Fatty acid and phospholipid selectivity of different phospholipase A_2 enzymes studied by using a mammalian membrane as substrate

Emilio DIEZ,*§ Floyd H. CHILTON,|| George STROUP,* Ruth J. MAYER,† James D. WINKLER‡ and Alfred N. FONTEH||¶

*Departments of Cell Sciences, †Medicinal Chemistry and ‡Inflammation and Respiratory Pharmacology, SmithKline Beecham Pharmaceuticals, King of Prussia, PA 19406, U.S.A., §Laboratorios Beecham S.A., Centro de Investigacion Basica, c/Santiago Grisolia 4, Tres Cantos, 28760 Madrid, Spain, and ||Division of Pulmonary and Critical Care Medicine, Department of Medicine, Bowman Gray School of Medicine, Winston-Salem, NC 27103, U.S.A.

Previous studies using phospholipid mixed vesicles have demonstrated that several types of phospholipase A_2 (PLA_2) enzymes exhibit different selectivity for fatty acids at the *sn*-2 position, for the type of chemical bond at the *sn*-1 position or for the phosphobase moiety at the *sn*-3 position of phospholipids. In the present study, we have utilized natural mammalian membranes from U937 monocytes to determine whether two purified 14 kDa PLA_2 isoenzymes (Type I, Type II) and a partially purified 110 kDa PLA_2 exhibit substrate selectivity for certain fatty acids or phospholipids. In these studies, arachidonic acid (AA) release from membranes was measured under conditions where the remodelling of AA mediated by CoA-independent transacylase (CoA-IT) activity has been eliminated. In agreement with the mixed-vesicle models, AA was the major unsaturated fatty acid hydrolysed from membranes by the 110 kDa PLA_2, suggesting that this PLA_2 is selective in releasing AA from natural membranes. By contrast, Type I and Type II PLA_2s were less selective in releasing AA from phospholipids and released a variety of unsaturated fatty acids at molar ratios that were proportional to the ratios of these fatty acids in U937 microsomal membranes. Examination of AA release from phospholipid classes indicated that all three enzymes released AA from the major AA-containing phospholipid classes (phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositol) of U937 membranes. The 110 kDa PLA_2 released AA from phospholipid subclasses in ratios that were proportional to the AA content within phospholipid classes and subclasses of U937 membranes. These data suggested that the 110 kDa PLA_2 shows no preference either for the *sn*-1 linkage or for the *sn*-3 phosphobase moiety of phospholipids. By contrast, Type I and Type II PLA_2s preferentially released AA from ethanolamine-containing phospholipids and appeared to prefer the 1-acyl-linked subclass. Taken together, these data indicate that the 110 kDa PLA_2 selectively releases AA from U937 membranes, whereas Type I and Type II PLA_2 release a variety of unsaturated fatty acids. Furthermore, the 110 kDa PLA_2 releases the same molar ratios of AA from all major phospholipid subclasses, whereas Type I and Type II PLA_2s show some specificity for phosphatidylethanolamine when these enzymes are incubated with a complex mammalian membrane substrate.

INTRODUCTION

In inflammatory cells, the release of arachidonic acid (AA) from membrane phospholipids by phospholipase A_2 (PLA_2) is a critical step in the generation of lipid mediators (Vadas and Pruzanski, 1990). AA mobilized by PLA_2, activity can be metabolized to bio-active compounds such as prostaglandins, thromboxane and leukotrienes (Salmon and Higgs, 1978; Holtzman, 1991). During the last decade, it has been demonstrated that inflammatory cells contain a wide array of arachidonate-containing phospholipid molecular species. To date, several mammalian PLA_2s which may play a role in the mobilization of AA from phospholipids have been described. For example, extracellular PLA_2s are found in mammalian fluids as digestive enzymes and as a consequence of inflammatory diseases (Vadas et al., 1985). These extracellular enzymes have a low molecular mass ($\sim$ 14 kDa) and a very rigid three-dimensional structure maintained by disulphide bridges (Kramer et al., 1989). They have an absolute requirement for Ca^{2+} for their catalytic activity (Waite, 1987). PLA_2 from human synovial fluid has been cloned and sequenced and has been shown to be a Type II enzyme, having 30–40% sequence similarity to the Type I pancreatic enzymes (Kramer et al., 1989; Seilhamer et al., 1989). Type I and II enzymes have been crystallized and shown to have very similar structural features (Dijkstra et al., 1981; Scott et al., 1990, 1991). When provided with phospholipids substrates (in vesicles or as *Escherichia coli* membranes) containing several fatty acids at the *sn*-2 position, Type II PLA_2 hydrolyses a variety of unsaturated fatty acids, including linoleic acid (LA), oleic acid (OA) and AA.

