_1]{k_{on}} \text{hmw-PLA}_2\text{•vesicle} \\ t_2 = 20\ \text{min} \downarrow \quad \text{vesicle}^* \\ \text{hmw-PLA}_2\text{•vesicle}^* + 20{:}4^* \end{array} \qquad (1)$$

Figure 1 shows that increasing the time of exposure of the enzyme to unlabeled vesicles causes a decrease in the amount of labeled 20:4 released during t_2. The reaction is ≥95% complete after t_1 = 5 min and follows first-order kinetics with a rate constant $k_{obs} = 0.02\ s^{-1}$. The decrease can be explained by the binding of enzyme to unlabeled product-containing vesicles during t_1; enzyme bound to unlabeled vesicles does not dissociate significantly during t_2. The maximum amount of hydrolysis of labeled vesicles was measured by simultaneously adding labeled and unlabeled vesicles to buffer containing enzyme (Figure 1, data for t_1 = 0). The small difference between the observed amount of ^{3}H-20:4 at t_1 = 5 min and the amount predicted by $k_{obs} = 0.02\ s^{-1}$ (solid line) is not significant within the error of the measurements (approximately 10%). An upper limit of $3 \times 10^{-4}\ s^{-1}$ is given for the rate constant k_{off}, which is the first-order rate constant that describes the dissociation of enzyme from the unlabeled vesicles.

The important conclusion from the data of Figure 1 is that catalytic turnover by the enzyme occurs without the enzyme dissociating from the interface. Such behavior, termed *scooting mode catalysis*, is observed for the action of numerous 14-kDa phospholipases A_2 acting on vesicles of anionic phospholipids such as 1,2-dimyristoylphosphatidylmethanol and OPPC vesicles containing either 5–10 mol % phosphatidic acid or phosphatidylmethanol (Ghomashchi et al., 1991; Jain et al., 1991).

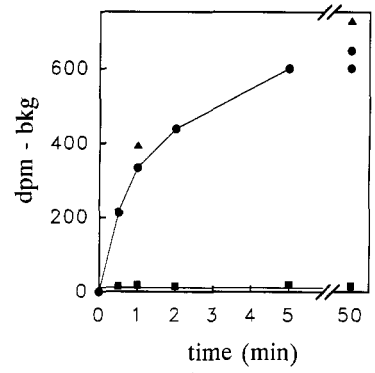

FIGURE 2: Hydrolysis of ^{3}H-SAG (squares) and ^{14}C-SAPC (circles, triangles) by the human hmw-PLA_2. Reaction mixtures contained 9 microunits of human hmw-PLA_2 in buffer A at a final volume of 0.15 mL at room temperature. The reactions were initiated by the 10-fold dilution of substrate vesicles to give final concentrations of either 4.2 μM OPPC, 0.6 μM S-lyso-PC, 0.6 μM 20:4, 0.6 μM ^{3}H-SAG (100 mCi/mmol), and 0.6 μM ^{14}C-SAPC (54 mCi/mmol) (circles and squares) or 4.8 μM OPPC, 0.6 μM S-lyso-PC, 0.6 μM 20:4, and 0.6 μM ^{14}C-SAPC (54 mCi/mmol) (triangles). The reactions were quenched at the times given in the figure and the amounts of ^{3}H-20:4 and ^{14}C-20:4 were measured as described under Materials and Methods. Blanks for the reactions did not contain enzyme and were quenched at t = 50 min. The values given at t = 50 min for hydrolysis of vesicles, which contained ^{3}H-SAG (circles) and ^{14}C-SAPC (squares), represent 0% and 8% hydrolysis, respectively, of the total ^{3}H-SAG and ^{14}C-SAPC in the vesicles. The value given at t = 50 min for vesicles which only contain ^{14}C-SAPC as the labeled substrate (triangles) represents 9% of the total ^{14}C-SAPC present in the vesicles.

Importance of Scooting Mode Analysis for the Evaluation of the Substrate Specificity of hmw-PLA_2. The evaluation of substrate preferences of PLA_2 enzymes is more difficult than with enzymes that act in a homogeneous solution-phase environment. The analysis is greatly simplified when the enzyme is studied in the scooting mode (Gelb et al., 1992; Ghomashchi et al., 1991). Having all of the enzyme bound to the interface of the substrate vesicle ensures that differences in observed reaction velocities are not due to differences in the fraction of total enzyme bound to interface. Furthermore, scooting ensures that the composition of each enzyme-containing vesicle changes uniformly with time; the observed formation of products is the sum of the products formed from each of the enzyme-containing vesicles. In the scooting mode, the relative rates of steady-state substrate hydrolysis are determined by the relative k_{cat}/K_M^* values for the different substrates in the interface. The asterisk denotes that the Michaelis constant is for the interaction at the interface between enzyme and phospholipid (Berg et al., 1991).

