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Human Plasma Platelet-activating Factor Acetylhydrolase

OXIDATIVELY FRAGMENTED PHOSPHOLIPIDS AS SUBSTRATES*

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Human plasma platelet-activating factor (PAF) acetylhydrolase hydrolyzes the *sn*-2 acetyl residue of PAF, but not phospholipids with long chain *sn*-2 residues. It is associated with low density lipoprotein (LDL) particles, and is the LDL-associated phospholipase A_2 activity that specifically degrades oxidatively damaged phospholipids (Stremler, K. E., Stafforini, D. M., Prescott, S. M., Zimmerman, G. A., and McIntyre, T. M. (1989) *J. Biol. Chem.* 264, 5331–5334). To identify potential substrates, we synthesized phosphatidylcholines with *sn*-2 residues from two to nine carbon atoms long, and found the *V/k* ratio decreased as the *sn*-2 residue was lengthened: the C_5 homolog was 50%, the C_6 20%, while the C_9 homolog was only 2% as efficient as PAF. However, the presence of an ω-oxo function radically affected hydrolysis: the half-life of the *sn*-2 9-aldehydic homolog was identical to that of PAF.

We oxidized [2-arachidonoyl]phosphatidylcholine and isolated a number of more polar phosphatidylcholines. We treated these with phospholipase C, derivatized the resulting diglycerides for gas chromatographic/mass spectroscopic analysis, and found a number of diglycerides where the *m/z* ratio was consistent with a series of short to medium length *sn*-2 residues. We treated the polar phosphatidylcholines with acetylhydrolase and derivatized the products for analysis by gas chromatography/mass spectroscopy. The liberated residues were more polar than straight chain standards and had *m/z* ratios from 129 to 296, consistent with short to medium chain residues. Therefore, oxidation fragments the *sn*-2 residue of phospholipids, and the acetylhydrolase specifically degrades such oxidatively fragmented phospholipids.

Platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, PAF[1]) is a potent phospholipid autacoid whose actions are mediated by receptors on the surface of target cells that specifically recognize PAF. At concentrations as low as 10^{-10} M it activates platelets, neutrophils, and other leukocytes, and it induces hypotension, increased vascular permeability, and shock in animals (reviewed in Refs. 1 and 2). The potency and nature of its effects suggest that the presence of PAF should be strictly controlled and, in fact, the enzymatic activities responsible for PAF biosynthesis are tightly regulated (1–3). Additionally, accumulation of PAF in some cells is controlled by its rate of degradation (4, 5). Degradation also plays a major role in the potential for PAF to circulate as a hormone or function as a locally acting autacoid due to the presence of plasma PAF acetylhydrolase. This enzyme inactivates PAF by hydrolyzing the *sn*-2 acetyl residue, and is exclusively responsible for the degradation of PAF in whole blood (6).

The majority (60–70%) of PAF acetylhydrolase in human plasma is associated with low density lipoprotein; the rest is associated with a subpopulation of high density lipoprotein that contains apolipoprotein E (HDL-with apoE) (6). The enzyme associated with LDL is immunologically equivalent to that in HDL and is transferred between the two lipoprotein particles in a pH-dependent fashion (6). There is a sharp deviation from Michaelis-Menten kinetics at low substrate concentrations for LDL-associated activity; hydrolysis of 1 nM PAF proceeds at only 1.7% of the predicted rate (7). HDL-associated activity is even less efficient than LDL-associated activity at low substrate concentrations, so that in whole plasma only LDL-associated activity appears to catalyze PAF hydrolysis (7). Lipoprotein-associated acetylhydrolase activity also rapidly degrades endothelial cell-associated PAF (8), indicating that stimulated endothelial cells express this PAF on their cell surface. It also shows that LDL can modulate

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[1] The abbreviations used are: PAF, platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine); GPC, *sn*-glycero-3-phosphocholine; HDL, high density lipoprotein; LDL, low density lipoprotein; *t*-BDMS, *tert*-butyldimethylsilyl; CV3988, *rac*-3-(*N*-*n*-octadecylcarbamoyloxy)-2-methoxypropyl-2-thiazolioethyl phosphate; Kadsurenone, 5-(prop-2-ene)-2(3,4-dimethoxyphenyl)-3a,α-methoxy-3-methyl-2,3,3a,6-tetrahydro-6-oxobenzofuran; L652,731, *trans*-2,5-bis(3,4,5-trimethoxyphenyl)tetrahydrofuran; WEB2086, 3-[4-(chlorophenyl)-9-methyl-11-thieno[3,2-*f*][1,2,4]triazolo-[4,3-*a*][1,4]diazepin-2-yl](4-morpholinyl)-1-propanone; L659,989, (±)-*trans*-2-(3-methoxy-5-methylsulfonyl-4-propoxyphenyl)-5-(3,4,5-trimethoxyphenyl)tetrahydrofuran; U66985, (1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphoric acid-6′-trimethylammoniumhexyl ester); WEB2170, 6-(2-chlorophenyl-8,9-dihydro-1-methyl-8-(4-morpholinylcarbonyl)-4H,7H-cyclopenta[4,5]thieno[3,2-*f*][1,2,4]triaozo[4,3-*a*][1,4]diazepine; SRI63441, *cis*-(±)-1-{2-[hydroxy({tetrahydro-5-[(octadecylaminocarbonoyl)oxy]methyl}furan-2-yl)methoxyphosphinyloxy]ethyl}quinolinium hydroxide (inner salt); HPLC, high performance liquid chromatography; PC, phosphatidylcholine; TLC, thin layer chromatography; GLC, gas liquid chromatography.