In the last few years, much interest has also been concentrated on intracellular PLA_2s. Among many intracellular enzymes that have been described, an 110 kDa PLA_2 is best characterized. This enzyme has been purified from different cell lines. It has been cloned and sequenced (Clark et al., 1991; Kramer et al., 1991), and its structure was shown to be completely unrelated to that of the low-molecular-mass PLA_2s. In the presence of Ca^{2+} (0.1–1 μM) this PLA_2 appears to translocate from the cytosol to cellular membranes (Diez and Mong, 1990; Channon and Leslie, 1990; Ramesha and Ives, 1993). When supplied with vesicles containing different phospholipids with a variety of fatty acids, this PLA_2 selectively hydrolyses AA-containing phospholipids (Diez and Mong, 1990; Kramer et al., 1991; Diez et al., 1992; Hanel et al., 1993). Ca^{2+}-independent PLA_2 activities that selectively release fatty acids from 1-*O*-alk-1′-enyl-phospholipid subclasses have also been characterized (Wolf and Gross, 1985; Hazen et al., 1990).

The aforementioned studies using various membrane systems (either artificially produced or from bacteria) suggest that some

Abbreviations used: LA, linoleic acid; OA, oleic acid; SA, stearic acid; DGLA, dihomo-γ-linoleic acid; EPA, eicosapentaenoic acid; AA, arachidonic acid; DHA, docosahexaenoic acid; GPC, *sn*-glycero-3-phosphocholine; GPE, *sn*-glycero-3-phosphoethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PLA_2, phospholipase A_2; CoA-IT, CoA-independent transacylase.

¶ To whom all correspondence should be addressed.

specificity by the different PLA_2s exists in terms of the fatty acid located at the *sn*-2 position. However, it is still not apparent whether these enzymes are selective in hydrolysing fatty acids from specific phospholipid classes or subclasses within mammalian membranes. Until now, a comparative study of the different enzymes using a natural substrate composed of the major phospholipid classes [phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI)] and subclasses (1-acyl-, 1-*O*-alkyl- and 1-*O*-alk-1′-enyl-linked) has not been performed. To address this issue, three well-characterized mammalian PLA_2s (Type I, Type II and 110 kDa) were incubated with membranes from the human monocytic leukaemia cell line U937. We than determined the selectivity of these PLA_2s for the radyl linkage in the *sn*-1 position of phospholipids, the fatty acid moiety in the *sn*-2 position of phospholipids, and the phosphobase moiety in the *sn*-3 position of phospholipids. Our results suggest that the 110 kDa PLA_2 is more selective in releasing AA from phospholipids than is either Type I or Type II PLA_2. In addition, the 110 kDa PLA_2 hydrolyses AA from all phospholipid subclasses in amounts that are proportional to the AA content within these subclasses. By contrast, Type I and Type II PLA_2 prefer to release AA from PE.

MATERIALS AND METHODS

Materials

[5,6,8,9,11,12,14,15-^{3}H]AA (76 Ci/mmol) was purchased from New England Nuclear Products (Boston, MA, U.S.A.). PC, PE, PI, phosphatidylserine and phosphatidic acid were purchased from Avanti Polar Lipids (Birmingham, AL, U.S.A.). Phospholipase C (*Bacillus cereus*, type XI) was purchased from Sigma (St. Louis, MO, U.S.A.). Acetylation-grade acetic anhydride and pyridine were purchased from Alltech/Applied Science Associate (Deerfield, IL, U.S.A.). All solvents were h.p.l.c. grade purchased from Fisher Scientific.