Scooting Mode Analysis of Substrate Specificity: Effect of the Polar Head Groups. Substrate specificity measurements were carried out with OPPC vesicles that contained 10 mol % products (1:1 mixture of S-lyso-PC and 20:4) and small amounts of two competing substrates, which had either ^{3}H or ^{14}C in their *sn*-2 arachidonyl chains. This dual radioisotope assay allows the measurement of the hydrolysis of two competing substrates that are present in a single population of vesicles. In the steady-state, the relative initial rates (v_i) of hydrolysis of the ^{3}H and ^{14}C radiolabeled substrates are determined by the ratio of the values of k_{cat}/K_M^* for the substrates and the ratio of the initial substrate concentrations:

Table I: Substrate Specificity Studies: Phospholipids with Different Polar Head Groups and a Plasmenylcholine Phospholipid[a]

	$(k_{cat}/K_M^*)_{SAPX}/(k_{cat}/K_M^*)_{SAPC}$				
	0.5 min	1 min	2 min	3 min	average
^{3}H-SAPC ^{14}C-SAPC	0.84[b]	0.85	0.85	nd[c]	0.85
^{3}H-SAPC ^{14}C-SAPC	1.02	0.78	0.79	nd	0.86
^{14}C-SAPI[d] ^{3}H-SAPC	nd	1.02	1.13	0.99	1.05
^{3}H-PlasAPC ^{14}C-SAPC	0.75	0.66	0.81	0.84	0.77
^{3}H-SAPE ^{14}C-SAPC	0.46	0.53	0.75	nd	0.58
^{3}H-SAPA ^{14}C-SAPC	0.30	0.21	0.38	nd	0.30
^{3}H-SAPS ^{14}C-SAPC	0.13	0.47	0.23	nd	0.28

[a] Reaction mixtures contained 9 microunits of purified rat kidney hmw-PLA_2 in a final volume of 0.15 mL of buffer A at room temperature. Reactions were initiated by the 10-fold dilution of vesicle stock solutions to give final concentrations of 4.8 μM OPPC, 0.6 μM S-lyso-PC, 0.6 μM 20:4, 0.6 μM ^{14}C-SAPC (54 mCi/mmol), and either 0.4 μM ^{3}H-PlasAPC, 0.3 μM ^{3}H-SAPC, 0.1 μM ^{3}H-SAPE, 0.1 μM ^{3}H-SAPA, or 0.2 μM ^{3}H-SAPS (400 mCi/mmol for each ^{3}H-phospholipid) except as noted below. The reactions were quenched at the times given at the top of the table and the radiolabeled products were isolated and measured as described under Materials and Methods. Reaction blanks did not contain enzyme and were quenched at t = 20 min. The first two lines of the table represent duplicate determinations. [b] The ratios of k_{cat}/K_m^* for the pairs of substrates given in the table were calculated using eq 2, which is given in the Results section. The cpm values that were measured at t = 0.5 and 1 min for the enzyme-catalyzed reactions were typically 1.5–4-fold above the background levels measured in the absence of enzyme. [c] Not determined. [d] Reactions were initiated by the 10-fold dilution of phospholipid vesicles to give final concentrations of 4.8 μM OPPC, 0.6 μM S-lyso-PC, 0.6 μM 20:4, 0.6 μM ^{14}C-SAPI (50 mCi/mmol), and 0.3 μM ^{3}H-SAPC (400 mCi/mmol) in the reaction mixture.

$$\frac{(k_{cat}/K_M^*)^{^3H}}{(k_{cat}/K_M^*)^{^{14}C}} = \frac{v_i^{^3H}[S^{^{14}C}]_{t=0}}{v_i^{^{14}C}[S^{^3H}]_{t=0}} \quad (2)$$

The data in Figure 2 show that the human hmw-PLA_2 does not hydrolyze the diglyceride ^{3}H-SAG. The OPPC/product-containing vesicles also contained 10 mol % ^{3}H-SAG and 10 mol % ^{14}C-SAPC. The reaction stops after 8–9% of the total ^{14}C-SAPC is hydrolyzed, and there is no detectable production of ^{3}H-20:4 from ^{3}H-SAG. Reactions with similar vesicles that do not contain ^{3}H-SAG give similar amounts of hydrolysis of ^{14}C-SAPC at times t = 1 and 50 min (Figure 2). These results show that SAG is not a substrate for the enzyme and that the presence of 10 mol % ^{3}H-SAG does not activate the enzyme to hydrolyze ^{14}C-SAPC.