this surface expression and thereby alter cellular interactions, such as neutrophil adhesion and activation, that is mediated by endothelial cell-associated PAF (8).

We have purified and characterized acetylhydrolase from human plasma (9) and have identified and partially characterized a distinct activity from human erythrocytes (10). Examination of the substrates utilized by plasma acetylhydrolase (9, 11–13) shows that it functions as a short chain acyl hydrolase. It will use substrates with either an ether or ester bond at the *sn*-1 position, and it demonstrates little specificity for the *sn*-3 headgroup (11). It does not, however, effectively utilize *sn*-2 fatty acyl residues that are longer than six carbon atoms in length. We fragmented 1-palmitoyl-2-arachidonoyl-GPC by ozonolysis to generate 1-palmitoyl-2-(5-oxovaleroyl)-GPC (11) and found that it was approximately half as effective a substrate as PAF for the acetylhydrolase. This suggests that other oxidatively generated species might be substrates for the acetylhydrolase, and, indeed, we found that uncontrolled oxidation of a phospholipid containing a polyunsaturated *sn*-2 residue generates a myriad of unidentified compounds, some of which are substrates for the acetylhydrolase (11). We found that the more polar of these species were better substrates than the less polar species, suggesting that the more polar species contained either shorter *sn*-2 residues, more oxygenated residues, or a combination of these two modifications. The restricted substrate specificity of the acetylhydrolase is unusual, but guarantees that only fragmented phospholipids are attacked without affecting the bulk of unoxidized parent phospholipids. This substrate specificity allows acetylhydrolase to circulate in a fully active state, and Stremler *et al.* (11) and Steinbrecher and Pritchard (13) postulate that this makes it likely that an additional physiological function of this enzyme is to degrade phospholipids that have been oxidatively fragmented. This is consistent with the observation (14) that oxidative modification of LDL results in the hydrolysis of as much as 40% of its phosphatidylcholine by an LDL-associated phospholipase A_2 activity.

PAF acetylhydrolase and the PAF receptor recognize the same phospholipid, and share a requirement for a short *sn*-2 residue. The PAF receptor also interacts with a variety of compounds (15) that competitively block PAF binding to, and activation of, this receptor. This raises the possibility that receptor antagonists might also inhibit acetylhydrolase activity. This could be an important issue from a physiological perspective, since, if receptor antagonists prevented PAF metabolism in plasma, the concentration of PAF would rise and tend to counteract the competitive blockade of the receptor. It would also suggest that receptor antagonists might interfere with the metabolism of oxidatively fragmented phospholipids and possibly prolong the half-life of these reactive lipids.

The goal of the experiments presented here was to define the potential role of acetylhydrolase in the metabolism of oxidatively fragmented phospholipids by examining the substrate specificity of plasma PAF acetylhydrolase in detail. This included a definition of the *sn*-2 residues recognized by the acetylhydrolase, identification of phospholipids derived from the oxidative fragmentation of arachidonoyl-containing phosphatidylcholine, and characterization of the hydrolytic products obtained after acetylhydrolase treatment of these oxidatively fragmented phospholipids. We also determined the effect of PAF receptor antagonists on the activity of PAF acetylhydrolase to determine if they might interfere with substrate degradation.