Enzymes

The cDNA of human Type II PLA_2 (14 kDa) was cloned from placenta mRNA and expressed in Chinese-hamster ovary cells. Authentic processed Type II PLA_2 was purified as previously described (Kramer et al., 1989). Human cytosolic PLA_2 (110 kDa) was partially purified from undifferentiated U937 cells by using a 'high-salt phenyl-Superose' column, followed by enzyme concentration on a Mono Q HR 5/5 column (Kramer et al., 1991). Protein amounts were determined by the BCA assay (Pierce Chemical Co.), and specific activities of both enzymes were then determined as previously described (Diez et al., 1992). Bovine pancreatic PLA_2 was obtained from Sigma.

Cells and labelled-membrane preparation

Human monocytic leukaemia cell line U937 was obtained from the American Type Culture Collection. The cells were grown in RPMI-1640 media, containing 10% fetal-calf serum, 10 mM L-glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin at 37 °C in a humidified-CO_2 (5%) incubator. Labelled membranes were prepared by adding [^{3}H]AA to the cell culture at a density of 5×10^6 cells/ml. After 24 h, cells were washed three times with fresh media, and twice with PBS. Finally, cells were resuspended in 10 mM Tris/HCl, pH 8.0, containing 0.25 M sucrose, 0.2 mM EGTA and protease inhibitors (0.2 mM phenylmethanesulphonyl fluoride, 0.7 mg/l pepstatin A, 0.5 mg/l leupeptin) at 4 °C. Cells were disrupted by nitrogen cavitation [4.14 MPa (600 lb/in^2) 15 min at 4 °C]. One volume of 2 M sucrose was added, and the homogenate was centrifuged at 800 *g* for 8 min. The supernatant was further centrifuged at 100000 *g* for 60 min. The pellet was resuspended in PBS containing 1 mM EGTA and 1 mg/ml BSA, and washed twice in this buffer. The pellet was finally resuspended in PBS at a concentration of 5–6 μg of protein/μl (10000 d.p.m./μl) and stored at −80 °C.

PLA_2 assay

PLA_2 activity was routinely determined in 50 mM Tris/HCl buffer, pH 7.1, containing 2 mg/ml BSA, 1 μM free Ca^{2+}, 100 μl of labelled U937-cell membranes, and Type I (4–5 μg), Type II (1–1.5 μg) or 110 kDa (100–200 μg) PLA_2 in a final volume of 250 μl. PLA_2 amounts that were added to U937 membranes were determined by titration, such that all three enzymes hydrolysed the same percentage of [^{3}H]AA (20–25%, within 30 min at 37 °C) from the membrane phospholipids. After incubation of enzymes with membranes, reactions were stopped with 0.75 ml of chloroform/methanol/HCl (100:100:1, by vol.) and lipids extracted as previously described (Bligh and Dyer, 1959). Phospholipids and AA were separated by t.l.c. on silica-gel G plates developed in n-hexane/diethyl ether/acetic acid (70:30:1, by vol.). The spots corresponding to AA and phospholipids were revealed by exposure to iodine vapour and then scraped into scintillation vials. The radioactivity in the silica powder was determined by liquid-scintillation counting.

Measurement of CoA-independent transacylase (CoA-IT) activity

CoA-IT activity can transfer molecules of AA between different subclasses of choline- and ethanolamine-containing glycerolipids (Kramer and Deykin, 1983; Sugiura et al., 1987). CoA-IT activity was measured by monitoring the acylation of [^{3}H]-1-*O*-alkyl-2-lyso-glycerophosphocholine (GPC) in the absence of CoA, as previously described (Winkler et al., 1991a). By using this assay, microsomes prepared from U937 cells were found to contain substantial amounts of CoA-IT activity (292 ± 12 pmol/min per mg). As experiments were performed to determine which glycerolipid subclasses were losing AA by the action of the different PLA_2s, this endogenous CoA-IT activity could confound the interpretation of the experiment by moving AA between the different glycerolipid subclasses. We have previously shown that CoA-IT activity could be eliminated by preincubation with heat (Winkler et al., 1991b). In the present studies, incubation of U937 membranes at 57 °C for 5 min before assay at 37 °C eliminated measurable CoA-IT activity.

Chromatographic analysis of glycerolipids

(a) Glycerolipid analysis by t.l.c.

Lipid extraction was carried out as described above (Bligh and Dyer, 1959). Phospholipids were fractionated by two-dimensional t.l.c.: silica-gel G plates were developed in chloroform/methanol/NH_4OH (65:25:4, by vol.) in the first dimension, dried under N_2, and then developed in the second dimension with chloroform/acetone/methanol/acetic acid/water (6:8:2:2:1, by vol.).