Competitive substrate studies were carried out with diacylphospholipids that differ in the structure of their polar head groups and with a 1-alkenyl-2-acylplasmenylcholine. The results of these studies with the rat kidney hmw-PLA_2 are summarized in Table I. The data are reported as the ratio of the k_{cat}/K_M^* values for the hydrolysis of either ^{3}H-PlasAPC or 1-stearoyl-2-[^{14}C or ^{3}H]arachidonylphospholipids with different polar head groups and the value of k_{cat}/K_M^* for the hydrolysis of either ^{3}H- or ^{14}C-SAPC. The ratios of v_i were estimated from the amounts of ^{3}H- and ^{14}C-labeled products formed after reaction times of 0.5, 1, 2, and 3 min. The ratio of initial substrate concentrations was measured by dual-channel scintillation counting of vesicles in the absence of enzyme. The first two entries of the table are controls in which two isotopic forms of the same substrate (^{3}H- and ^{14}C-SAPC) were used, and the ratios of k_{cat}/K_M^* values are close to 1 as expected. The data show that the enzyme does not discriminate between SAPC, PlasAPC, and SAPI and that SAPC is 2-fold preferred over SAPE. The largest differences that are observed are between SAPC and the anionic phospholipids SAPA and SAPS. The discrimination is only 3-fold in favor of SAPC, which is a zwitterionic phospholipid.

Table II: Substrate Specificity Studies with $SX_{nat}PC$[a]

X_{nat}	$(k_{cat}/K_M^*)_{SX_{nat}PC}/(k_{cat}/K_M^*)_{SAPC}$
cis-9-16:1	<0.04[b]
cis-9-18:1	0.04
cis-9,12-18:2	0.1
cis-9,12,15-18:3	0.7

[a] Reaction mixtures contained 27 μM $SX_{nat}PC$, 10^6 dpm of ^{3}H-SAPC (200 Ci/mmol), 3 μM S-lyso-PC, and 3 μM ^{2}H-20:4 in a total volume of 10 mL of buffer A at room temperature. The hydrolysis reactions were initiated by the addition of 300 microunits of human hmw-PLA_2 and quenched after 1 min. The internal standard 17:0 (2.5 μg) was added, and the fatty acid reaction products were isolated and prepared as their corresponding methyl esters as described under Materials and Methods. Background levels of fatty acids at t = 50 min were measured in reaction mixtures that did not contain enzyme. Levels of nondeuterated arachidonic acid methyl ester from the reactions were measured by GC/mass spectrometry, and all other fatty acid methyl esters were measured by flame-ionization detection GC. The percent hydrolysis of SAPC (11 mol % of total $SX_{nat}PC$) at time t = 1 min was 4.2%, as determined by GC/mass spectrometry, and this value was used to calculate the values given in the table. Radiometric measurements of the percent hydrolysis of SAPC at t = 1 min gave a value of 7.6%. [b] Measurement of 16:1 by flame-ionization detection GC analysis was not possible because of high background levels in the sample where 16:1 eluted. There was no detectable 16:1 hydrolysis product present in the sample as determined by GC/mass spectrometry. The value is given as an upper limit and is calculated from the smallest amount of 16:1 that could reliably be detected by GC/mass spectrometry.

The results summarized in Figure 2 and Table I demonstrate an important requirement of the hmw-PLA_2 for substrate hydrolysis: the phosphate group of phospholipids is essential for the enzyme-catalyzed lipolysis reaction. This fact follows from the observations that SAG is not hydrolyzed by the enzyme and that SAPA, which is similar to SAG except that a phosphate group is present at carbon 3, is a substrate of the enzyme. The enzyme does discriminate between phospholipids with different polar head groups, although the level of discrimination is not large.

Scooting Mode Analysis of Substrate Specificity: Effect of the sn-2 Acyl Chain. A mixture of phosphatidylcholines, which contain different fatty acyl chains instead of 20:4 at the *sn*-2 position, was prepared in a one-pot synthetic reaction. S-lyso-PC was coupled to a mixture of fatty acids that are present in mammalian phospholipids (Table II). This mixed phospholipid material ($SX_{nat}PC$) was incorporated into vesicles that contained 10 mol % reaction products (1:1 mixture of S-lyso-PC and 20:4) and a small amount of ^{3}H-SAPC (10^6 dpm, 200 Ci/mmol). Exhaustive methanolysis of $SX_{nat}PC$ vesicles followed by high-resolution capillary GC analysis of the mixture of fatty acid methyl esters gave the relative amounts of the different *sn*-2 acyl chains that made up the phospholipid mixture. The vesicles were incubated with human hmw-PLA_2, and the relative amounts of fatty acids hydrolyzed at an early time point in the progress of the reaction (1 min) were determined by GC analysis of the corresponding methyl esters. This information was used to calculate the values of k_{cat}/K_M^* for the competing substrates relative to the value of k_{cat}/K_M^* for SAPC using eq 2. The presence of ^{3}H-SAPC in vesicles permitted the estimation of the amount of enzyme-generated 20:4 in the presence of 10 mol % unlabeled 20:4 in the vesicles. An independent estimate of the hydrolysis of SAPC was made by incorporating 10 mol % ^{2}H-20:4 instead of 20:4 into the vesicles and using combined GC/mass