EXPERIMENTAL PROCEDURES[2]

Materials—1-Palmitoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine was purchased from Du Pont-New England Nuclear and 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine from Amersham Corp. 1-Palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, and 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine were obtained from Avanti Polar Lipids (Birmingham, AL). 4-(*N*,*N*-Dimethylamino)-pyridine, propionic anhydride, and succinic anhydride were obtained from Aldrich. Butyric anhydride, valeric anhydride, and hexanoic anhydride were purchased from Eastman Kodak (Rochester, NY). Nonanoic anhydride and all short chain fatty acids (C_3 through C_7 (C_n represents a straight carbon chain n atoms long)) were obtained from Sigma. *N*-Methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide was purchased from Pierce Chemical Co. CV3988 was a gift from M. Nishikawa, Central Research Division, Takeda Chemical Industries Ltd. Kadsurenone, L652,731, and L659,989 were kindly provided by J. C. Chabala, Merck Sharp and Dohme Research Laboratories. WEB2086 and WEB2170 were the kind gifts of Peggy Ganong, Boehringer Ingelheim Pharmaceuticals, Inc. BN50726 was kindly provided by P. Braquet, Institut Henri Beaufour. D. J. Hanahan kindly provided U66985, a product of The Upjohn Company. SRI63441 was a gift from D. A. Handley, Sandoz Research Institute. The PAF acetylhydrolase was purified from human plasma as described (9).

Synthesis of Phospholipids—The majority of the *sn*-2 short chain phospholipids were synthesized by the coupling method of Gupta *et al.* (16) and purified using a modification (11) of the reversed-phase HPLC procedure described by Brash (17). The *sn*-2 aldehydic phosphatidylcholines were prepared by reductive ozonolysis (18). The general procedures utilized for reductive ozonolysis and the coupling are presented below; spectral characterization and experimental details for each compound may be found in the supplementary material.

General Procedure for Preparation of Phospholipids Containing an sn-2 Aldehydic Chain—Reactions were carried out by ozone treatment of the starting phospholipid, either 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (64–128 μmol of the unlabeled or 10 μCi of the *sn*-2 1-^{14}C-labeled compounds) dissolved in anhydrous dichloromethane at −78 °C, until a blue color persisted. Excess ozone was removed by exposing the solution to room temperature until it became clear. The solution was rechilled to −78 °C, and 50 μl of dimethyl sulfide was added. Solvent and excess dimethyl sulfide were removed under nitrogen flow. The resulting residue was purified by reversed-phase HPLC (5-μm ODS Ultrasphere, column A = 0.46 × 25 cm or column B = 1.0 × 25 cm; 905:70:25 methanol/water/acetonitrile containing 20 mM choline chloride). Appropriate fractions based on the radioactivity profile of the eluant were combined, reduced in volume under nitrogen flow, and extracted to remove the choline chloride using the method of Bligh and Dyer (19). The resulting product was stored in dichloromethane at −20 °C.

General Procedure for Preparation of Phospholipids Containing an sn-2 Short Chain Residue—1-Palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine or the 1-[1-^{14}C]palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (1 equivalent) was dissolved in anhydrous chloroform/pyridine, 4:1 (v/v). These solvents were dried over phosphorus pentoxide and calcium hydride, respectively, and distilled immediately prior to use. After addition of 4-(*N*,*N*-dimethylamino)pyridine (16) and the fatty acid anhydride (1.5–3 equivalents of each compound for unlabeled syntheses and 50–100 equivalents of each for the ^{14}C-labeled compounds) the reaction flask was flushed with nitrogen and the resulting solution was allowed to stir at room temperature for 26–36 h. The solvent was then removed under nitrogen flow, and the resulting residue was purified by reverse-phase HPLC (11), as above. Appropriate fractions based on the radioactivity profile of the eluant were combined, reduced in volume under nitrogen flow, and extracted to remove the choline chloride using the method of Bligh and Dyer (19). The resulting product was stored in dichloromethane at −20 °C. For the 1-palmitoyl-2-succinyl-GPC ([2-succinoyl]-PC) the reversed-phase HPLC solvent contained 25 mM ammonium formate instead of choline chloride. Appropriate fractions were combined, reduced in

[2] Portions of this paper (including part of "Experimental Procedures") are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

volume under nitrogen flow, and the ammonium formate was removed by lyophilization or acidic extraction (19). The resulting product was stored in dichloromethane at −20 °C.

Assay of PAF Acetylhydrolase Activity—PAF acetylhydrolase activity was determined as previously described (6). Determination of half-time ($t_{½}$) of PAF degradation at subsaturating concentrations of substrate has been described in detail elsewhere (7). Kinetic parameters for hydrolysis of *sn*-2 short chain phospholipids by purified plasma PAF acetylhydrolase were determined by varying the substrate concentration between 0 and 40 μM. The phospholipids were labeled either at the carboxyl group of the *sn*-2 residue or at the carboxyl group of the *sn*-1 palmitoyl residue. At the end of the incubation, the amount of radioactively labeled fatty acid released from *sn*-2-labeled phospholipids was determined after separation from remaining substrate by either reversed-phase chromatography on C_{18} Baker columns or by a Bligh-Dyer extraction (19). Both methods gave comparable results. For the *sn*-1 labeled substrates, the amount of 1-[1-^{14}C]palmitoyl-2-hydroxy-GPC produced was determined after extraction, separation by TLC (20), and liquid scintillation spectrometry.