(b) Isolation of glycerolipid classes by h.p.l.c.

Glycerolipid classes were isolated by normal-phase h.p.l.c. (Chilton, 1990). The Bligh and Dyer extracts were loaded on to an Ultrasphere Silica column (4.6 mm × 250 mm: Rainin Instrument Co., Woburn, MA, U.S.A.) and eluted with hexane/propan-2-ol/ethanol/phosphate buffer (pH 7.4)/acetic acid (490:367:100:30:0.6, by vol.) for 5 min at a flow rate of 1 ml/min. The amount of phosphate buffer in the eluting solvent

was increased from 3% to 5% over 5 min, and this solvent composition was maintained until all the major phospholipid classes had been eluted from the column (30–40 min). Fractions (1 ml) were collected for liquid-scintillation counting.

(c) Separation of PC and PE subclasses

PC and PE fractions from normal-phase h.p.l.c. were hydrolysed with 25 units of *B. cereus* phospholipase C (Sigma) for 6 h. Diradylglycerols obtained from phospholipase C hydrolysis were converted into diradylglycerol acetates by using acetic anhydride/pyridine (9:1, v/v; 12 h at 37 °C). 1-Acyl, 1-*O*-alkyl and 1-*O*-alk-1′-enyl subclasses were separated by t.l.c. on silica-gel G developed in benzene/hexane/ether (50:25:4, by vol.) (Nakagawa et al., 1982). Radioactivity in phospholipid subclasses was determined by liquid-scintillation counting.

Fatty acid composition of U937 membranes

[$^2H_3$]Stearic acid ([$^2H_3$]SA; 100 ng) as an internal standard for the analysis of LA and OA, and [$^2H_8$]AA (100 ng) as an internal standard for the analysis of eicosapentaenoic acid (EPA), dihomo-γ-linoleic acid (DGLA), docosahexaenoic acid (DHA) and AA, were added to a portion (20 μl) of U937 membranes. Subsequently, fatty acyl chains were hydrolysed with 2 M KOH in 75% (v/v) ethanol (30 min, 60 °C). The reaction was stopped by addition of an equal amount of water, and the pH of the reaction mixture was adjusted to 3 with 6 M HCl. Non-esterified fatty acids were extracted with hexane/ether (1:1, v/v). Solvents were then removed by using a stream of N_2, and a fatty-acid-enriched fraction was obtained by solid-phase extraction. Briefly, lipids were suspended in 4 ml of hexane before application on to a Bakerbond silica-gel extraction column (J. T. Baker Inc., Phillipsburg, NJ, U.S.A.) that had been conditioned with 4 ml of hexane. After washing the columns with 4 ml of 10% ether in hexane, a fatty-acid-enriched fraction was eluted from the columns with 4 ml of hexane/ether (1:1, v/v). Non-esterified fatty acids were converted into their pentafluorobenzyl esters and analysed by g.l.c./m.s. using selected ion-monitoring techniques (Chilton and Murphy, 1987). Carboxylate anions at *m/z* 279, 281, 286, 301, 303, 305, 327 and 311 for LA, OA, [$^2H_3$]SA, EPA, AA, DGLA, DHA and [$^2H_8$]AA respectively were monitored with a Hewlett–Packard mass spectrometer (HP 5989 A) that was interfaced to an Hewlett–Packard series II gas chromatograph (HP 5890). Mole quantities of fatty acids were determined from standard curves obtained for each fatty acid by using [$^2H_3$]SA and [$^2H_8$]AA as internal standards.

Determination of non-esterified fatty acid release by g.l.c./m.s.

After addition of 100 ng of [$^2H_8$]AA as an internal standard to the neutral-lipid fraction from normal-phase h.p.l.c., a fatty-acid-enriched fraction was obtained by using Bakerbond silica-gel disposable columns as described above. Fatty acids were then converted into their pentafluorobenzyl esters, and carboxylate anions at *m/z* 279, 281, 301, 303, 305, 327 and 311 for LA, OA, EPA, AA, DGLA, DHA and [$^2H_8$]AA respectively were monitored by negative-ion chemical-ionization g.l.c./m.s. as described above. Mole quantities of fatty acids released by PLA_2 isoenzymes were then calculated from standard curves as described above.