Table III: Substrate Specificity Studies with $SX_{20}PC$[a]

Figure 3 elution order[b]	X_{20} fatty acid	$(k_{cat}/K_M^*)_{SX_{20}PC}/(k_{cat}/K_M^*)_{SAPC}$ 1 min, hmw-PLA_2	10 min, nps-PLA_2
1	20:0	0.07	1.25
2	*cis*-5-20:1	0.17	1.53
3	*cis*-8-20:1	0.10	1.21
4	*cis*-11-20:1	0.12 (0.04)[c]	1.53
6	*cis*-14-20:1	0.13[d]	1.42
5	*cis*-5,8-20:2	0.21	1.67
6	*cis*-5,11-20:2	0.20[d]	1.81
7	*cis*-5,14-20:2	0.25	1.76
8	*cis*-8,11-20:2	0.15	1.20
9	*cis*-8,14-20:2	0.25	1.84
11	*cis*-11,14-20:2	0.14 (0.10)	2.48
10	*cis*-5,8,11-20:3	0.63	2.96
12	*cis*-5,8,14-20:3	0.48	1.72
13	*cis*-5,11,14-20:3	0.31	1.90
14	*cis*-8,11,14-20:3	0.22 (0.20)	1.49
15	20:4	1	1

[a] The reactions were carried out as described in the legend of Figure 3 and were quenched at the times given in the table. [b] The order of elution is given for the GC profiles shown in Figure 3, and the numbers given in the table increase with increasing retention times of the products. [c] The values given in parentheses were determined from experiments with $SX_{nat}PC$, which are described in the legend of Table II. They are given here for comparative purposes. [d] The products *cis*-14-20:1 and *cis*-5,11-20:2 were not resolved by GC. The amounts released by the enzyme were estimated by determining the average amounts of the resolved 20:1 and 20:2 products formed in the reactions and assigning these average values for the production of *cis*-14-20:1 and *cis*-5,11-20:2, respectively. The sum of the two calculated average values was in good agreement with the measured amount of the two unresolved products. The mol % of *cis*-14-20:1 and *cis*-5,11-20:2 in the $SX_{20}PC$ mixture was estimated by determining the average mol % for the resolved 20:1 and 20:2 species in the substrate mixture and assigning the average values for the mol % of *cis*-14-20:1 and *cis*-5,11-20:2, respectively. The sum of the calculated average values was in good agreement with the measured mol % of the two unresolved fatty acids.

spectrometry to analyze the amount of nondeuterated 20:4 produced by the enzyme. Both methods give similar results (Table II).

The 16:1 *sn*-2 acyl group was also present in the phospholipid mixture, but it could not be measured as a hydrolysis product by flame-ionization GC analysis because of large background values in the region of the chromatogram where the methyl ester of 16:1 elutes. However, the parent ion of the methyl ester of 16:1 was not detected by GC/mass spectrometry at reaction times of 1 and 50 min.

The results of the studies with $SX_{nat}PC$ are summarized in Table II. The relative values of k_{cat}/K_M^* are listed as the ratio of the value of k_{cat}/K_M^* for a particular *sn*-2 fatty acyl species to the value of k_{cat}/K_M^* for SAPC. The data establish a rank order of substrate preference by the enzyme of 20:4 > 18:3 > 18:2 > 18:1 ≥16:1.

The phospholipid mixture $SX_{nat}PC$ also contained the 20-carbon fatty acids *cis*-11-20:1, *cis*-11,14-20:2, and *cis*-8,11,14-20:3 esterified at the *sn*-2 position. These acyl chains were included in the mixture to determine their specificities as substrates relative to 20:4 when present in vesicles, which also contained phospholipids that had 16- and 18-carbon *sn*-2 acyl chains. The relative values of k_{cat}/K_M^* for these 20-carbon *sn*-2 acyl chains are similar to the values given in Table III for phospholipid substrates that contain only 20-carbon *sn*-2 acyl chains; the values obtained with $SX_{nat}PC$ are given in Table III (see below) for comparative purposes. These results demonstrate that the presence of *sn*-2 acyl chains of different lengths in the vesicles does not affect the substrate specificity of the enzyme.