Oxidation of 1-Palmitoyl-2-arachidonoyl-sn-GPC and Analysis of Phospholipid Products—1-Palmitoyl-2-[1-^{14}C]arachidonoyl-GPC (2.0 μCi, 6.4 μmol) was incubated for 60 min at 25 °C with 7.5 μmol of sodium deoxycholate, 4.62×10^5 units of soybean lipoxygenase (Type V, EC 1.13.11.12) in 1.5 ml of 0.2 M borate buffer (pH 9), as described (17). The phospholipids were extracted, solvent was removed under nitrogen flow, and the resulting thin film was stored under air at −20 °C for 7–10 days, allowing further oxidation to occur. Following oxidation, the samples were examined by reversed-phase HPLC (11). Fractions were combined based on the radioactivity profile, extracted, and then analyzed by positive ion fast atom bombardment mass spectrometry or derivatized for further separation and analysis. Further separation of the components of these HPLC fractions was obtained by treatment with diazomethane (21) or not, followed by hydrolysis with phospholipase C, and conversion to *tert*-butyldimethylsilyl derivatives (22) suitable for gas chromatography. The *tert*-butyldimethylsilyl standards were prepared from synthetic *sn*-2 short chain fatty acyl phosphatidylcholines using the same hydrolysis and derivatization procedure. The *tert*-butyldimethylsilyl derivatives of these standards and unknowns were analyzed using a mass spectrometer (VG Micromass 7050 double focusing high resolution mass spectrometer with a VG data system) coupled to a gas chromatograph with a DB-5 capillary column (developed 210 °C for 10 min, 10 °C/min, 280 °C for 15 min). Spectral characterization of these compounds may be found in the Miniprint Supplement.

Hydrolysis of Oxidized Phospholipids by PAF Acetylhydrolase—Following oxidation of 37–70 μmol of 1-palmitoyl-2-[1-^{14}C]arachidonoyl-GPC (0.03–0.05 μCi/μmol) and reversed-phase HPLC separation as described above, the oxidation products (fractions I through IV) were incubated with PAF acetylhydrolase (68 units) for 18 h at 37 °C. The solution was then acidified (pH 2) and extracted (19). Blanks were prepared by incubating oxidized products and PAF acetylhydrolase separately under these same conditions. The apolar phases from the extraction were adjusted to pH 8 before the solvent was removed by evaporation under aspirator vacuum. The *sn*-2 acyl chains released by PAF acetylhydrolase, or present in the untreated control samples, were extracted from the residue with methanol. The extracts and short chain fatty acid standards were analyzed using a mass spectrometer coupled to an OV-351 capillary column, developed at 70 °C for 1 min, 3 °C/min, 200 °C for 10 min. Spectral characterization of these compounds may be found in the Miniprint Supplement.

RESULTS

Effect of sn-2 Chain Length and Composition on the Substrate Specificity of PAF Acetylhydrolase—Oxidation of 1-palmitoyl-2-arachidonoyl-GPC generates a number of unidentified compounds that are hydrolyzed by the plasma acetylhydrolase (11). Since oxidation of unesterified polyunsaturated fatty acids generates fragments of varying size with varying oxygen functions (23), oxidation and fragmentation of phospholipids containing polyunsaturated fatty acyl residues should generate phospholipids with varying *sn*-2 residues. To determine which of these compounds plasma PAF acetylhydrolase might attack, we determined the kinetic parameters for the hydrolysis of a series of synthetic *sn*-2 homologs. Homologs of 1-[1-^{14}C]palmitoyl-2-acyl-GPC containing *sn*-2 fatty acyl residues of increasing chain length were synthesized and tested as substrates for the purified PAF acetylhydrolase. Hydrolysis of each compound was examined at 12 concentrations, and the kinetic constants were determined from a double-reciprocal plot. The results (Fig. 1) show that PAF acetylhydrolase was sensitive to the length of the *sn*-2 residue, but that it hydrolyzed *sn*-2 acyl groups of up to five carbons in length without a dramatic reduction in catalytic efficiency, *i.e.* the V/k ratio was reduced only about 2-fold for the valeroyl homolog. However, when the *sn*-2 residue was extended to 9 carbon atoms the catalytic efficiency was reduced to 2% of that of PAF. This substrate specificity showed that PAF acetylhydrolase behaved as an efficient short chain acyl hydrolase.