RESULTS

All experiments described in this paper were carried out at 1 μM free calcium. This value is close to intracellular Ca^{2+} levels in stimulated cells, and the three PLA_2s used in this study were active under these conditions (Marshall and McCarte-Roshak, 1992).

PLA_2-induced hydrolysis of fatty acids from U937-cell membranes

Initial experiments were designed to determine the fatty acid composition of U937 membranes. Portions (20 μl) of U937 membranes were subjected to base hydrolysis and determination of non-esterified fatty acids by negative-ion chemical-ionization g.l.c./m.s. Unsaturated fatty acid composition (pmol) of the membranes was as follows: 53.4±4.1 LA; 1230.9±47.9 OA; 94.4±1.5 DGLA; 65.1±16.5 EPA; 388.3±6.5 AA and 125.6±5.6 DHA (means±S.E.M., $n = 4$).

In subsequent studies, 100 μl of U937 membrane preparation was incubated with different PLA_2 and non-esterified fatty acid levels compared with those of membranes that had not been exposed to any PLA_2. As shown in Table 1, both Type I and Type II enzymes hydrolysed various fatty acids from natural membranes. As a ratio of mole quantities of fatty acids released, AA accounts for 0.28 and 0.22 of unsaturated fatty acids hydrolysed by Type I and Type II PLA_2 respectively. Both Type I and Type II PLA_2 released significant quantities of other unsaturated fatty acids from natural membranes. By contrast, the 110 kDa PLA_2 was selective for hydrolysis of AA. The selectivity for AA is enhanced when it is considered that AA is only a small proportion of the total unsaturated fatty acids found in membranes of U937 cells, and yet accounted for most (~66%) of all unsaturated fatty acids hydrolysed by the 110 kDa PLA_2. This is in agreement with previously published results (Clark et al., 1991).

Distribution of [^{3}H]AA in U937-cell membrane phospholipids

When U937-cell membranes were labelled for 24 h as described in the Materials and methods section, more than 90% of the [^{3}H]AA was incorporated into the cells, and of this more than 80% was present in the 100000 *g* membrane pellet. In order to perform specificity studies of the loss of [^{3}H]AA from different phospholipid classes and subclasses and correlate them with losses in mass of AA when using this membrane preparation, it was very important to ensure that the quantity of [^{3}H]AA in all phospholipid classes and subclasses was representative of the mole quantities of AA in these phospholipid (i.e. isotopic

Table 1 Fatty acid release from heat-treated U937 membranes by different PLA_2 enzymes

Heat-treated membranes (100 μl) from U937 cells were incubated with Type I PLA_2, Type II PLA_2 or 110 kDa PLA_2 as described in the Materials and methods section. The mole quantities of LA, OA, DGLA, EPA, AA and DHA released as non-esterified fatty acids from the membrane were determined by g.l.c./m.s. These data are expressed as the difference between membranes that were incubated with different PLA_2 enzymes and control membranes (no PLA_2) and are means±S.E.M. of four separate experiments.

		Amount of fatty acids released (pmol/600 μg of protein)		
Fatty acid	PLA_2...	Type I	Type II	110 kDa
LA		14.7±2.3	109.0±92.1	507±50.7
OA		732.0±561.0	1246±406.0	126.0±304.0
DGLA		52.7±12.4	54.3±7.1	7.0±4.0
EPA		49.7±9.9	9.7±0.3	9.3±2.9
AA		450.3±86.9	427.3±100.5	391.0±73.7
DHA		283.0±50.4	54.3±7.1	8.3±8.3

Table 2 Specific radioactivity of [^{3}H]AA in phospholipid classes

U937 cells were labelled with [^{3}H]AA for 24 h and membranes prepared as described in the Materials and methods section. Phospholipids were extracted from 20 μl of membrane preparation, and individual phospholipid classes were isolated by two-dimensional t.l.c. The amount of radioactivity and the mole quantities of arachidonate was measured in each phospholipid class. These data are means ± S.D. of two experiments each performed in duplicate. Abbreviations: PS, phosphatidylserine; ND, not determined.