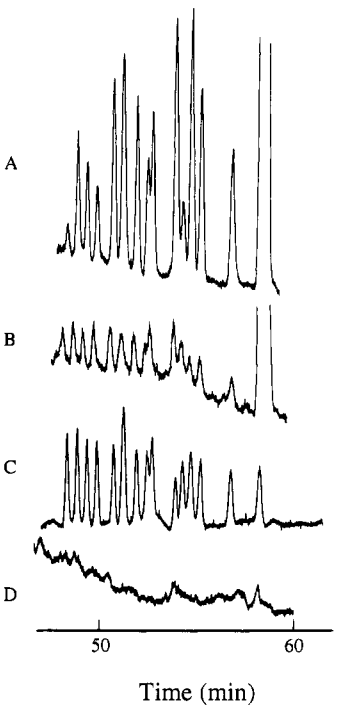

FIGURE 3: GC analysis of the methyl ester derivatives of hydrolysis products isolated from reactions of human hmw-PLA_2 and human nps-PLA_2 enzymes with $SX_{20}PC$. Trace A is for the human hmw-PLA_2 after a reaction time of 1 min. Trace B is for the human nps-PLA_2 after a reaction time of 10 min. Trace C is for the exhaustive methanolysis of $SX_{20}PC$ and gives the relative amounts of the individual hydrogenation products of 20:4 that are esterified at the *sn*-2 position of $SX_{nat}PC$. Trace D shows the amounts of the fatty acids isolated from a reaction mixture that did not contain enzyme. For traces C and D, mixtures of $SX_{20}PC$ in buffer were incubated for 75 min and then extracted. Reaction mixtures contained either 27 μM $SX_{20}PC$, 10^6 dpm of ^{3}H-SAPC (200 Ci/mmol), 3 μM S-lyso-PC, 3 μM 20:4, and 300 microunits of purified human PLA_2 (trace A), 27 μM $SX_{20}PC$, 10^6 dpm of ^{3}H-SAPC (200 Ci/mmol), 3 μM S-lyso-PC, 3 μM 20:4, and 0.4 μg of human nps-PLA_2 (trace B), or 30 μM $SX_{20}PC$ only (traces C and D) in 10 mL of buffer A at room temperature. The $SX_{20}PC$ contains 5.2 mol % SAPC. The reactions were quenched and the internal standard 17:0 was added to each sample. The total phospholipids and fatty acids were extracted, and the fatty acids were isolated and converted to the corresponding methyl esters as described under Materials and Methods. The relative amounts of internal standard that were measured for panels A, B, C, and D are 18, 36, 1, and 11, respectively, and are given for comparative purposes.

The specificities of the human hmw-PLA_2 and the human nps-PLA_2 for phosphatidylcholines, which contain 20:4 and all of its partially and fully hydrogenated forms, were investigated. Reduction of 20:4 with hydrazine (see Materials and Methods) leads to a mixture of all possible 20:3, 20:2, and 20:1 species along with 20:0. This mixture of 20-carbon fatty acids was coupled to S-lyso-PC in a one-pot synthetic reaction. Vesicles made from this mixture ($SX_{20}PC$) and 10 mol % reaction products were submitted to the same analysis that is described above for $SX_{nat}PC$.

Panel A of Figure 3 shows a typical chromatogram of the methyl ester derivatives of the products isolated from hmw-PLA_2-catalyzed hydrolysis reaction mixtures after 1 min. Panel B gives the data obtained with the human nps-PLA_2 after a reaction time of 10 min. The GC procedure that was used separates 20-carbon fatty acid methyl esters on the basis of polarity; esters of increasing polarity are retained longer by the column. Twelve of the 14 possible 20-carbon partial hydrogenation products, as well as 20:0 and 20:4, can be resolved to give a total of 15 peaks for the analysis. The amount of 20:4 produced from product-containing vesicles

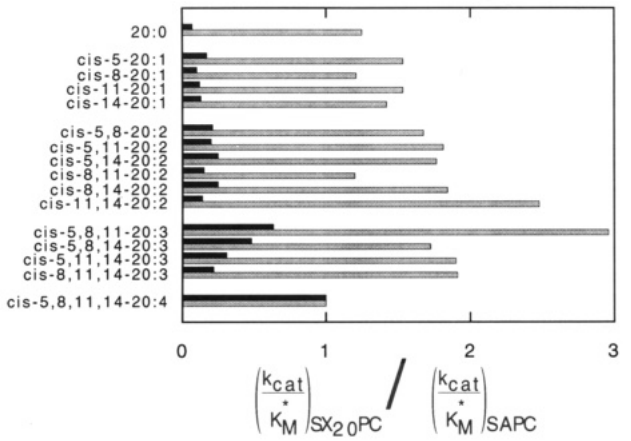

FIGURE 4: Hydrolysis of $SX_{20}PC$ by the human hmw-PLA_2 (dark bars) and the human nps-PLA_2 (light bars). The reaction conditions are described in the legend of Figure 3. The reaction with the human hmw-PLA2 was quenched at 1 min, and 5% of the total SAPC was hydrolyzed. The reaction with the human nps-PLA2 was quenched at 10 min, and 0.5% of the total SAPC was hydrolyzed.