We next determined the effect that an ω-oxy function had on catalytic efficiency by synthesizing phosphatidylcholine homologs with five-carbon *sn*-2 residues terminated with either an aldehydic or a carboxylic function. We found that the [2-(5-oxovaleroyl)]PC homolog was about two-thirds as efficient a substrate as PAF itself (Fig. 1), consistent with our previous results (11). We also found that the substrate with an ω-terminal carboxylic function was 56% as efficient as PAF, and that the valeroyl homolog was 51% as efficient a substrate as PAF. In contrast to our prediction that acetylhydrolase might preferentially recognize oxidized residues, it appeared that it was insensitive to ω-terminal oxy functions. However, when we extended the length of the *sn*-2 residue to nine carbon atoms, we found that the oxo function strongly affected catalysis. Although phosphatidylcholine with an *sn*-2 nonanoyl residue was an extremely poor substrate for the enzyme (its catalytic efficiency was only 2% of that of PAF), its nine-aldehydic homolog had a catalytic efficiency 21-fold higher (Fig. 1). This enhancement of hydrolysis by the ω-oxo

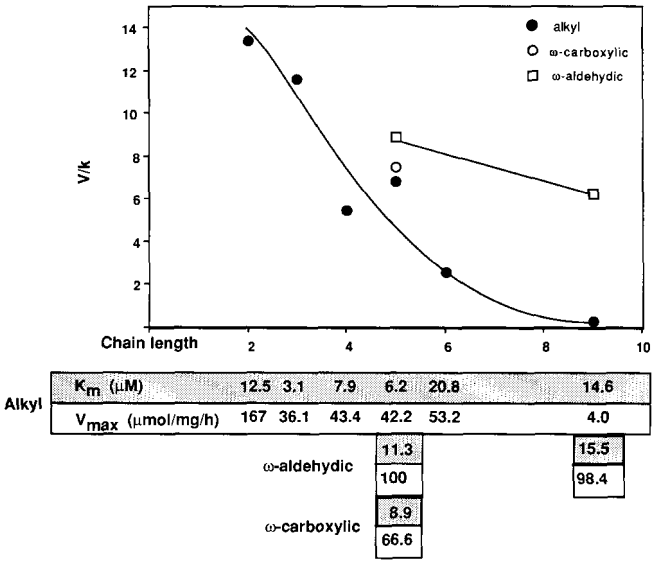

FIG. 1. **Effect of *sn*-2 chain length and oxidation state on catalytic efficiency of 1-palmitoyl-2-acyl-GPC as a substrate for plasma acetylhydrolase.** Purified plasma PAF acetylhydrolase (2.5×10^{-3} units) was incubated for 30 min at 37 °C with a series of homologs of 1-[1-^{14}C]palmitoyl-2-acyl-GPC, where the *sn*-2 residue was *n* carbon atoms in length, at concentrations ranging from 0 to 40 μM. The amount of [1-^{14}C]palmitoyl-GPC produced by this incubation was determined as described under "Experimental Procedures." Alternatively, an equal amount of enzyme from the same preparation was incubated with [*acetyl*-^{3}H]PAF, 1-palmitoyl-2-([1-^{14}C]5-oxovaleroyl)-GPC, or 1-palmitoyl-2-([1-^{14}C]9-oxononanoyl)-GPC, and the amount of radioactivity released from the phospholipid was determined by phase/phase extraction (19).

function overcomes much of the severe restriction on the length of the *sn*-2 residue and expands the number of potential substrates for acetylhydrolase.

Acetylhydrolase activity in plasma does not follow Michaelis-Menten kinetics at low substrate concentrations and deviation from calculated hydrolytic rates can be quite large: the rate of hydrolysis of 1 nM PAF is 60-fold less than that predicted from the kinetic constants (7). Since only subsaturating concentrations of substrates are likely to be encountered *in vivo*, we determined whether hydrolysis of the homologs with varying *sn*-2 residues also deviated from the predicted rate of hydrolysis under these conditions. We measured their half-life in whole plasma, and found that the relative rate of hydrolysis differed somewhat from that catalyzed by the purified acetylhydrolase (Fig. 2). For instance, the half-life of the *sn*-2 propionoyl homolog was shorter than that of PAF even though its V/k ratio was smaller than that of PAF. However, overall the half-lives of these homologs decreased with increasing chain length, and the ratio of half-lives of each compound relative to PAF was very similar to their ratios of V/k. For example, the half-life of the valeroyl homolog was 39% of that of PAF, while its V/k ratio was 51% of that of PAF. We found that, as with the V/k ratio, addition of an ω-terminal aldehydic or carboxylic function to the valeroyl homolog did not affect the half-life in whole plasma. These results showed that each of these compounds behaved like PAF, and therefore showed a large deviation from the predicted rate of hydrolysis at low concentrations in whole plasma.