Lipid class	^{3}H (d.p.m./μg of protein)	10^2 × AA (pmol/μg of protein)	[^{3}H]AA (d.p.m.)/ pmol of AA
PC	642 ± 31	360 ± 14	178
PE	1905 ± 76	945 ± 101	202
PI	446 ± 17	242 ± 5	184
PS	295 ± 22	118 ± 28	250
Others	130 + 12	ND	ND

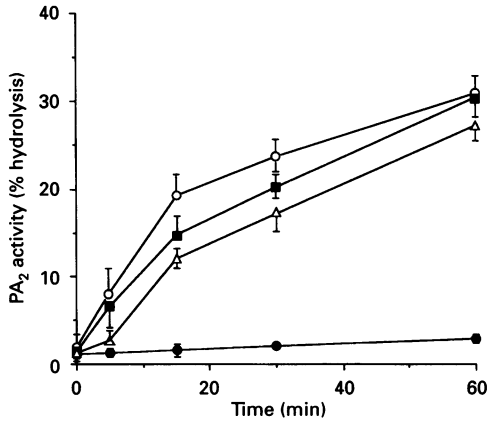

Figure 1 Time course of PLA_2-induced hydrolysis

[^{3}H]AA-labelled membranes from U937 cells were incubated without PLA_2 (●, no enzyme), Type I PLA_2 (○), Type II PLA_2 (■) or the 110 kDa PLA_2 (△) as described in the Materials and methods section. Reactions were stopped at different time points and [^{3}H]AA was isolated by t.l.c. Radioactivity in phospholipids and non-esterified fatty acids was determined by liquid-scintillation counting, and PLA_2 activity was expressed as the percentage hydrolysis of the labelled membrane substrate. These results are means ± S.E.M. of three separate experiments performed in duplicate.

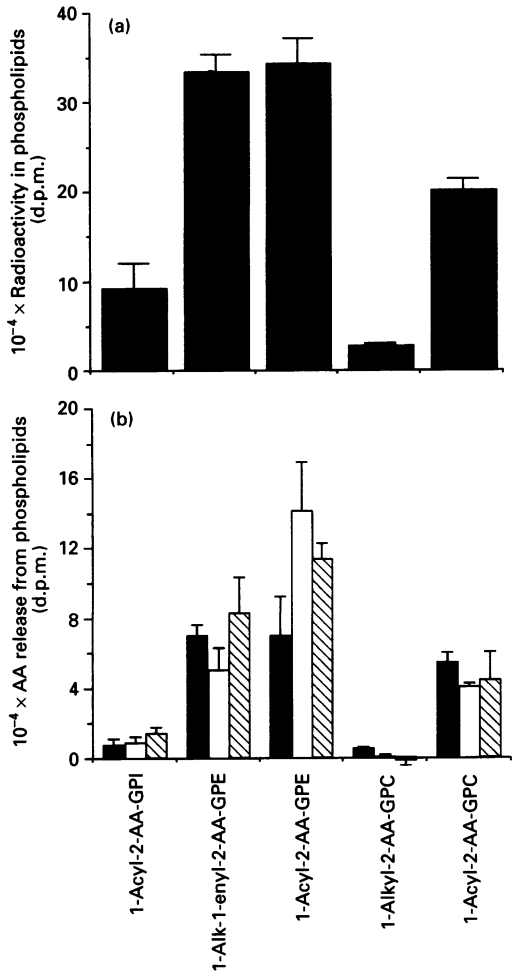

Figure 2 Distribution and PLA_2-enzyme-induced release of AA from U937-cell membranes

(**a**) AA distribution among phospholipid subclasses. Membranes (100 μl) from U937 cells that had been labelled with [^{3}H]AA were prepared as described in the Materials and methods section. Phospholipids were isolated by normal-phase h.p.l.c. PC and PE were converted into diradylglycerol acetates, which were separated into 1-acyl, 1-*O*-alkyl and 1-*O*-alk-1′-enyl subclasses by t.l.c. as described in the Materials and methods section. The radioactivity in each subclass was determined by liquid-scintillation counting after scraping the appropriate zones. These data are expressed as the total radioactivity found in phospholipid subclasses and are means ± S.E.M. of four separate experiments. (**b**) AA release by PLA_2 enzymes. [^{3}H]AA-labelled heat-treated U937 membranes (100 μl) were incubated with different PLA_2s (▨, Type I; □, Type II; or ■, 110 kDa), as described in the Materials and methods section. Membrane lipids were extracted by the method of Bligh and Dyer (1959), and phospholipid classes were isolated as described above. The radioactivity in each subclass was determined by liquid-scintillation counting after scraping the zones. These data are expressed as the difference in total radioactivity ([^{3}H]AA) released by each PLA_2 and [^{3}H]AA recovered from untreated (control) U937 membranes, and are means ± S.E.M. of four separate experiments. Abbreviation: GPI, *sn*-glycero-3-phosphoinositol.