was measured radiometrically as described in Materials and Methods. The relative elution order of the individual species of the monoene, diene, and triene classes is given by Sebedio and Ackman (1982), and the relative elution order of species of different classes were determined by GC/mass spectrometry using the parent ion molecular masses to differentiate the species of the different classes. The order of elution with increasing time of the individual hydrogenation products is given in Table III. The specificity is reported as the ratio of the k_{cat}/K_M^* value for each species present in $SX_{20}PC$ to the k_{cat}/K_M^* value of SAPC (Table III and Figure 4).

DISCUSSION

The analysis of PLA_2-catalyzed reactions is greatly simplified when the enzyme is operating in the processive scooting mode. The results of this study establish that the rat and human hmw-PLA_2 enzymes dissociate slowly from phosphatidylcholine vesicles that contain 10 mol % of a 1:1 mixture of S-lyso-PC and 20:4 ($k_{off} \leq 3 \times 10^{-4}$ s^{-1}, Figure 1). Furthermore, the enzyme is capable of undergoing processive catalytic turnover while remaining bound to the interface. The hydrolysis of the outer layer of the substrate vesicles is not complete before the reaction stops (Figure 2; Ghomashchi et al., 1992; Leslie, 1991; Diez et al., 1992). The reason why the reaction stops prematurely is not understood. However, the enzyme does not become irreversibly inactivated during turnover (Ghomashchi et al., 1992). In contrast, the 14-kDa PLA_2 enzymes are capable of hydrolyzing the entire outer layer of a substrate vesicle (Berg et al., 1991).

Analysis of the hmw-PLA_2 in the scooting mode allows the kinetics of interfacial catalysis to be analyzed in terms of classical steady-state rate equations, although the rate and equilibrium parameters are based on the concentrations of substrate(s) and enzyme at the surface of the vesicle rather than in bulk solution. Substrate specificities for the hmw-PLA_2 enzymes were analyzed in terms of eq 2, which is given in the Results section.

The results of this study show that the hmw-PLA_2 does not significantly discriminate between phospholipids that differ in the structure of their polar head groups (Table I). There is no discrimination between SAPC and SAPI by the enzyme. This result is similar to the findings reported for the action of the hmw-PLA_2 enzyme from human U937 cells on vesicles of 1,2-dimyristoylphosphatidylmethanol, which contain small amounts of *sn*-2 arachidonyl substrates (Diez et al., 1992), and for the action of the hmw-PLA_2 enzyme from the J774 mouse macrophage cell line on mixed SAPC/SAPI vesicles (Wijkander & Sundler, 1991). It is noted, however, that an analysis of substrate specificities under conditions of nonprocessive catalysis would give the same result if the ratio of k_{cat}/K_M^* for competing substrates is close to unity. The results of this study also show that SAPC is approximately 2-fold preferred over SAPE and approximately 3-fold preferred over SAPS and SAPA (Table I).

The lack of detectable hydrolysis of the diglyceride SAG (Figure 2) is important when interpreting proposed models for the biosynthesis of eicosanoids that describe the release of 20:4 from membranes either by the direct action of a phospholipase A_2 enzyme(s) on membrane phospholipids or by the sequential action of a phospholipase C followed by a diglyceride lipase. (Chilton, 1989; Irvine, 1982). Diacylglycerols have been reported to activate the human platelet hmw-PLA_2 in assays with phosphatidylcholine vesicles (Kramer et al., 1987). Figure 2 shows that no increase in activity is detected for the hydrolysis of SAPC when the enzyme is tightly bound to the interface of substrate vesicles which contain 10 mol % SAG. This result suggests that diacylglycerols at the interface do not activate the enzyme at the interface by a direct protein–lipid interaction. Previous studies have shown that the presence of diacylglycerol in substrate vesicles increases the extent of the hydrolytic reaction and that this increase is apparently due to a change in the physical properties of the vesicles (Ghomashchi et al., 1992).