We unexpectedly found that 1-palmitoyl-2-(9-oxononanoyl)-GPC was not hydrolyzed when added at low concentrations to whole plasma. Since purified acetylhydrolase did use this phospholipid as a substrate (Fig. 1), this result either was due to a failure of this phospholipid to localize to the appropriate compartment in plasma, presumably LDL, or the result of a very large deviation from the predicted rate of hydrolysis. We examined the latter possibility by asking whether purified acetylhydrolase could hydrolyze the *sn*-2 nine-carbonaldehydic residue at low substrate concentrations. We found that the purified acetylhydrolase not only hydrolyzed 1-palmitoyl-2-(9-oxononanoyl)-GPC at a concentration of 1 μM, it did so at a rate that was undistinguishable from that of PAF (Fig. 3). The very large effect of the ω-oxo function is apparent as the 2-nonanoyl homolog was not hydrolyzed under these conditions. Therefore, addition of an ω-oxo function overcame the effect of *sn*-2 chain elongation, even at low substrate concentrations, but a plasma component(s) prevented access of the substrate to the enzyme. We do not know why the 5-oxovaleroyl homolog did not demonstrate the same phenomenon, but it is clear that phospholipids with intermediate length *sn*-2 residues are acetylhydrolase substrates, provided that they contain an ω-oxo function.

Identification of Phospholipids derived from Oxidative Fragmentation of 1-Palmitoyl-2-[1-^{14}C]arachidonoyl-GPC—Our next goal was to determine what types of phosphatidylcholines were generated from an uncontrolled oxidative attack on a phosphatidylcholine containing an *sn*-2 polyunsaturated fatty acyl residue to define potential acetylhydrolase substrates. Phosphatidylcholine containing an *sn*-2 arachidonoyl residue was oxidized with soybean 15-lipoxygenase, to generate a few percent of the corresponding hydroperoxyphospholipid, and then allowed to oxidize in air for several days as a thin film. When lipids from this oxidation were analyzed by reversed-phase HPLC, we found an unresolved mixture of radiolabeled material that migrated in a position that showed they were more polar than the starting phospholipid (Fig. 4). Some of this material also absorbed light at 235 nm, indicative of bond rearrangement to a conjugated system. We collected the material in fractions 15 through 52 and chromatographed it on straight phase thin layer plates, where it again migrated as material more polar than phosphatidylcholine (not shown). We found that these lipids could be visualized with molybdic acid reagent or Dragendorff reagent (24), indicating the presence of phosphate and choline, respectively. The presence of ^{14}C radioactivity, derived from the carboxyl carbon of the *sn*-2 residue, showed that the *sn*-2 position remained esterified with at least a portion of the arachidonoyl residue.

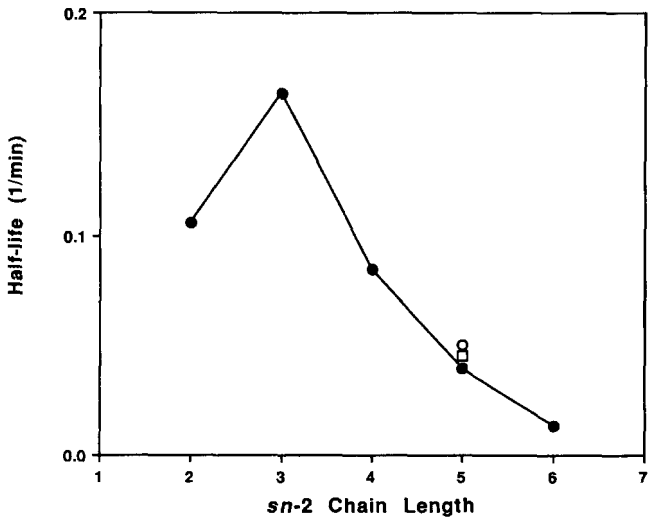

FIG. 2. **Effect of *sn*-2 chain length and oxidation state on the half-life of 1-palmitoyl-2-acyl-GPC in plasma.** Reaction mixtures contained 250 μl of plasma and 10^{-6}–10^{-9} M [*acetyl*-^{3}H]PAF or 1-[1-^{14}C]palmitoyl-2-acyl-GPC (●), 1-[1-^{14}C]palmitoyl-2-glutaroyl)-GPC (○), or 1-palmitoyl-2-(5-[1-^{14}C]oxovaleroyl-GPC (□) in a total volume of 300 μl. The half-life was independent of substrate concentration over these ranges. Aliquots (50 μl) were removed at various times (7), and the lipids were extracted and separated by thin layer chromatography. The loss of substrate was then quantitated by liquid scintillation counting by scraping the appropriate area of the plate.