equilibrium) (Ramesha and Taylor, 1991). Total phospholipids were extracted from the membranes, separated by two-dimensional t.l.c. and analysed for both [^{3}H]AA content and AA mass. The distribution of AA in phospholipids was of the order PE (66.1 ± 3.2%) ≫ PC (22.3 ± 1.3%) ≫ PI (8.8 ± 1.8%). In PE, the main AA-containing subclasses were 1-acyl-2-AA-glycerophosphoethanolamine (GPE) and 1-*O*-alk-1′-enyl-2-AA-GPE, containing 32.9 ± 2.6% and 31 ± 1.8% of total AA respectively. 1-Acyl-2-AA-GPC, containing 19.2 ± 1.3% of total AA of U937 membranes, was the main [^{3}H]AA reservoir of PC. The results shown in Table 2 indicate that the specific radioactivity of different phospholipids is similar, suggesting that the distribution of radioactivity mimicked the mass distribution of AA among phospholipids. We also analysed the different subclasses of PC and PE, and the results indicate that these subclasses were also labelled to isotopic equilibrium (Diez, E, unpublished work). These results allow us to conclude that changes of [^{3}H]AA in different phospholipids under this labelling condition accurately reflect losses of AA by mass from these phospholipids.

Kinetics of [^{3}H]AA release from U937-cell membranes

Labelled membranes were incubated with different PLA_2s and the reaction was monitored by the release of [^{3}H]AA. The enzymes were titrated to give similar amounts of AA hydrolysis. Figure 1 shows that all three enzymes hydrolysed AA in a progressive manner. At the concentrations of enzymes used, the

reactions were linear for the first 15 min, followed by a gradual decrease in the rate of AA release from 15 to 60 min. We obtained ~ 20% hydrolysis of substrate by the different enzymes in a 30 min incubation period. By mass, Type I, Type II and the 110 kDa PLA_2 released 450 ± 87, 427 ± 100 and 391 ± 74 pmol of AA (means ± S.E.M., $n = 4$), respectively, when incubated with 100 μl (600 μg of protein) of U937 membranes for 30 min.

Hydrolysis of [^{3}H]AA from different phospholipid subclasses

The distribution of [^{3}H]AA among phospholipids subclasses in control conditions is shown in Figure 2(a). When labelled U937 membranes were treated with the different PLA_2s under the same conditions as those described above, the three enzymes released AA from different phospholipid subclasses (Figure 2b). Three major conclusions can be drawn from these experiments. First, the 110 kDa PLA_2 hydrolysed AA from all phospholipid subclasses in proportion to the AA content within these same subclasses in the natural membrane. Secondly, Type I and Type II PLA_2s showed more preference for PE subclasses than for PC subclasses and PI. Thirdly, Type II PLA_2 was more selective for 1-acyl-2-AA-GPE than for 1-*O*-alk-1′-enyl-2-AA-GPE. Taken together, these data suggest that the 110 kDa PLA_2 does not discriminate between phospholipid subclasses when hydrolysing AA, whereas Type I or II PLA_2s show preference for phospholipid subclasses based on the nature of the phospholipid head group when presented in a complex mammalian substrate system.

DISCUSSION

Evidence has accumulated from numerous studies that various PLA_2 enzymes exhibit substrate specificity in regard to fatty acids or to phospholipid classes (Kim et al., 1990; Schalkwijk et at., 1990; Wijkander and Sundler, 1991; Diez et al., 1992; Mayer and Marshall, 1993). Most of these studies have utilized artificial membranes or bacterial membranes that have different lipid profiles from those of complex mammalian cell membranes. To define clearly the role of different PLA_2 enzymes in fatty acid metabolism in mammalian cells, we have utilized a natural membrane system derived from U937 cells. U937 membranes are ideal for these studies, since they contain significant amounts of AA that is distributed between several major phospholipid subclasses (1-acyl, 1-*O*-alkyl and 1-*O*-alk-1′-enyl) of PC, PE and PI. In addition, membranes can be prepared from U937 cells labelled such that [^{3}H]AA is distributed in phospholipid subclasses in a manner that mimics the mole quantities of AA in these subclasses.