The hmw-PLA_2 does not discriminate between SAPC and the plasmenylcholine PlasAPC (Table I). Plasmenylcholines are abundant arachidonic acid-containing phospholipids of the sarcolemma of heart muscle, and a calcium-independent phospholipase A_2 that preferentially hydrolyzes arachidonic acid of the *sn*-2 position of plasmenylcholine molecules has been identified (Gross, 1992). Selective release of arachidonic acid from myocardial membranes occurs during acute myocardial ischemia, and it is possible that the hmw-PLA_2 is responsible, in part, for the mobilization of arachidonic acid during this disease state.

The approach used in this study to define the structural requirements of the *sn*-2 acyl chain for recognition by the hmw-PLA2 was to prepare two mixtures of phospholipids with different *sn*-2 acyl chains ($SX_{nat}PC$ and $SX_{20}PC$) using a simple one-step synthetic method. Vesicles formed from these mixtures were incubated with PLA_2 enzymes, and the relative amounts of the different fatty acid hydrolysis products were measured by GC. Tables II and III and Figure 4 illustrate a trend in the substrate preferences of the enzyme. The rate of hydrolysis of 18- and 20-carbon *sn*-2 fatty acyl chains increases when the number of double bonds of the acyl chain increases. This result is consistent with previous findings that show that oleoyl (18:1) and linoleoyl (18:2) chains are 5–10-fold less preferred than the arachidonyl chain (Clark et al., 1990; Diez et al., 1992; Leslie et al., 1988; Takayama et al., 1991; Wijkander & Sundler, 1991) and that the saturated palmitoyl chain is >10-fold less preferred than 20:4 (Clark et al., 1990; Diez et al., 1992; Leslie et al., 1988).

A clear statement regarding the effect of acyl chain length on substrate preferences cannot be made from the data of Table II by comparing the preferences of the enzyme for 16:1 and 18:1. This is because a signal for the 16:1 hydrolysis product could not be detected by flame-ionization detection GC over the background noise of the sample. The limit that is given in Table II is based on the amount of 16:1 in the

sample that could be reliably measured by GC/mass spectrometry. GC/mass spectrometry measurements showed no evidence for hydrolysis of 16:1 at reaction times of 1 and 50 min. This observation could indicate that the specificity decreases with decreasing acyl chain length.

The structural features of 20:4 that are important for recognition by the hmw-PLA_2 have not been previously determined. Experiments of this study address the effect of both the number and the location of double bonds of 20-carbon *sn*-2 acyl chains of phosphatidylcholine on the relative rates of hydrolysis of the acyl chains by the hmw-PLA_2. The data in Table III, which are shown graphically in Figure 4, show that 20:4 is preferred by the enzyme over all of the partially and fully hydrogenated analogues of 20:4. In all cases, except when comparing *cis*-11,14-20:2 with *cis*-8,11,14-20:3, it was found that the rate of hydrolysis increases with an increase in the number of double bonds of the 20-carbon *sn*-2 acyl chain. The following additional observations are noted. Within the class of 20:3 fatty acyl chains, there is greater hydrolysis of the positional isomers that have the double bonds closer to the enzyme-susceptible ester bond. The double bond at the 5-position is apparently more important for recognition of the substrate by the enzyme than is the double bond at the 8-position. The same trend for the positions of the double bonds is observed for the 20:2 chains but the results are not as dramatic as those observed for the 20:3 class. The position of the single double bond of the 20:1 class of acyl chains does not significantly affect the rates of hydrolysis of these substrates.

In contrast, the human nps-PLA_2 does not discriminate significantly between the different classes of 20-carbon *sn*-2 acyl chains (Figure 4); the substrates are hydrolyzed equally well. The human nps-PLA_2 has previously been shown not to discriminate significantly between phosphatidylcholines that contain *sn*-2-palmitoyl, -oleoyl, or -arachidonyl acyl chains (Diez et al., 1992). The results of Figure 4 are important because they demonstrate that the discrimination that is observed for the human hmw-PLA_2 is caused by the enzyme and not by the composition of the vesicles. Similar discrimination by the two enzymes would be expected if the composition of the vesicles alone determined the rates of the hydrolysis reactions. The results obtained with the human nps-PLA_2 indicate that the substrate molecules are symmetrically distributed between the inner and outer layers of the vesicles; an asymmetric distribution would result in different concentrations of the substrates in the outer layer, which in turn would affect the measured rates of hydrolysis. The results obtained with the human nps-PLA_2 and for the hydrolysis of 20-carbon *sn*-2 acyl chains of $SX_{nat}PC$ by the human hmw-PLA_2 (Table III) also suggest that ground-state free energies of the substrates are equivalent. The values of k_{cat}/K_M^* that were determined for the $SX_{20}PC$ substrates with the human nps-PLA_2 are approximately equal. Therefore, any differences in ground-state free energies would have to be compensated by corresponding differences in the transition-state free energies for the reactions; this is unlikely. The similar values of k_{cat}/K_M^* for 20-carbon substrates of $SX_{nat}PC$ and $SX_{20}PC$ obtained with the human hmw-PLA_2 suggest that the presence of 16- and 18-carbon substrates in the vesicle does not affect the ground-state free energy of the 20-carbon substrates.