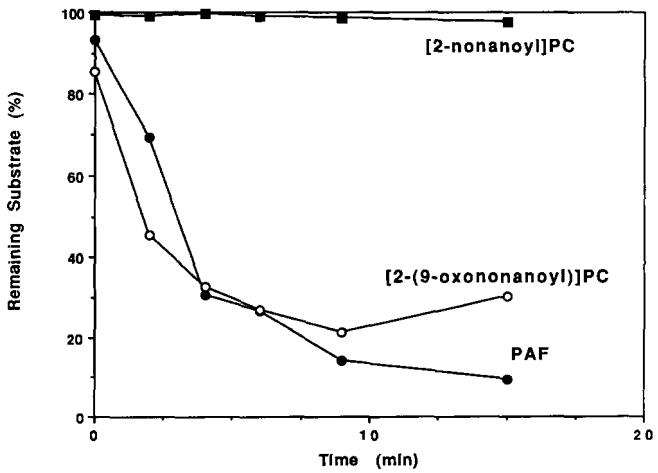

FIG. 3. **Effect of an ω-oxo function on the half-life of 1-palmitoyl-2-nonanoyl-GPC.** [*acetyl*-^{3}H]PAF or 1-palmitoyl-2-(9-[1-^{14}C]oxononanoyl)-GPC at a concentration of 1 μM was incubated with 0.18 unit of purified plasma acetylhydrolase. At the stated times, aliquots were removed to quantitate the amount of substrate hydrolyzed as described under Fig. 2. When 1-palmitoyl-2-nonanoyl-GPC was the substrate, the sensitivity of the assay was increased by decreasing the substrate concentration to 1 nM and increasing the amount of enzyme to 0.75 unit.

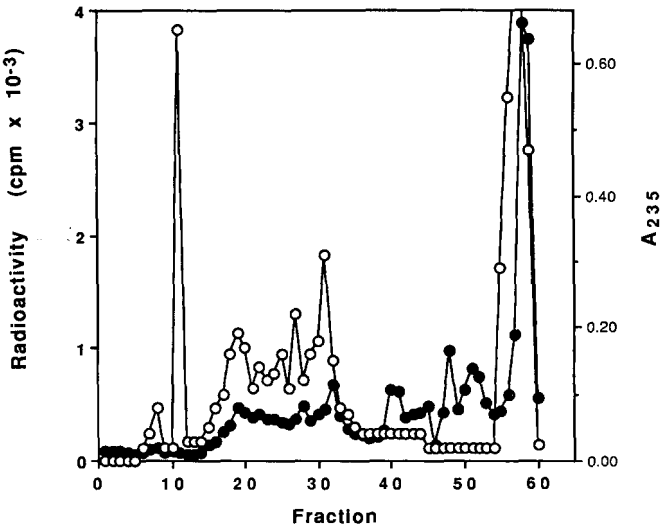

FIG. 4. **Reversed-phase HPLC chromatogram of products obtained from uncontrolled oxidation of phosphatidylcholine.** 1-Palmitoyl-2-[1-^{14}C]arachidonoyl-GPC was oxidized by 15-lipoxygenase to the *sn*-2 [1-^{14}C]15-hydroperoxyeicosatetraenoyl species, which comprises the largest radioactive and UV-absorbtive peak in the chromatogram. This product was allowed to oxidize by exposure to air as a thin film at −20 °C before lipid products were recovered by extraction (19). These lipids were separated by chromatography over an octadecylsilica column developed isocratically at 0.8 ml per min with methanol/water/acetonitrile (930:70:50) containing 20 mM choline chloride. The effluent was monitored for UV absorption at 235 nm (○) with a flow-through detector before fractions (1 ml) were collected, and radioactivity (●) estimated by removing aliquots for liquid scintillation spectroscopy. Subsequently, fractions were combined as follows: I, HPLC *fractions 15–21*; II, HPLC *fractions 22–28*; III, HPLC *fractions 29–34*; IV, HPLC *fractions 35–52*.