Results of this study indicate that different PLA_2 enzymes show different patterns of specificity for fatty acids released from U937 membranes. In agreement with previous studies, the 110 kDa PLA_2 was specific in releasing predominantly AA from U937 membranes. By contrast with the 110 kDa PLA_2, Type I and Type II low-molecular-mass PLA_2s were not selective for AA release from U937 membranes. These studies imply that the 110 kDa PLA_2 may mobilize AA that can function as a second messenger or can be utilized for eicosanoid formation. Although the 110 kDa PLA_2 was more selective for AA, Type I and Type II PLA_2 could clearly release large quantities of AA from natural membranes. Recent studies have demonstrated that exogenous application of Type I or Type II PLA_2 to different whole-cell systems results in enhanced formation of eicosanoids (Murakami et al., 1993). Moreover, current studies in our laboratory using whole bone-marrow mast cells suggest that incubation of whole cells with Type I or Type II PLA_2 results in the selective release of AA from these cells. The difference in results obtained between these systems may revolve around the fact that secretory PLA_2 (Type I or II) may have specific binding sites on cell surfaces (Tohkin et al., 1993). Such sites may be rich in AA, and thus will account for the specific release of AA from whole cells, but not from the broken-cell preparations. In summary, these data suggest that in inflammatory cells that contain both 110 kDa PLA_2 and Type II PLA_2, the activities of both enzymes may contribute in the generation of eicosanoid by releasing significant quantities of AA.

To understand whether these PLA_2 enzymes recognize portions of the phospholipid other than the *sn*-2 position, we examined their ability to mobilize AA from different phospholipid subclasses found in natural membranes. In whole cells (Fonteh and Chilton, 1992) and in membrane systems (Kramer and Deykin, 1983; Sugiura et al., 1987), AA has been shown to be remodelled between different phospholipid subclasses by the action of CoA-IT. To define clearly the phospholipid sources of AA, we designed a model system in which CoA-IT activity was completely eliminated by heat inactivation. In these studies, the 110 kDa PLA_2 hydrolysed AA in a manner that represented the natural occurrence of AA within membrane phospholipids. For example, the release of AA from 1-acyl-2-AA-GPC, 1-acyl-2-AA-GPE and 1-*O*-alk-1′-enyl-2-AA-GPE by the 110 kDa PLA_2 mimicked the cellular distribution of AA among these same subclasses, indicating that this enzyme has no preference for the radyl group (acyl, alkyl or alk-1′-enyl) at the *sn*-1 position or the type of polar head group at the *sn*-3 position of phospholipids. Thus the fatty acid moiety at the *sn*-2 position of phospholipids seems to be the determining factor for a particular phospholipid to be utilized as a substrate by the 110 kDa PLA_2. By contrast with the 110 kDa PLA_2, Type I and Type II PLA_2 showed preference for PE over PC. Within PE subclasses, both low-molecular-mass PLA_2s showed some preference in AA release from 1-acyl-2-AA-GPE over 1-*O*-alk-1′-enyl-2-AA-GPE.

In conclusion, these studies suggest that different PLA_2 enzymes display unique patterns of substrate selectivities when examined by using a complex mammalian membrane system. Previous PLA_2 studies have typically used defined substrates of a few phospholipid subclasses of defined fatty acid composition. We believe that the complex natural membrane utilized in this study more accurately reflects the natural competition for substrates that cannot easily be duplicated with artificially defined substrates. Moreover, a large amount of information is obtained about the substrate preferences for many different fatty acids and phospholipids in one assay. Results from more complex natural membrane systems must, however, be viewed with some caution, as complicating factors, such as the presence of transacylase enzymes and different accessibility of PLA_2 to various phospholipids, could mislead interpretation of the results. The ultimate test of our understanding of PLA_2 substrate selectivity and specificity will be the explanation of how these enzymes act in the intact cell.

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