The kinetic parameter k_{cat}/K_M^* is a measure of the difference in free energy between the enzyme–substrate transition-state complex and the free enzyme and substrate in their respective ground states. Enzymes catalyze reactions, in part, by stabilizing the transition state of the reaction. The results of this study indicate that increasing the number of double bonds of the substrate increases the favorable interactions between the enzyme and substrate in the transition state. Furthermore, the closer the double bonds are to the ester bond that is cleaved by the enzyme, the more favorable the interactions are in the transition state between the enzyme and substrate. Two possible reasons for why the number and position of the double bonds are important for transition-state stabilization are as follows. The added rigidity of substrates which have 3–4 double bonds may help to position the substrate on the enzyme when going to the transition state. There would be a greater loss of entropy associated with the more saturated substrates going to the transition state. This would result in a corresponding increase in the free energy for the reaction. Alternatively, increasing the number of double bonds of the substrate increases the polarity of the substrate, and this increased polarity may help to stabilize the transition-state complex. It is reasonable to assume that a combination of these two effects would contribute to the stabilization of the transition state of the reaction.

Rate and Equilibrium Constants for Binding of hmw-PLA2 to Vesicles. The first-order rate constant of 0.02 s^{-1} that describes the binding of the hmw-PLA_2 to the OPPC/10 mol % product-containing vesicles of Figure 1 is a pseudo-first-order rate constant. The second-order rate constant for the binding reaction, k_{on} (eq 1), is calculated from k_{obs} and the concentration of unlabeled vesicles in the assay. The concentration of vesicles in the experiment[2] was ~0.9 nM, and the second-order rate constant for the binding of the enzyme to product-containing vesicles is $\sim 2 \times 10^7$ M^{-1} s^{-1} (M is defined as the molarity of vesicles).

The equilibrium dissociation constant for the binding reaction $K_D \leq 1.5 \times 10^{-11}$ M is estimated from the ratio of $k_{off} \leq 3 \times 10^{-4}$ s^{-1} and $k_{on} = \sim 2 \times 10^7$ M^{-1} s^{-1}. High-affinity binding of the hmw-PLA_2 to product-containing vesicles is largely the result of slow dissociation of the enzyme from the vesicle since the binding step of the reaction occurs with a rate constant that is near the diffusion limit.[3] Affinities of 0.1 pM have been reported for the 14-kDa PLA_2 enzymes binding to vesicles of 1,2-dimyristoylphosphatidylmethanol (Berg et al., 1991).

ACKNOWLEDGMENTS

We thank Drs. Brian Kennedy, Ian Street, and Phillip Weech of Merck Frosst Canada for providing purified human hmw-PLA_2 and Dr. Richard Gross of Washington University for providing the lysoplasmenylcholine Plas-lyso-PC. We are

[2] The binding reactions described in Figure 1 were carried out with a total concentration of phospholipid during t_1 of 6.6 μM. The concentration of vesicles is estimated from the concentration of phospholipids in the reaction mixture and the number of phospholipids that comprise a single vesicle. Although the vesicles used in this study have not been carefully sized, similar sonicated vesicles of OPPC, which contain 4–15 mol % dioleoylphosphatidic acid, contain ~4600 phospholipids in their outer monolayer, and this represents ~60% of the total phospholipid of the vesicle (~7700 total phospholipids per vesicle) (Ghomashchi et al., 1991).

[3] Values between 3×10^8 and 7×10^9 M^{-1} s^{-1} for k_{on} describe the diffusion-limited binding of the hmw-PLA_2 to the surface of a vesicle. The values are based on a model (Schmitz & Schurr, 1972) that accounts for radii of the enzyme and vesicle and the proper orientation of the enzyme relative to the surface of the vesicle. The rate constants are calculated using radii for the vesicle and enzyme of 1.3×10^{-6} and 3.2×10^{-7} cm (based on the MW of 85 000), respectively, and a range of angles of 10–50° is used for the proper orientation of the enzyme. The latter is the cone angle that defines the patch on the enzyme surface that must contact the bilayer for productive interfacial binding.

also grateful to Professor Mahendra Jain for helpful discussions.

SUPPLEMENTARY MATERIAL AVAILABLE

Description of the synthesis and purification of ^{3}H-SAPC, ^{3}H-PlasAPC, ^{3}H-SAPA, ^{3}H-SAPE, ^{3}H-SAPS, and ^{3}H-SAG (5 pages). Ordering information is given on any current masthead page.

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