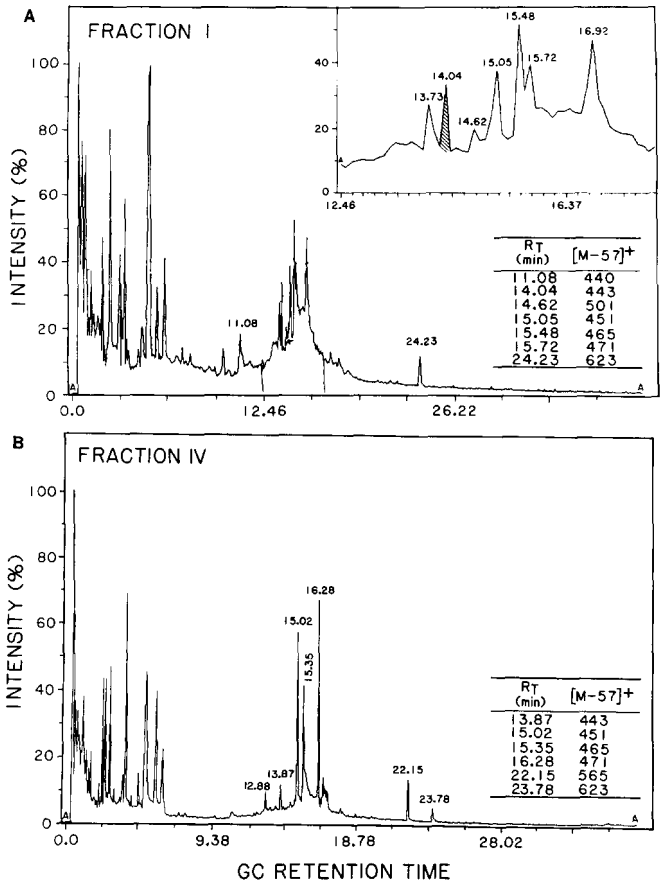

FIG. 5. **GLC chromatogram of methylated, *t*-BDMS derivatives of material contained in reversed-phase HPLC fractions I and IV.** Material derived from the oxidation of 1-palmitoyl-2-arachidonoyl-GPC that eluted in fractions 15–21 (fraction I) and 35–52 (fraction IV) were extracted (19) to remove choline chloride. These fractions were treated with diazomethane (or not, data not shown), incubated with phospholipase C, and then converted to *t*-BDMS derivatives as described under "Experimental Procedures." These derivatives were injected onto a DB-5 column that was then developed at 210 °C for 10 min, 10 °C/min, 280 °C for 15 min. Peaks with retention times of 11.08–24.23 min were analyzed by mass spectrometry and found to contain material consistent with *t*-BDMS derivatives of diglycerides with *sn*-2 residues ranging from three to 15 carbons in length. Peaks with retention times of 12.88, 13.73, and 16.92 min appeared to be a mixture of compounds, so a value for $[M-57]^+$ is not listed. Molecular ions and fragmentation patterns for peaks with retention times shorter than 6 min were consistent with *t*-BDMS derivatives of the *sn*-1 and -2 fatty acyl residues, as well as the derivatized glycerol backbone. Treatment with diazomethane produced, in each combined fraction, three to five additional peaks with retention times shorter than 6 min. *A*, methylated, *t*-BDMS derivative of fraction I; *B*, methylated, *t*-BDMS derivative of fraction IV.

We analyzed these polar phospholipids by mass spectrometry. Four fractions corresponding to fractions 15–21, 22–28, 29–34, and 35–52 of the HPLC chromatogram (Fig. 4) were combined, extracted by an acidic Bligh and Dyer procedure, and subjected to fast atom bombardment mass spectrometry. The resulting spectra were complex, but were consistent with the presence of a mixture of phospholipids with shortened *sn*-2 residues. The four combined fractions were methylated with diazomethane, or not, and then treated with phospholipase C. The resulting diglycerides were then converted to their *t*-butyldimethylsilyl derivatives. These were separated by gas chromatography on a DB-5 capillary column and analyzed by mass spectrometry. The gas chromatogram obtained from the analysis of methylated fraction I (Fig. 5*A*) shows the presence of numerous peaks. Material that eluted prior to 11 min was not analyzed further, although some of these fractions contained material with a molecular weight and fragmentation pattern indicative of the *t*-BDMS derivative of 1-palmitoyl-GPC. The materials that eluted between 13 and 17 min were sufficiently resolved (Fig. 5, *inset*) to produce spectra consistent with the presence of a single major component. The $[M-57]^+$ value listed for each peak (except those eluting at 13.73 and 16.92 min which appeared to be a mixture of compounds) appeared to be the molecular ion of the *t*-BDMS derivative based on isotope and fragmentation pattern analysis. The material migrating with a retention time of 14.62 min was only present in the chromatogram of the methylated sample (not shown) and was present only when the lipids were extracted (19) with an acidified system. This behavior is similar to that observed by Tokumura *et al.* (25) during their examination of phosphatidylcholines, derived from bovine brain, that contained short ω-carboxylic *sn*-2 residues. The material eluting at 14.62 min had a molecular ion consistent with the methyl ester of the *t*-BDMS derivative of [2-succinoyl]phosphatidylcholine (see Miniprint Supplement), and a fragmentation pattern like that of the methyl ester of the *t*-BDMS derivative of [2-glutaroyl]phosphatidylcholine, except that the *m/z* of the fragments containing the *sn*-2 residue was 14 less. This strongly suggests that the parent lipid was 1-palmitoyl-2-succinoyl-GPC. We conclude, therefore, that oxidation of [arachidonoyl]phosphatidylcholine fragments the *sn*-2 residue, and that the resulting products include phosphatidylcholines with short *sn*-2 dicarboxylic acyl residues.

We next analyzed fraction IV, the least polar fraction obtained from reversed-phase HPLC, in a similar fashion to

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